Microbial Ecology in States of Health and Disease: Workshop Summary

ISBN
978-0-309-29062-3

548 pages
6 x 9
PAPERBACK (2014)

Eileen R. Choffnes, LeighAnne Olsen, and Alison Mack, Rapporteurs; Forum on Microbial Health; Board on Global Health; Institute of Medicine

MICROBIAL ECOLOGY IN STATES OF HEALTH AND DISEASE

WORKSHOP SUMMARY

Eileen R. Choffnes, LeighAnne Olsen, and Alison Mack,
Rapporteurs

Forum on Microbial Threats

Board on Global Health

INSTITUTE OF MEDICINE
OF THE NATIONAL ACADEMIES

THE NATIONAL ACADEMIES PRESS
Washington, D.C.
www.nap.edu

THE NATIONAL ACADEMIES PRESS 500 Fifth Street, NW Washington, DC 20001

NOTICE: The workshop that is the subject of this workshop summary was approved by the Governing Board of the National Research Council, whose members are drawn from the councils of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

Financial support for this activity was provided by the U.S. Department of Health and Human Services: National Institutes of Health, National Institute of Allergy and Infectious Diseases, Centers for Disease Control and Prevention, Food and Drug Administration, and the Fogarty International Center; U.S. Department of Defense: Armed Forces Health Surveillance Center, Defense Threat Reduction Agency, and Medical Research and Materiel Command; U.S. Department of Justice: Federal Bureau of Investigation; U.S. Department of Veterans Affairs; U.S. Department of Homeland Security; U.S. Agency for International Development; Uniformed Services University of the Health Sciences; Alfred P. Sloan Foundation; American Society for Microbiology; Burroughs Wellcome Fund; GlaxoSmithKline; Infectious Diseases Society of America; Johnson & Johnson; Merck Company Foundation; and sanofi pasteur. The views presented in this publication do not necessarily reflect the views of the organizations or agencies that provided support for this activity.

International Standard Book Number-13: 978-0-309-29062-3
International Standard Book Number-10: 0-309-29062-7

Additional copies of this workshop summary are available from the National Academies Press, 500 Fifth Street, NW, Keck 360, Washington, DC 20001; (800) 624-6242 or (202) 334-3313; http://www.nap.edu.

For more information about the Institute of Medicine, visit the IOM home page at: **www.iom.edu.**

Printed in the United States of America

The serpent has been a symbol of long life, healing, and knowledge among almost all cultures and religions since the beginning of recorded history. The serpent adopted as a logotype by the Institute of Medicine is a relief carving from ancient Greece, now held by the Staatliche Museen in Berlin.

Cover images: (Front, upper) Medical illustration of methicillin-resistant *Staphylococcus aureus* (MRSA); (Front, lower) Medical Illustration of *Clostridium difficile*; (Back) Medical illustration of *Streptococcus pneumoniae*. Photo Credits: All photos courtesy of the Centers for Disease Control and Prevention, www.cdc.gov.

Suggested citation: IOM (Institute of Medicine). 2014. *Microbial ecology in states of health and disease: Workshop summary.* Washington, DC: The National Academies Press.

"Knowing is not enough; we must apply.
Willing is not enough; we must do."
—Goethe

INSTITUTE OF MEDICINE
OF THE NATIONAL ACADEMIES

Advising the Nation. Improving Health.

THE NATIONAL ACADEMIES

Advisers to the Nation on Science, Engineering, and Medicine

The **National Academy of Sciences** is a private, nonprofit, self-perpetuating society of distinguished scholars engaged in scientific and engineering research, dedicated to the furtherance of science and technology and to their use for the general welfare. Upon the authority of the charter granted to it by the Congress in 1863, the Academy has a mandate that requires it to advise the federal government on scientific and technical matters. Dr. Ralph J. Cicerone is president of the National Academy of Sciences.

The **National Academy of Engineering** was established in 1964, under the charter of the National Academy of Sciences, as a parallel organization of outstanding engineers. It is autonomous in its administration and in the selection of its members, sharing with the National Academy of Sciences the responsibility for advising the federal government. The National Academy of Engineering also sponsors engineering programs aimed at meeting national needs, encourages education and research, and recognizes the superior achievements of engineers. Dr. C. D. Mote, Jr., is president of the National Academy of Engineering.

The **Institute of Medicine** was established in 1970 by the National Academy of Sciences to secure the services of eminent members of appropriate professions in the examination of policy matters pertaining to the health of the public. The Institute acts under the responsibility given to the National Academy of Sciences by its congressional charter to be an adviser to the federal government and, upon its own initiative, to identify issues of medical care, research, and education. Dr. Harvey V. Fineberg is president of the Institute of Medicine.

The **National Research Council** was organized by the National Academy of Sciences in 1916 to associate the broad community of science and technology with the Academy's purposes of furthering knowledge and advising the federal government. Functioning in accordance with general policies determined by the Academy, the Council has become the principal operating agency of both the National Academy of Sciences and the National Academy of Engineering in providing services to the government, the public, and the scientific and engineering communities. The Council is administered jointly by both Academies and the Institute of Medicine. Dr. Ralph J. Cicerone and Dr. C. D. Mote, Jr., are chair and vice chair, respectively, of the National Research Council.

www.national-academies.org

PLANNING COMMITTEE FOR A WORKSHOP ON MICROBIAL ECOLOGY IN STATES OF HEALTH AND DISEASE[1]

ARTURO CASADEVALL, Albert Einstein College of Medicine, Bronx, New York
EDUARDO GOTUZZO, Instituto de Medicina Tropical–Alexander von Humboldt, Universidad Peruaña Cayetano Heredia, Lima, Peru
JO HANDELSMAN, Yale University, New Haven, Connecticut
JAMES M. HUGHES, Emory University, Atlanta, Georgia
DAVID A. RELMAN, Stanford University and Veterans Affairs Palo Alto Health Care System, Palo Alto, California
P. FREDERICK SPARLING, University of North Carolina, Chapel Hill, North Carolina

[1] Institute of Medicine planning committees are solely responsible for organizing the workshop, identifying topics, and choosing speakers. The responsibility for the published workshop summary rests solely with the workshop rapporteurs and the institution.

FORUM ON MICROBIAL THREATS[1]

DAVID A. RELMAN (*Chair*), Stanford University, and Veterans Affairs Palo Alto Health Care System, Palo Alto, California
JAMES M. HUGHES (*Vice-Chair*), Global Infectious Diseases Program, Emory University, Atlanta, Georgia
LONNIE J. KING (*Vice-Chair*), Ohio State University, Columbus, Ohio
KEVIN ANDERSON, Biological and Chemical Defense Division, Science and Technology Directorate, Department of Homeland Security, Washington, DC
ENRIQUETA C. BOND, Burroughs Wellcome Fund (Emeritus), QE Philanthropic Advisors, Marshall, Virginia
ROGER G. BREEZE, Lawrence Livermore National Laboratory, Livermore, California
JOHN E. BURRIS,[2] Burroughs Wellcome Fund, Research Triangle Park, North Carolina
ARTURO CASADEVALL, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York
ANDREW CLEMENTS, U.S. Agency for International Development, Washington, DC
PETER DASZAK, EcoHealth Alliance, New York, New York
JEFFREY S. DUCHIN, Public Health–Seattle and King County, Seattle, Washington
JONATHAN EISEN, Genome Center, University of California, Davis, California
RALPH L. ERICKSON,[3] Walter Reed Army Institute of Research, Silver Spring, Maryland
MARK B. FEINBERG, Merck Vaccine Division, Merck & Co., Inc., West Point, Pennsylvania
JACQUELINE FLETCHER, Oklahoma State University, Stillwater, Oklahoma
CLAIRE FRASER, Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, Maryland
JESSE L. GOODMAN, Food and Drug Administration, Rockville, Maryland
EDUARDO GOTUZZO, Instituto de Medicina Tropical–Alexander von Humboldt, Universidad Peruaña Cayetano Heredia, Lima, Peru
CAROLE A. HEILMAN, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
DAVID L. HEYMANN, Public Health England, London, United Kingdom

[1] Institute of Medicine forums and roundtables do not issue, review, or approve individual documents. The responsibility for the published workshop summary rests with the workshop rapporteurs and the institution.
[2] Forum member until October 18, 2013.
[3] Forum member until June 30, 2013.

ZHI HONG,[4] GlaxoSmithKline, Research Triangle Park, North Carolina
PHILIP HOSBACH, sanofi pasteur, Swiftwater, Pennsylvania
STEPHEN ALBERT JOHNSTON, Arizona BioDesign Institute, Arizona State University, Tempe, Arizona
KENT KESTER,[5] Uniformed Services University of the Health Sciences, Bethesda, Maryland
GERALD T. KEUSCH, Boston University School of Medicine and Boston University School of Public Health, Boston, Massachusetts
RIMA F. KHABBAZ, Centers for Disease Control and Prevention, Atlanta, Georgia
MARK KORTEPETER,[6] Uniformed Services University of the Health Sciences, Bethesda, Maryland
STANLEY M. LEMON, University of North Carolina, Chapel Hill, North Carolina
MARGARET J. McFALL-NGAI, University of Wisconsin, Madison, Wisconsin
EDWARD McSWEEGAN, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
PAULA J. OLSIEWSKI, Alfred P. Sloan Foundation, New York, New York
JULIE PAVLIN, Armed Forces Health Surveillance Center, Silver Spring, Maryland
GEORGE POSTE, Complex Adaptive Systems Initiative, Arizona State University, SkySong, Scottsdale, Arizona
DAVID RIZZO, University of California, Davis, California
GARY A. ROSELLE, Veterans Health Administration, Department of Veterans Affairs, Cincinnati, Ohio
KEVIN RUSSELL, Armed Forces Health Surveillance Center, Silver Spring, Maryland
JANET SHOEMAKER, American Society for Microbiology, Washington, DC
JAY P. SIEGEL,[7] Johnson & Johnson, Radnor, Pennsylvania
P. FREDERICK SPARLING, University of North Carolina, Chapel Hill, North Carolina
MARY E. WILSON, Harvard School of Public Health, Harvard University, Boston, Massachusetts
EDWARD H. YOU,[8] Federal Bureau of Investigation, Washington, DC

[4] Forum member until October 18, 2013.
[5] Retired as of December 31, 2013.
[6] Forum member since January 15, 2014.
[7] Forum member since January 15, 2014.
[8] Forum member since July 1, 2013.

IOM Staff

EILEEN CHOFFNES, Scholar and Director
LEIGHANNE OLSEN,[9] Program Officer
KATHERINE McCLURE, Associate Program Officer
REBEKAH HUTTON, Research Associate
CHARLEE ALEXANDER,[10] Senior Program Assistant
JOANNA ROBERTS,[11] Senior Program Assistant (Temp)

[9] Staff member until August 15, 2013.
[10] Staff member from April 1 to November 15, 2013.
[11] Staff member since November 1, 2013.

BOARD ON GLOBAL HEALTH[1]

THOMAS C. QUINN (*Chair*), Associate Director for International Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Professor of Medicine, International Health, Epidemiology, and Molecular Biology and Immunology, Johns Hopkins University School of Medicine, Baltimore, Maryland

JO IVEY BOUFFORD (*IOM Foreign Secretary*), President, New York Academy of Medicine, New York

CLAIRE V. BROOME, Adjunct Professor, Division of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia

JACQUELYN C. CAMPBELL, Anna D. Wolf Chair, and Professor, Johns Hopkins University School of Nursing, Baltimore, Maryland

THOMAS J. COATES, Michael and Sue Steinberg Professor of Global AIDS, Research Co-Director, UC Global Health Institute, David Geffen School of Medicine, University of California, Los Angeles, California

GARY DARMSTADT, Director, Family Health Division, Global Health Program, Bill & Melinda Gates Foundation, Seattle, Washington

VALENTIN FUSTER, Director, Wiener Cardiovascular Institute, Kravis Cardiovascular Health Center, Professor of Cardiology, Mount Sinai School of Medicine, Mount Sinai Medical Center, New York, New York

JACOB A. GAYLE, Vice President, Community Affairs, Executive Director, Medtronic Foundation, Minneapolis, Minnesota

GLENDA E. GRAY, Executive Director, Perinatal HIV Research Unit, Chris Hani Baragwanath Hospital, University of the Witwatersrand, Diepkloof, South Africa

STEPHEN W. HARGARTEN, Associate Dean, Global Health Program, Medical College of Wisconsin, Milwaukee, Wisconsin

PETER J. HOTEZ, Professor of Pediatrics and Molecular Virology and Microbiology, Texas Children's Hospital Endowed Chair of Tropical Pediatrics, President, Sabin Vaccine Institute and Texas Children's Center for Vaccine Development, Dean, National School of Tropical Medicine, Baylor College of Medicine, Houston, Texas

CLARION JOHNSON, Consultant, Exxon Mobil, Fairfax, Virginia

FITZHUGH MULLAN, Professor, Department of Health Policy, The George Washington University, Washington, DC

OLUFUNMILAYO F. OLOPADE, Walter L. Palmer Distinguished Service Professor of Medicine, The University of Chicago, Chicago, Illinois

[1] Institute of Medicine boards do not review or approve individual workshop summaries. The responsibility for the content of the workshop summary rests with the workshop rapporteurs and the institution.

GUY PALMER, Regents Professor of Pathology and Infectious Diseases, Director of the School for Global Animal Health, Washington State University, Pullman, Washington

IOM Staff

PATRICK KELLEY, Director
ANGELA CHRISTIAN, Program Associate

Reviewers

This workshop summary has been reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise, in accordance with procedures approved by the National Research Council's Report Review Committee. The purpose of this independent review is to provide candid and critical comments that will assist the institution in making its published workshop summary as sound as possible and to ensure that the workshop summary meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the process. We wish to thank the following individuals for their review of this workshop summary:

Jeffrey L. Dangl, University of North Carolina, Chapel Hill, North Carolina
Maria Dominguez-Bello, New York University Langone Medical Center, New York, New York
Jesse L. Goodman, Food and Drug Administration, Rockville, Maryland
Margaret J. McFall-Ngai, University of Wisconsin, Madison, Wisconsin

Although the reviewers listed above have provided many constructive comments and suggestions, they did not see the final draft of the workshop summary before its release. The review of this workshop summary was overseen by **Melvin Worth.** Appointed by the Institute of Medicine, he was responsible for making certain that an independent examination of this workshop summary was carried out in accordance with institutional procedures and that all review comments were carefully considered. Responsibility for the final content of this workshop summary rests entirely with the rapporteurs and the institution.

Acknowledgments

The Forum on Emerging Infections was created by the Institute of Medicine (IOM) in 1996 in response to a request from the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH). The purpose of the Forum is to provide structured opportunities for leaders from government, academia, and industry to regularly meet and examine issues of shared concern regarding research, prevention, detection, and management of emerging, reemerging, and novel infectious diseases in humans, plants, and animals. In pursuing this task, the Forum provides a venue to foster the exchange of information and ideas, identify areas in need of greater attention, clarify policy issues by enhancing knowledge and identifying points of agreement, and inform decision makers about science and policy issues. The Forum seeks to illuminate issues rather than resolve them. For this reason, it does not provide advice or recommendations on any specific policy initiative pending before any agency or organization. Its value derives instead from the diversity of its membership and from the contributions that individual members make throughout the activities of the Forum. In September 2003, the Forum changed its name to the Forum on Microbial Threats.

The Forum on Microbial Threats and the IOM wish to express their warmest appreciation to the individuals and organizations who gave their valuable time to provide information and advice to the Forum through their participation in the planning and execution of this workshop. A full list of presenters, and their biographical information, may be found in Appendixes B and E, respectively.

The forum gratefully acknowledges the contributions of the members of the planning committee[1]: Arturo Casadevall (Albert Einstein College of Medicine),

[1] Institute of Medicine planning committees are solely responsible for organizing the workshop, identifying topics, and choosing speakers. The responsibility for the published workshop summary rests solely with the workshop rapporteurs and the institution.

Eduardo Gotuzzo (Universidad Peruaña Cayetano Heredia), Jo Handelsman (Yale University), James M. Hughes (Emory University), David A. Relman (Stanford University), and P. Frederick Sparling (University of North Carolina, Chapel Hill).

The Forum is also indebted to the IOM staff who tirelessly contributed throughout the planning and execution of the workshop and the production of this workshop summary report. On behalf of the Forum, we gratefully acknowledge these efforts led by Dr. Eileen Choffnes, Scholar and Director of the Forum; Dr. LeighAnne Olsen,[2] Program Officer; Katherine McClure, Associate Program Officer; Rebekah Hutton, Research Associate; and Charlee Alexander,[3] Senior Program Assistant for dedicating much effort and time to developing this workshop's agenda and for their thoughtful and insightful approach and skill in planning for the workshop and in translating the workshop's proceedings and discussion into this workshop summary report. We would also like to thank the following IOM staff and consultants for their valuable contributions to this activity: Daniel Bethea, Laura Harbold DeStefano, Chelsea Frakes, Alison Mack, Erika Vijh, and Julie Wiltshire.

Finally, the Forum wishes to recognize the sponsors that supported this activity. Financial support for this project was provided by the U.S. Department of Health and Human Services: National Institutes of Health, National Institute of Allergy and Infectious Diseases, Centers for Disease Control and Prevention, Food and Drug Administration, and the Fogarty International Center; U.S. Department of Defense: Armed Forces Health Surveillance Center, Defense Threat Reduction Agency, and Medical Research and Materiel Command; U.S. Department of Justice: Federal Bureau of Investigation; U.S. Department of Veterans Affairs; U.S. Department of Homeland Security; U.S. Agency for International Development; Uniformed Services University of the Health Sciences; Alfred P. Sloan Foundation; American Society for Microbiology; Burroughs Wellcome Fund;[4] GlaxoSmithKline;[5] Infectious Diseases Society of America; Johnson & Johnson; Merck Company Foundation; and sanofi pasteur. The views presented in this workshop summary are those of the workshop participants and have been summarized by the rapporteurs. They do not necessarily reflect the views of the Forum on Microbial Threats, its sponsors, or the IOM.

[2] Staff member until August 15, 2013.
[3] Staff member from April 1 to November 15, 2013.
[4] Sponsor until October 18, 2013.
[5] Sponsor until October 18, 2013.

Contents

Tables, Figures, and Boxes

TABLES

FIGURES

BOXES

Workshop Overview[1]

Perhaps one of the most important changes we can make is to supersede the 20th-century metaphor of war for describing the relationship between people and infectious agents. A more ecologically informed metaphor, which includes the germs'-eye view of infection, might be more fruitful. Consider that microbes occupy all of our body surfaces. Besides the disease-engendering colonizers of our skin, gut, and mucous membranes, we are host to a poorly cataloged ensemble of symbionts to which we pay scant attention. Yet they are equally part of the superorganism genome with which we engage the rest of the biosphere.

—Joshua Lederberg, "Infectious History" (2000)

MICROBIAL ECOLOGY IN STATES OF HEALTH AND DISEASE

Introduction

Individually and collectively, resident microbes play important roles in host health and survival. Shaping and shaped by their host environments, these microorganisms form intricate communities that are in a state of dynamic equilibrium.

[1] The planning committee's role was limited to planning the workshop, and the workshop summary has been prepared by the workshop rapporteurs (with the assistance of Charlee Alexander, Rebekah Hutton, and Katherine McClure) as a factual summary of what occurred at the workshop. Statements, recommendations, and opinions expressed are those of individual presenters and participants and are not necessarily endorsed or verified by the Forum, the Institute of Medicine, or the National Research Council, and they should not be construed as reflecting any group consensus.

This ecologic and dynamic view of host–microbe interactions is rapidly redefining our view of health and disease. It is now accepted that the vast majority of microbes are, for the most part, not intrinsically harmful, but rather become established as persistent, co-adapted colonists in equilibrium with their environment, providing useful goods and services to their hosts while deriving benefits from these host associations. Disruption of such alliances may have consequences for host health, and investigations in a wide variety of organisms have begun to illuminate the complex and dynamic network of interactions—across the spectrum of hosts, microbes, and environmental niches—that influence the formation, function, and stability of host-associated microbial communities (Dethlefsen et al., 2007; Turnbaugh et al., 2007; Robinson et al., 2010; IOM, 2012).

From the microbiota[2] on the surface of our skin to those that inhabit the mucus-covered lining of our gut, we are deeply embedded in a microbial world—an observation that extends to most, if not all, plant and animal life on Earth. By the time we reach adulthood, more than 100 trillion microorganisms—including Archaea, Bacteria, Fungi, Protozoa, and Viruses—inhabit specialized environmental niches in and on our body surfaces, forming complex communities that contribute to the nutrition, defense, and development of the intricate, microbe-dominated ecosystems that we humans call "ourselves." Indeed, we are more accurately viewed as superorganisms—compilations composed of human and microbial cells that are "yoked into a chimera of sorts'" (Lederberg, 2000; Hooper and Gordon, 2001; Xu and Gordon, 2003).

Recent studies of the human gut microbiota have suggested intriguing associations between "dysbiosis" (a general term[3] denoting alterations to the composition and dynamics of our microbiota) and a variety of chronic conditions not thought to have a microbial etiology—including severe acute malnutrition, obesity, cardiovascular disease, asthma, adult-onset diabetes, and the inflammatory bowel diseases (Ley et al., 2005; Petersen et al., 2008; Han et al. 2012; Karlsson et al., 2012, 2013; Qin et al., 2012; Ridaura et al., 2013; Smith et al., 2013; Tang et al., 2013). Scientists have yet to determine whether these associations reflect a causal relationship, and many have urged caution about "overselling" the importance of these initial observations. Still, the dramatic rise in the global pervasiveness of many of these apparently noncommunicable diseases over the past half-century has fueled intense interest in the possibility that local and global alterations in our microbial ecology may be contributing to the

[2] For the purposes of this workshop overview, microbiota is a collection of microorganisms—including Archaea, Bacteria, Fungi, Protozoa, and Viruses—that exist in the same place at the same time (see Robinson et al., 2010). The terms resident, endogenous, or indigenous microorganisms describe host-associated microbiota.

[3] Coined by Mechnikoff in the early 1900s, *dysbiosis* describes a state of microbial imbalance in the gut and now refers to a change in the structural and/or functional configuration of the microbiota that produces a disruption in the homeostasis between a host and its indigenous microbes (Gordon, 2012).

development or progression of a wide variety of complex diseases (Blaser and Falkow, 2009).

In addition to the varied landscapes provided by animals and plants, microbial communities inhabit Earth's soil, water, and air, where they drive important geochemical and biological processes. Indeed, microbial communities form the "heart of all ecosystems" (Shade et al., 2012), and their exploration promises to transform our understanding of the natural world (IOM, 2006, 2009, 2012; Shade et al., 2012). Taking a more holistic view of "who [and what] we are" that includes consideration of the role that our resident microbiota plays in influencing states of health and disease may also revolutionize clinical approaches to the diagnosis, treatment, and, ultimately, prevention of disease (Shade et al., 2012).

Statement of Task

On March 18 and 19, 2013, the Institute of Medicine's (IOM's) Forum on Microbial Threats hosted a public workshop, in Washington, DC, to explore the scientific and therapeutic implications of microbial ecology in states of health and disease. Participants explored host–microbe interactions in humans, animals, and plants; emerging insights into how microbes may influence the development and maintenance of states of health and disease; the effects of environmental change(s) on the formation, function, and stability of microbial communities; and research challenges and opportunities for this emerging field of inquiry. This meeting built and expanded upon many of the topics explored at a 2002 Forum workshop, The Infectious Etiology of Chronic Diseases (IOM, 2004).

Organization of the Workshop Summary

This workshop summary was prepared by the rapporteurs for the Forum's members and includes a collection of individually authored papers and commentary. The contents of the unattributed sections of this summary report provide a technical context for the reader to appreciate the presentations and discussions that occurred over the 2 days of this workshop and do not represent the views of the members of the Forum on Microbial Threats, its sponsors, or the IOM.

The summary is organized into sections as a topic-by-topic distillation of the presentations and discussions that took place at the workshop. Its purpose is to present information from relevant experience, to delineate a range of pivotal issues and their respective challenges, and to offer differing perspectives on the topic as discussed and described by the workshop participants. Manuscripts and reprinted articles submitted by workshop participants may be found, in alphabetical order by author, in Appendix A.

Although this workshop summary provides a description of the individual presentations, it also reflects an important aspect of the Forum's philosophy. The workshop functions as a dialogue among representatives from different sectors

and perspectives that allows them to present their views about which areas, in their opinion, merit further study. This report only summarizes the statements of participants over the course of the workshop. This summary is not intended to be an exhaustive exploration of the subject matter, nor does it represent the findings, conclusions, or recommendations of a consensus committee process.

HUMANS, ANIMALS, AND PLANTS IN A MICROBIAL WORLD

Co-evolution, co-adaptation, and codependency are all features of our relationships with our indigenous microbiota.
—Blaser and Falkow (2009)

Like all forms of life on Earth, microorganisms exist within often complex communities. In every habitat studied—from acidic hot springs to the external and internal surfaces of plants, animals, and humans—microorganisms interact with and influence one another and their environment (IOM, 2012). As noted by David Relman, chair of the Forum on Microbial Threats, the important biology accomplished by the net actions of interacting microbial communities is really the norm on our planet, yet we are only just beginning to explore and appreciate the principles that might define these communities.

Most studies of the microbial world, until fairly recently, isolated microorganisms from their natural ecological settings and studied in sterile monoculture.[4] Guided by a reductionist strategy of reductionism that relied upon the isolation and growth of single microbial species in pure culture, this approach limited observations to a narrow range of species that could be isolated and grown under controlled conditions[5] (IOM, 2012). Molecular, sequence-based approaches[6]—pioneered by environmental microbiologists in the late 20th century—ultimately alleviated these constraints and allowed scientists to characterize communities of bacteria and Archaea in a wide range of environments. These studies dramatically expanded our understanding of the natural world by revealing the vast, and previously unseen, diversity of the microbial world (Pace, 1997; Whitman et al., 1998; Handelsman, 2004). Today, genomic methods are being

[4] Most studies of microbes were performed on organisms isolated in the laboratory and apart from their natural environmental contexts.

[5] Culturing single cells of a particular microbial type is a useful approach to learn about the biology of a particular organism. It is an unnatural environment for most microorganisms because cells are grown in isolation and under controlled conditions. However, by some estimates greater than 99 percent of the microbial world is or may be unculturable (Robinson et al., 2010).

[6] Because all cell-based organisms possess rRNA genes, these gene sequences were used as a culture-independent means for organism detection. Researchers used sequences shared by all rRNA it is an unnatural environment for most microorganisms because to amplify each rRNA gene sequence present in a sample and then analyzed subtle differences between rRNA gene sequences to infer the types of organisms present. Today, sequence-based techniques can be applied directly to DNA isolated from an environmental sample to characterize entire genomes of organisms present (Eisen, 2007).

developed to extend these ecological surveys to fungi and viruses (see Virgin et al., 2009; Findley et al., 2013).

Over the past decade, investigations of the ecology of host-associated microbial communities in a variety of contexts have flourished because of the lower cost, increased speed, and greater capacities of nucleic acid sequencing and other analytic techniques, coupled with advances in bioinformatics and computational biology. In addition to metagenomic surveys of the taxa and genomic content present in a microbial community (discovering "who is there?"), it is increasingly possible to examine their function ("what are they doing and why are they doing it?") by probing gene expression (metatranscriptomics), protein synthesis (proteomics), and production of small molecular weight compounds (metabolomics). Coupling these techniques with concepts developed in the field of macroecology, researchers are able to create a rich, multidimensional picture of the ecology of microbial communities (Robinson et al., 2010; Boyle and Gill, 2012). Terminology commonly used in these studies are defined in Table WO-1.

Microbes in a Microbial World: Exploring Host–Microbe Ecosystems

The idea that disruption of host–microbe interactions or alterations to community structure may lead to disease raises fundamental questions about the ways in which host–microbe, and microbe–microbe, associations are formed and maintained. Such questions have been the basis of a rich body of research on symbiotic interactions, in which disparate organisms form beneficial (mutualistic), neutral (commensal), or harmful (parasitic) associations that often persist over the lifetime of the host. While such symbiotic associations were once considered to be exceptions, symbioses are now known to be the rule in biotic and abiotic systems. Microbial symbionts are not only a normal part of the life cycle of plants and animals, they are often integral to host development and evolution (Fraune and Bosch, 2010; Gilbert et al., 2012).

Studies of symbioses in a wide variety of organisms have provided important insights into the factors that drive the formation, function, and stability of host–microbe associations and a means to pursue the deeper question of whether universal rules and mechanisms govern these processes (IOM, 2009, 2012; Fraune and Bosch, 2010). As noted by Forum member Margaret McFall-Ngai of University of Wisconsin, Madison, "Nature and evolution have done phenomenal experiments from which we can learn, and although humans often forget that they are part of the environment, we are . . . highly linked, and, more importantly, we are products of our evolutionary history." McFall-Ngai went on to observe that comparative investigations of host–microbe associations in a variety of systems will help to define the "very basic rules by which animals and plants interact with microorganisms."

TABLE WO-1 Microbial Ecology Definitions

Term	Definition
Biogeography	The study of biodiversity in space and time
Diversity	A measure of how much variety is present in a community, irrespective of the identities of the organisms present; consists of richness and evenness
Evenness	The distribution of individuals across types
Function	An activity or "behavior" associated with a community (e.g., nitrogen fixation or resistance to invasion)
Invasion	An ecological event characterized by the establishment of a foreign organism in a new community and the persistence and spread of this organism
Metagenomics	A culture-independent method used for functional and sequence-based analysis of total environmental (community) DNA (note that this is not the same as amplifying, cloning, and sequencing the 16S rRNA-encoding gene, although metagenomic sequences, such as those generated via modern sequencing methods, can be probed for 16S rRNA-encoding genes or other phylogenetic markers)
Microbiome	The gene complement of a community
Microbiota/community	A collection of microorganisms existing in the same place at the same time
Resilience	The rate at which a community recovers to its native structure following a perturbation
Resistance	The ability of a community to resist change to its structure after an ecological challenge
Richness	Number of types (e.g., species) in a community
Similarity	A measure that determines the similarity of two or more communities, typically based on shared members, total richness, and sometimes abundance of members
Structure	The composition of the community and the abundance of individual members
Temporal stability	The ability of a community to maintain its native structure

SOURCE: Robinson et al. (2010).

Examples of Host–Microbe Communication, Colonization, and Development

Symbiotic associations can be quite specific, as illustrated in the following examples, suggesting co-evolution of host and microbe over long periods of time. This shared evolutionary past is also reflected in the chemical dialog that mediates these associations (McFall-Ngai et al., 2013). In addition to long-term selective forces that hone microbe–host interdependencies, indigenous microbes are subject to shifting environmental conditions over the lifetime of the host. Early patterns of niche colonization and community assembly can strongly—and in some cases, irreversibly—influence the developing host environment and microbiota.

The bacterium and the squid Studied for more than 25 years, the persistent association between the Hawaiian bobtail squid *Euprymna scolopes* and the gram-negative, luminescent bacterium *Vibrio fischeri* continues to reveal important insights into host–microbe associations. An early and exclusive association between the squid and a single species of bacteria (*V. fischeri*) triggers tissue maturation within the squid's body cavity to form a specialized light-emitting organ. Luminescence emitted by the bacteria resembles moonlight and starlight filtering through ocean waters, camouflaging the squid from predators swimming below (Nyholm and McFall-Ngai, 2004).

Bacterial colonization begins within hours of the squid's hatching (Figure WO-1). The squid acquires *V. fischeri* from its environment, and upon the initiation of colonization the juvenile squid selectively recruits *V. fischeri* in a glycan-rich mucus, separating it from the rich mixture of seawater microbes. It has now been reported that first contact within the squid-*vibrio* symbiosis triggers profound molecular and chemical changes that are crucial for bacterial colonization[7] (Kremer et al., 2013). Once colonized, the squid undergoes dramatic morphological changes—including programmed cell death that eliminates "symbiont-recruiting" structures and the remodeling of tissue to favor the maintenance of *V. fischeri* within the mature light organ. Host tissue recognition of two non-specific bacterial products—peptidoglycan and lipopolysaccharide—triggers theses developmental events in the squid (Nyholm and McFall-Ngai, 2004). These molecules are members of a broad class of microbe-associated molecular patterns[8] (MAMPs) that have now been shown to trigger developmental processes in a wide variety of animals and plants (see Koropatnick et al., 2004, and McFall-Ngai et al., 2013).

Plant–microbe interactions in the rhizosphere Like the development of the bobtail squid's light organ, one of the best-characterized plant–microbe interactions features a symbiotic association that triggers tissue development in leguminous plants. Nitrogen-fixing bacteria called rhizobia, attracted by plant-secreted flavonoid compounds, colonize legume roots and release chemicals called nodulation (Nod) factors. These factors trigger gene expression within plant roots that results in the uptake of bacteria by plant tissues to form root nodules. The captured bacteria provide a critical nutrient for the plant and in

[7] This exquisitely sensitive response to the host's specific symbiotic partner includes the upregulation of a host endochitinase, whose activity hydrolyzes polymeric chitin in the mucus into chitobiose, thereby priming the symbiont and also producing a hemoattractant gradient that promotes *V. fischeri* migration into host tissues. Thus, the host responds transcriptionally upon initial symbiont contact, which facilitates subsequent colonization (Kremer et al., 2013).

[8] Microbe-associated molecular patterns (MAMPs) are essential structures on features of microbes that are recognized by the innate immune system (Koropatnick et al., 2004). They are recognized by toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) in plants and animals. MAMPs are often referred to as pathogen-associated molecular patterns (PAMPs). However these motifs are shared among microbes.

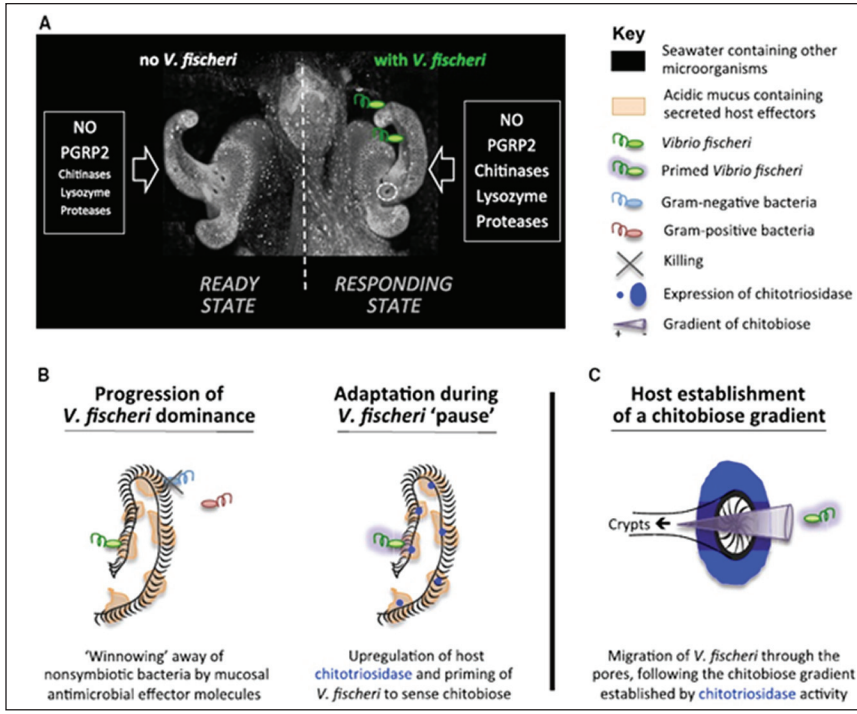


FIGURE WO-1 Model for early colonization. (A) The initial contact of *V. fischeri* with host tissues induces the expression of several genes (e.g., proteases, chitinases such as EsChitotriosidase, and lysozyme) whose products, when supplemented with components already present in the mucus (NO and EsPGRP2), affect the chemistry of the mucus matrix, shaping the specificity and preparing for future colonization events. (B) Course of events that allow selective colonization by *V. fischeri*. Left: Antimicrobial compounds (e.g., lysozyme and PGRP2) are activated by acidic proteases in the low-pH environment and participate in the selective exclusion of nonsymbiotic bacteria. Right: While *V. fischeri* cells are "pausing" in the aggregate, the upregulation of EsChitotriosidase in the ciliated field of the light organ hydrolyzes chitin into chitobiose, which prepares *V. fischeri* to sense and be attracted toward chitobiose. (C) EsChitotriosidase, which is highly expressed close to the pores and optimally active at low pH, degrades chitin produced by the host into chitobiose, thereby establishing a chitobiose gradient extending out of the pores. Primed *V. fischeri* cells are attracted by the chitobiose gradient and migrate through the pores (Mandel et al., 2012).
SOURCE: Mandel et al. (2012) in Kremer et al. (2013).

return are fed by the plant's roots. The roots of most higher-plant species form similar, flavonoid-mediated symbiotic associations with mycorrhizal fungi (IOM, 2006, 2009; Desbrosses and Stougaard, 2011).

Distinct microbiota colonize the rhizosphere (root–soil interface) and endosphere (endophytic compartment within plant root tissues), both of which differ in composition from those found in the surrounding soil. Plant roots provide a structured and nonhomogeneous habitat for tens of thousands of species of microbes. Within this complex environment, microhabitats of nutrient, water, pH, and oxygen gradients shape—and are shaped by—root-associated microbial communities (Ramirez-Puebla et al., 2013). Explorations of this complex environment have illuminated a variety of small molecules (including MAMPs) and chemical signaling networks that mediate plant–microbe and microbe–microbe interactions. The intricate chemical and genetic "cross-talk" between plants and their associated microbiota supports a variety of functions—including plant growth, nutrition, productivity, carbon sequestration, phytoremediation,[9] and protection (Figure WO-2) (Badri et al., 2009; Berendsen et al., 2012; Bulgarelli et al., 2012).

Community Assembly and Dynamics in a Model Vertebrate

Microbial colonization is a crucial event in the development of the vertebrate gut, and early colonization events appears to be important to the normal maturation and functioning of the immune and digestive systems. In a recent study in the *Proceedings of the National Academy of Sciences of the United States of America*, Everard et al. (2013) appear to have demonstrated a causal relationship between Akkarmansia carriage and obesity. Speakers Karen Guillemin and Brendan Bohannan, both of the University of Oregon, described wide-ranging work with the zebrafish *Danio rerio*, which Guillemin described as "a model vertebrate that is allowing us to explore the complex systems biology of host–microbe interacting systems" (Dr. Guillemin's contribution may be found on pages 323-346 in Appendix A; Dr. Bohannan's contribution may be found on pages 164-184 in Appendix A). Zebrafish have several advantages as a model system, Guillemin explained: their guts and immune systems resemble those of other mammals, including humans; they develop rapidly; they are transparent, so their digestive tract can be easily visualized; they are readily amenable to genetic manipulation; and, perhaps most importantly, germ-free zebrafish have been developed, along with methods to associate them with different bacterial communities.

Patterns of colonization Guillemin described her use of germ-free zebrafish and methods to associate them with different bacterial communities. "What our models allow us to do is to build up these systems and look at increasing complexity, starting with the germ-free animal," she said. "We can then associate them with very

[9] The use of green plants to decontaminate polluted soil or water.

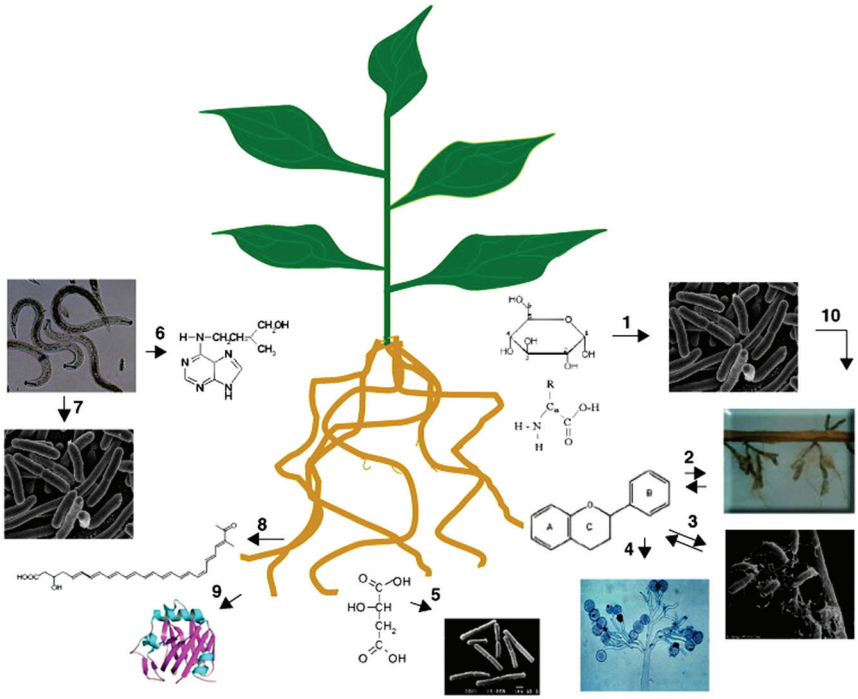


FIGURE WO-2 Illustration of the chemical communication that exists between plant roots and other organisms in the complex rhizosphere. Plant roots secrete a wide range of compounds; among those are sugars and amino acids that are engaged in attracting (chemotaxis) microbes (1), flavonoids act as signaling molecules to initiate interactions with mycorrhiza (AM fungi) (2), rhizobium and (3) pathogenic fungi (oomycetes) (4), aliphatic acids (e.g., malic acid) are involved in recruiting specific plant growth promoting rhizobacteria (*Bacillus subtilis*) (5), nematodes secrete growth regulators (cytokinins) that are involved in establishing feeding sites in plant roots (6), and nematodes secrete other compounds (organic acids, amino acids, and sugars) involved in attracting bacteria and in bacterial quorum sensing (7). Knowledge of the roles of other types of compounds, such as fatty acids (8) and proteins (9), secreted by roots in the rhizosphere and other multipartite interactions (10), remains unknown.
SOURCE: Badri et al. (2009).

simple communities, or we can allow them to be colonized with complex natural communities . . . [to] look at this whole spectrum of complexity" (Figure WO-3). The recently developed light sheet microscope provides Guillemin and coworkers with high-resolution, real-time imaging of the colonization and growth of bacteria in the developing zebrafish gut (Taormina et al., 2012).

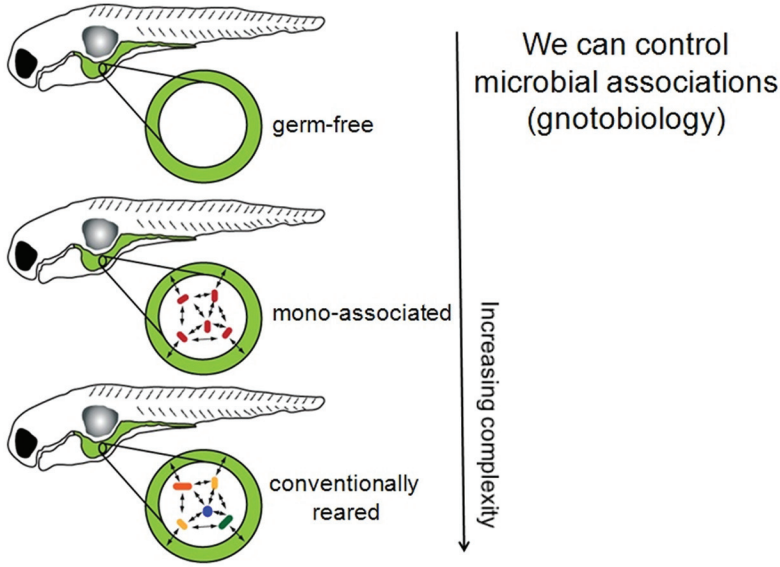


FIGURE WO-3 Host–microbe systems biology. In germ-free model systems, microbial associations may be manipulated to explore host–microbe interactions of increasing complexity. Gnotobiology comprises the study of germ-free plants and animals, as well as living things in which specific microorganisms, added by experimental methods, are known to be present.
SOURCE: Guillemin (2013).

Using this method in germ-free animals, the researchers observed that "early colonizers"—those bacteria that first reach the digestive tract—tend to dominate the developing community (Guillemin and Parthasarathy, unpublished). "We're exploring the possibility that those first colonizers might have access to certain privileged niches within the gut, and . . . whether there are changes in bacterial physiology upon colonization, perhaps in conjunction with changes in the host environment upon colonization," she said.

Dynamic interactions Bacterial colonization of the zebrafish gut triggers an innate immune response by the host, in the form of neutrophils—which are not present in the intestinal tracts of germ-free fish, Guillemin noted (Bates et al., 2007). Through experiments in which different species of bacteria that naturally populate the zebrafish gut were individually introduced to germ-free animals, she and coworkers found that the host response (as measured by neutrophil influx) was both varied and species specific; some isolates were very pro-inflammatory, inducing a large number of neutrophils, while others had very little effect on the

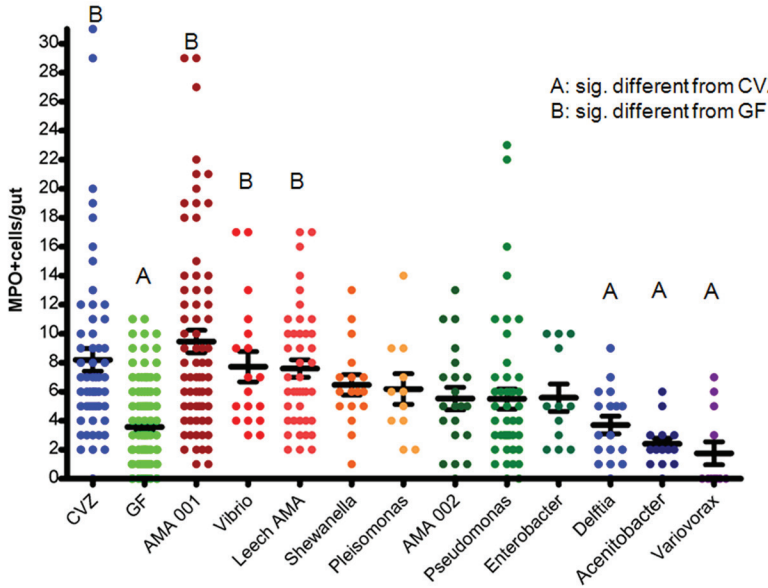


FIGURE WO-4 Monoassociation with zebrafish gut bacterial isolates elicits a wide range of neutrophil influx (MPO+ cells/gut).
NOTE: CVZ = conventionally raised; GF = germ free.
SOURCE: Guillemin (2013).

population of neutrophils (Figure WO-4). Positive, neutral, and negative correlations were observed for the number of neutrophils and specific strains of bacteria.

These findings led the researchers to ask whether host response to a community of bacteria would be a predictable sum of responses to its constituent species or an unpredictable "emergent property" of a complex system. To investigate these possibilities, researchers colonized germ-free fish with individual and paired combinations of *Aeromonas*, *Vibrio*, and *Shewanella* strains. When paired with *Aeromonas*, *Vibrio* dominated the community and shaped the neutrophil response. When paired with *Vibrio*, *Shewanella* growth was suppressed as compared with monoculture, while *Vibrio* growth was unchanged; however, neutrophil response appeared to be dictated by *Shewanella*, despite its minority.

"We're starting to learn that there are some really interesting kinds of interactions that we can detect between members of the microbiota, and that those relationships impact the host," Guillemin concluded. "The sum of those interactions could not simply be predicted by each individual member's effect alone. There are emergent properties that come out of even these very simple systems."

Drivers of community variation Identifying the mechanisms that produce variation among communities is a fundamental goal of ecology and an important step toward revealing how host-associated microbiota influence host health (Costello et al., 2012). In addition to the several advantages of the zebrafish model described by Guillemin, Bohannan noted that these aquarium fish can readily be raised in the large numbers and controlled environments required to study community variation.

Inquiry into the sources of variation in the zebrafish gut microbiome are informed by ideas developed over decades by plant and animal ecologists. According to one influential theoretical framework, three basic processes drive community variation over short time frames and coarse taxonomic resolutions:

- Dispersal (movement in space),
- Ecological drift (the stochastic loss of organisms from a community and their replacement from within or without), and
- Ecological selection (the differential fitness among organisms due to the community or environment) (Vellend, 2010).

Bohannan's work explores how these forces interact to drive community variation, in an effort to predict their dynamics, and, ultimately, ways to manage those dynamics that benefit host health, he explained. The zebrafish system enabled him and coworkers to conduct a longitudinal study[10] to track gut colonization in germ-free, nearly isogenic,[11] fish hatched into a common source of nonsterile water, thereby eliminating potential contributions to microbial community variation from dispersal or selection based on host genotype. Twelve fish were sampled at each of six stages of development from hatching to sexual maturity; sequencing of gut bacterial 16S rDNA determined the species present in each individual microbial community (Figure WO-5).[12]

Analyses of the abundance of colonizing microbial species in the gut relative to those available in the surrounding water demonstrated both positive and negative selection for certain taxa, Bohannan noted (Figure WO-6).

Bohannan reported that mathematical projections of variation caused entirely by drift accounted for more than 60 to 80 percent (approximately) of the variation observed among individual fish at various time points in the study. The remainder he attributed to nongenetically encoded host factors, such as adaptive immunity, or to interactions among the colonizing microbes—a possibility they are now testing in mutant zebrafish that lack functioning adaptive or innate immune function. One way to interpret changes in the fit of the "drift" model relative to observed

[10] Study in which subjects are followed over time with continuous or repeated monitoring of study variables or outcomes.

[11] Organisms having the same or closely similar genotypes.

[12] For the experiments discussed by Bohannan, operational taxonomic unit (OTU) variation was defined at 97 percent.

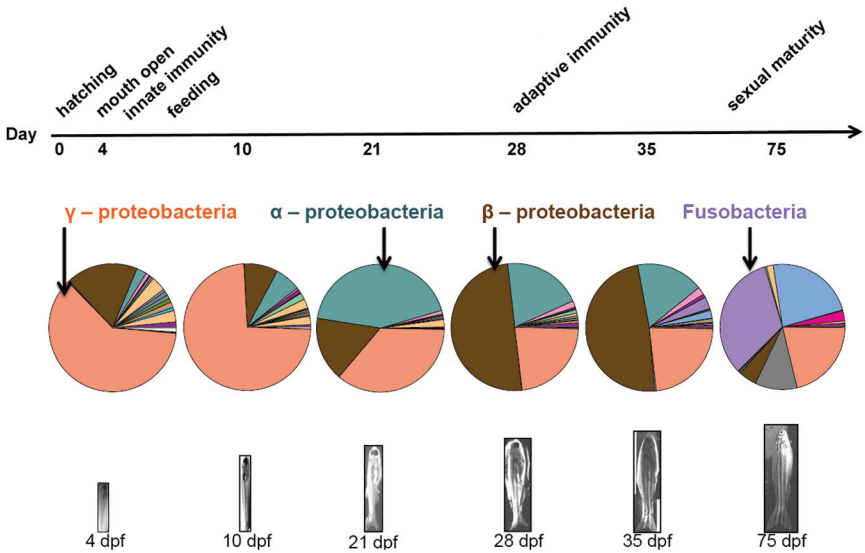


FIGURE WO-5 The gut microbial community composition changes over developmental time.
SOURCE: Bohannan (2013).

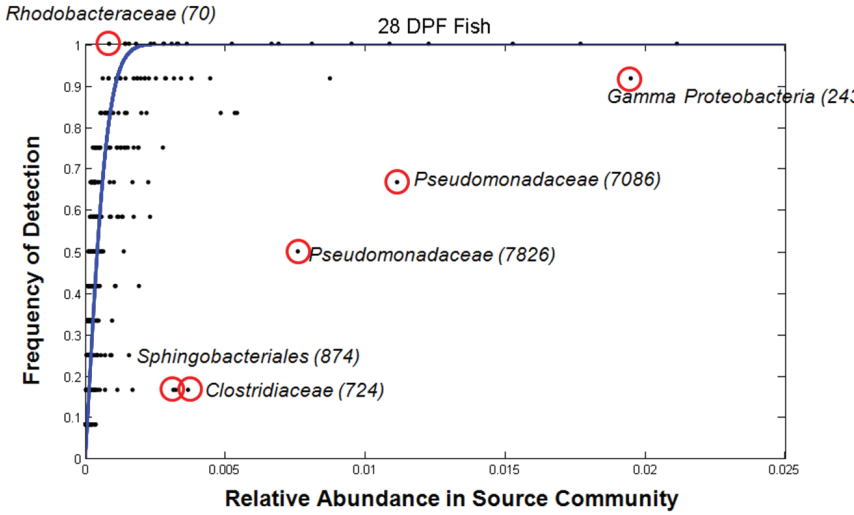


FIGURE WO-6 Positive and negative selection of colonizing microbial species in the gut. The outliers that lie above the blue line are likely taxa that are positively selected regardless of their relative abundance in the source community. Those below the line are taxa that are likely negatively selected despite their commonness in the source community.
SOURCE: Bohannan (2013).

variation over the course of the experiment is that the strength of the selective pressures on the microbiota varies with particular developmental milestones, including the initiation of innate or adaptive immunity and the onset of sexual maturity; he and coworkers are currently testing this hypothesis.

Microbiota Composition and Function in a Plant Root System

Speaker Gerald Tuskan and his team from Oak Ridge National Laboratory (ORNL) use a member of the Salicaceae family as a model system to explore plant–microbe interactions in the rhizosphere and endosphere (Dr. Tuskan's contribution may be found on pages 412-435 in Appendix A).[13] Tuskan's research originally focused on the *Populus*[14] genome. The catalyst for his research began with an interest in using trees as a source of cellulose for biofuel and of lignin for carbon fibers, and as living "sinks" for carbon dioxide to offset the effects of climate change (Tuskan et al., 2006). When modified plant genotypes transplanted into an "open" environment did not behave as they did in the greenhouse or in the laboratory, Tuskan and colleagues discovered that "it wasn't solely about the host plant." The difference was the microbiome, Tuskan said.

In his presentation, Tuskan described his group's efforts to characterize the sources of genetic diversity among these microbial communities and to relate community composition to function (Figure WO-7). "We want to understand the communication, the chemical signaling that occurs between the bacteria, the fungi, and the host, to see if we can then ultimately reconstruct the community in a predictive manner," Tuskan explained. By studying the genetic and functional diversity of the *Populus* microbiome, the investigators ultimately hope to understand its influence on cell wall synthesis and to use this knowledge to increase cellulose and lignin production.

Over the course of several seasons, Tuskan and coworkers sampled *Populus* microbiomes of multiple host genotypes across a range of microenvironments along two southeastern rivers and among 1,000 individual genotypes collected throughout the Pacific Northwest and transplanted to one of four common gardens.[15] Sequencing the microbial communities in their extensive sample set revealed that about 70 percent of the identified OTUs were shared by both rhizosphere and endosphere—although the abundance of a given OTU often varied between the two compartments (Gottel et al., 2011). After separating out differences according to compartment, the second largest driver of diversity was host genotype. The least important variables were year and season: "When we

[13] Microbial communities that live within the tissues (endophytic compartment) of plant roots.

[14] Populus is a genus of 25–35 species of deciduous flowering plants in the family Salicaceae, native to most of the Northern Hemisphere. English names variously applied to different species include poplar, aspen, and cottonwood.

[15] Controlled setting for experiments in which one or more organisms are moved from one environment to another.

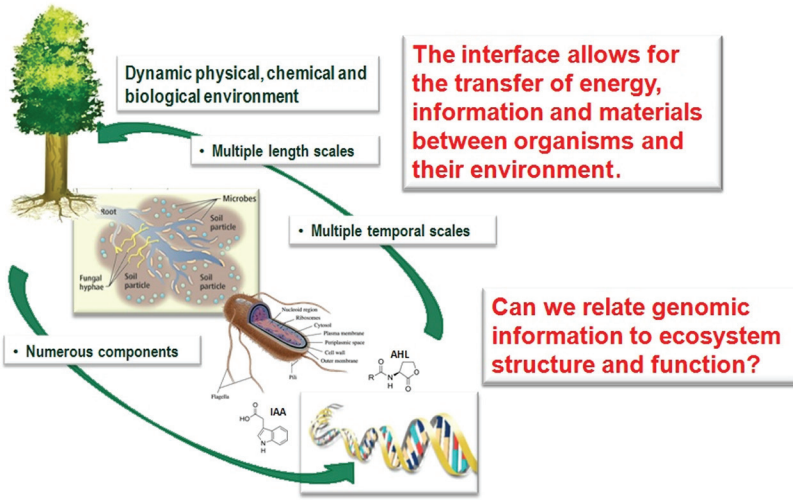


FIGURE WO-7 The dynamic interfaces that exist between plants, microbes, and the environment.
SOURCE: Tuskan (2013).

went back to the same trees and the same root systems over multiple seasons over multiple years we isolated basically the same broad microbiome both from the rhizosphere and the endosphere," Tuskan reported.

In terms of community diversity, "the endosphere turns out to be a subcomponent of the rhizosphere," Tuskan concluded (Gottel et al., 2011). This observation supports a conception of microbiota acquisition that he called the "lottery hypothesis" where plants recruit microbial inhabitants of the root endosphere from the wider range of microbes present in the immediate environment of the rhizosphere—which, in turn, represents a subset of the far larger pool of potential endosymbionts available in the various environments in which the plant grows. Tuskan likened this scenario to that of shoppers in open markets that are populated with many kiosks selling similar goods: rather than sampling all of the kiosks, shoppers typically make their purchases from the first or second merchant they encounter. A similar pattern emerged from their analysis of fungal diversity in the *Populus* endosphere and rhizosphere, reinforcing the notion that the *Populus* selects members of its endophytic community from the rhizosphere.

Tuskan described several experiments investigating how individual and communities of microorganisms may contribute to important functions. Based on the culture, isolation, and sequencing of about 2,800 representative bacteria from three compartments of the *Populus* environment—the nearby soil, the soil in contact with the root system, and the endosphere—the researchers determined

that the endosphere microbiota was rich in *Pseudomonas* species, according to Tuskan. From these *Pseudomonas* isolates,[16] they identified the individual genes that could confer functional advantages to a colonizing bacterium through their effects on such processes as quorum sensing[17] and biofilm formation.

In addition, certain endophytic bacteria are known to partner with the mycorrhizal fungi that form symbiotic associations with plant roots. Through pairwise inoculation experiments with *Populus* fungal isolates, the ORNL team identified "mycorrhizal helper bacteria" among their collections of *Populus* bacterial isolates. "When we take those helper bacteria in combination with the mycorrhizal fungi and we inoculate the host we see positive differential effects upon the host," he explained. "There appears to be a combinatorial interaction between the bacteria, the fungi, and then ultimately the host genotype." Based on this observation, the researchers have identified regions of the *Populus* genome that control colonization by *Laccaria bicolor*, a mycorrhizal fungus, and ultimately, a gene locus favoring colonization by the bacterium.

Bringing together the knowledge they have gained from these analyses, Tuskan's group can now assess how promising combinations of host genotypes possessing favorable signaling and colonization profiles interact with the numerous bacteria–fungi combinations populating the endosphere (Weston et al., 2012). According to Tuskan, 21 days after inoculating *Populus* cuttings with five phylogenetically diverse bacterial isolates representing distinct functional types, some plants outgrew controls by as much 30 percent. In these cases, he said, "We see a favorable enhancement in growth, which we had predicted based on functional assays, quorum sensing, biofilm formation, chemotaxis, etc., as well as the host genotype" (Figure WO-8). The diverse nature of these reconstructed communities suggests that the functional abilities of the constituents of the endosphere microbiota in *Populus*—and not the specific bacterial and fungal taxa—are crucial to host growth and biomass productivity, he concluded. "There's really not much to be learned [through pyrosequencing] other than phylogenetic taxonomy. We need to study individual organisms and their interaction with their host in order to gain predictive power in terms of function at both the bacterial and fungal levels."

Coevolved Metabolic Networks

Speaker Angela Douglas of Cornell University discussed the implications of animal signaling networks that evolved within the context of preexisting interactions with the microbial world (Dr. Douglas' contribution may be found on

[16] As an illustration of the importance of going beyond taxonomic definitions to understanding the genomic content of host-associated microbes, Tuskan noted that for the 21 isolates of *Pseudomonas*, there were on the order of 3,000 shared among all isolates (core genes) but more than 11,000 genes unique across isolates (the pan genome).

[17] Cell–cell communication system that allows bacteria to monitor population density and control of specific genes in a density-dependent manner (IOM, 2012).

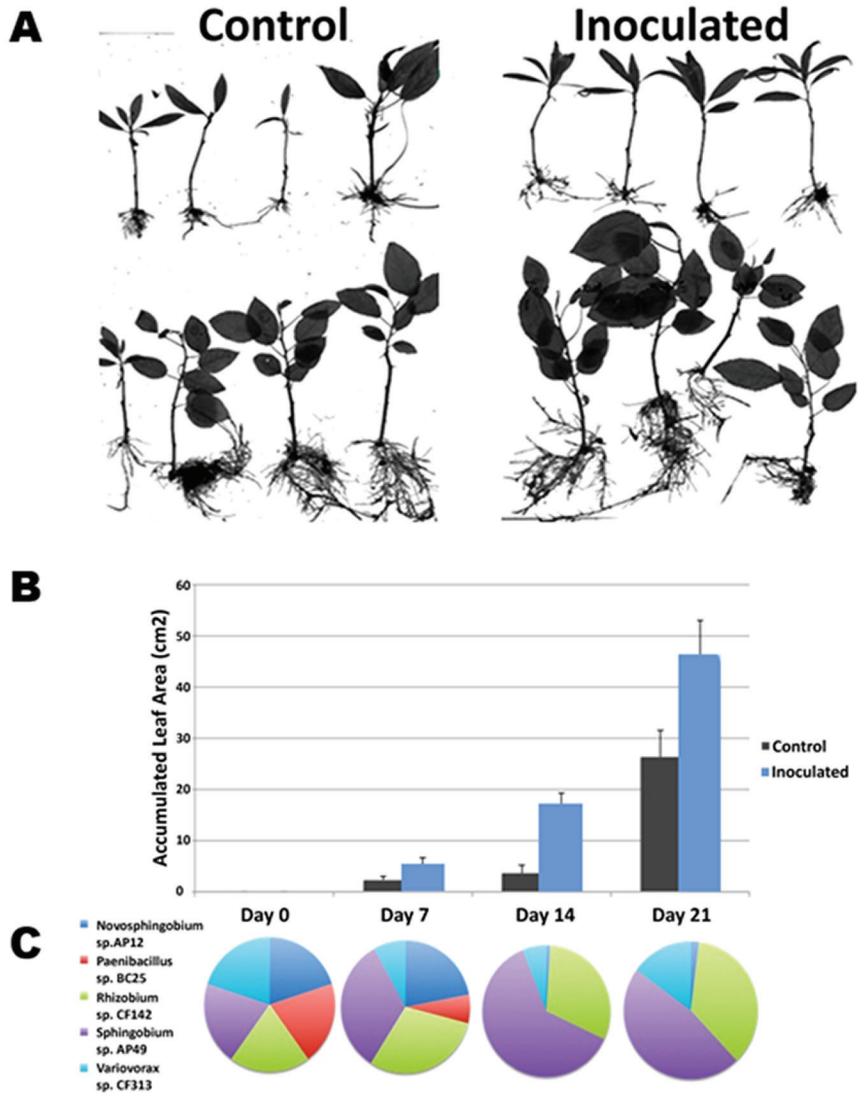


FIGURE WO-8 Constructed communities based on functional diversity. Five phylogenetically diverse bacterial isolates that each brings an alternate set of function types. The *Populus* host—both *trichocarpa* (panel A, top) and *deltoides* (panel A, bottom)—were inoculated and monitored over a 21-day period. Both species exhibited increased growth versus the control over the 21 days (panels A and B). Community composition changed over time (panel C).
SOURCE: Tuskan (2013).

pages 207-224 in Appendix A). Animals "were multiorganismal before they were multicellular" she said, and their evolution was accompanied by a "dramatic flowering of variation of new genes." Accounting for approximately 16 percent of genes in the human genome, these genes are largely associated with cell signaling and communication and are hotspots for disease-related genes (Figure WO-9) (Domazet-Loso and Tautz, 2008). Consequently all animals—including humans—may share certain fundamental principles of interaction with their resident microbiota, Douglas said.

To explore these principles, Douglas and her group make use of insect symbioses, which, she has noted "offer various clear-cut exemplars of processes

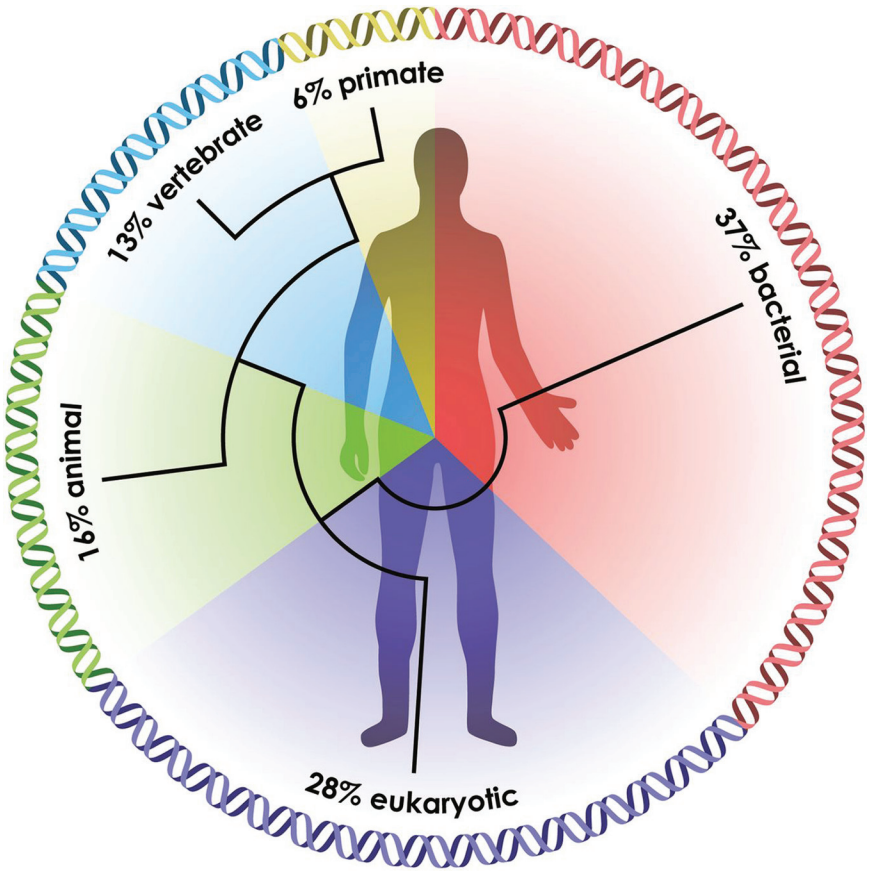


FIGURE WO-9 The ancestry of humans reflected in the genomic signature. A phylogenetic analysis of the human genes reveals the relative percentage of the genome that arose at a series of stages in biological evolution (Domazet-Loso and Tautz, 2008).
SOURCE: McFall-Ngai et al. (2013).

underlying interactions between animals and their resident microbiota," including "spectacular examples of coevolution with major consequences for the health and well-being of the animal host" (Douglas, 2011).

Her workshop presentation featured studies of two such model systems: in the fruit fly *Drosophila melanogaster* and in the pea aphid *Acyrthosiphon pisum*. Douglas observed that compared to a typical mammal, the fruit fly—like most other insects investigated—has a much less diverse microbiome (Douglas, 2011; Wong et al., 2011, 2013). Consisting of 20 to 50 taxa, it is dominated by two species of *Acetobacter* (particularly *A. pomorum*) and three species of *Lactobacillus* (particularly *L. fructivorans*). Germ-free flies achieve similar body weights compared to conventional flies, but store energy as triglycerides and glucose at much higher levels (Ridley et al., 2012, 2013). "Our interpretation of these data is that carbohydrate and energy sensing and signaling networks in the animal are clearly modulated by the microbiota," she said.

Douglas has used the pea aphid in order to explore how different bacteria may interact with the nutritional biology of its host. The pea aphid is dependant upon its bacterial endosymbiont, *Buchnera aphidicola*, for the supply of "essential amino acids"—those amino acids that contribute to protein synthesis but that the animal cannot synthesize on its own. The essential amino acids are in short supply in the aphid diet of plant sap. Housed within specialized cells known as bacteriocytes, the bacterium engages in a dynamic flow of nutrients with the surrounding cytoplasm, Douglas observed. From a survey of metabolism-related genes in *B. aphidicola*, the researchers inferred the number of reactions and metabolites involved in its exchange with aphids and used that information to characterize the "metabolic network" that connects the two organisms (Figure WO-10) (Thomas et al., 2009; MacDonald et al., 2011). Further studies (Wilson et al., 2010; Poliakov et al., 2011) have revealed the loss of *Buchnera* genes encoding reactions that are also present in the insect and the dedicated expression of the compensatory host genes in the host cells, she added. "We now have evidence of shared metabolic pathways [between aphid and bacterium] in the synthesis of 5 out of the 10 essential amino acids," Douglas reported. This metabolite exchange between animal and resident microbiota is underpinned by coevolved animal and microbial metabolic networks, a phenomenon that "may be general to many more complex types of associations," concluded Douglas.

Hibernation-Related Dynamics of a Vertebrate Gut Microbiome

Mammals found in all classes hibernate during periods of unfavorable environmental conditions, during which they experience profound changes in physiology, morphology, and behavior (Carey et al., 2003). According to speaker Hannah Carey of the University of Wisconsin, Madison, the examination of the transitions between extremes of feeding and fasting in an animal model of hibernation may

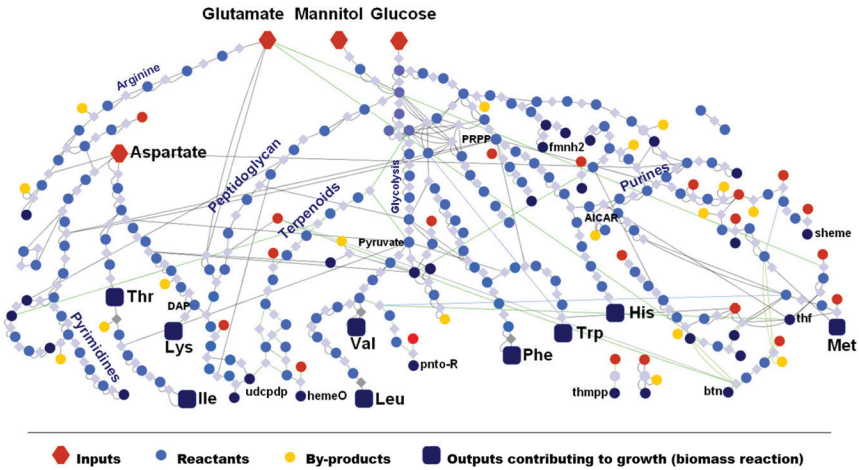


FIGURE WO-10 The metabolic network of *Buchnera aphidicola* APS1, illustrating the flow of carbon from the main precursors (red diamonds: glucose, mannitol, etc.) to essential amino acids (blue squares: Thr, Lys, etc.).
SOURCE: Thomas et al. (2009).

reveal insights into host–gut microbe dynamics (Dr. Carey's contribution may be found on pages 184-206 in Appendix A). Dietary and environmental changes experienced by animals during this annual cycle are extreme but can also be "viewed as a normal, recurring perturbation that is well tolerated by the host and its microbial symbionts" (Carey et al., 2013).

Carey's group studies the 13-lined ground squirrel *Ictidomys tridecemlineatus*, which undergoes an annual hibernation cycle (see Figure WO-11). The squirrels are lean when they emerge from hibernation in the spring and feed voraciously throughout the summer until they reach near-obesity in the fall, she said. These squirrels voluntarily stop eating as hibernation begins. Much remains to be learned about the appetite signals that control the squirrels' feeding behavior, which coincides with a shift from homeothermy (maintaining a constant, high body temperature) to heterothermy (alternating periods of high body temperature with periods of lower temperatures closer to the surrounding environment). During hibernation, the animals undergo bouts of torpor, during which metabolism is extremely slow and body temperatures dip toward the freezing point, punctuated with periods of arousal to normal (~37°C) body temperature.

Once normal digestive processes cease, the squirrel's intestinal mucosa atrophy. "The animals aren't eating, so they don't need a robust mucosa to finish digestion and undergo absorption of the food," Carey explained. However, she added, the gut must still act as a barrier to restrict bacteria and their metabolites

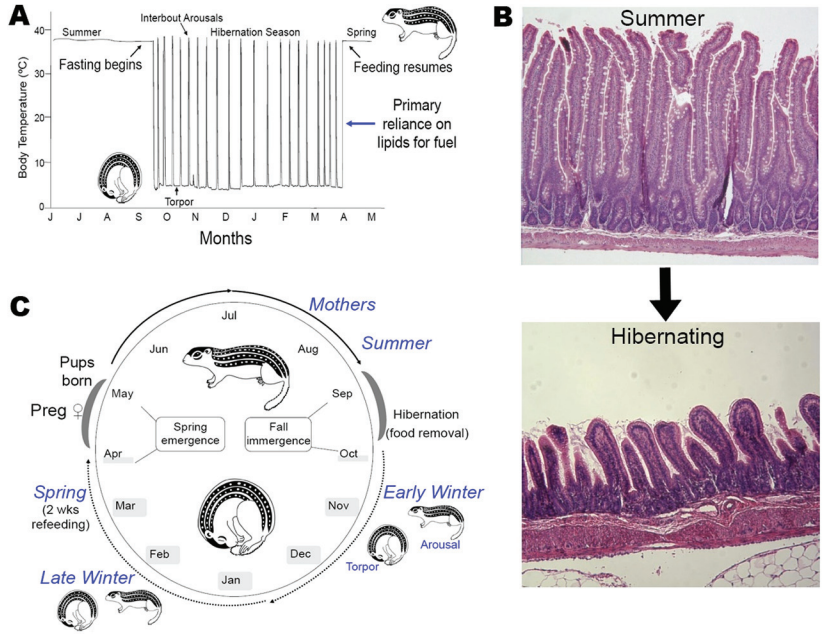


FIGURE WO-11 Annual cycles of body temperature, gut morphology, and feeding behavior. Panel A, body temperature during annual hibernation cycle. Panel B, winter fasting induces mucosal atrophy. Food processing and absorptive functions are largely absent. However, there is a continued need for barrier function and a mutualistic relationship with bacteria. Panel C, annual hibernation cycle.
SOURCE: Carey (2013).

to the lumen and thereby prevent disease. To explore how the gut microbiota responds to the extreme transitions during the annual hibernation cycle, she and her group compared the sequences of 16S rRNA genes from the microbiota occupying the squirrels' intestinal lumen during summer, early winter, late winter, and spring (Carey et al., 2013) (Figure WO-11). Their results demonstrate that intestinal microbes also follow a diet-dependent annual cycle, with different taxa dominating when the animal is feeding than when it relies on stored energy reserves during its hibernation fast.

These investigators are currently studying whether a similar pattern occurs among the microbiota of the intestinal mucosa, which is more intimately associated with the host, Carey said. They are also interested in the possible role of the microbiota in maintaining host energy and protein stores. "It has long been suggested in the literature of hibernation that protein conservation, which is absolutely important in hibernation, is helped by nitrogen recycling due to gut

microbes," Carey observed, "but there is very little data that demonstrate that directly."

Several physiological features of hibernation have implications for gut–microbiome interactions, Carey noted. In the squirrel, as well as in other animals, the gut becomes more permeable during hibernation, which can increase translocation of bacterial products across the epithelial surface, where they may cause widespread deleterious immune effects (Carey, 1990; Carey et al., 1992). "There is a pretty significant remodeling of the intestinal immune system during hibernation, perhaps in response to this leakier gut . . . [or to] moderate levels of bacterial product movement across the epithelium," she reported. These changes—including an increase in the numbers of intra-epithelial lymphocytes considered to be the "first line of defense" against bacterial pathogens; an elevation in anti-inflammatory cytokines; and an apparent increase in the expression of proteins that maintain tight junctions between the cells of the intestinal epithelium—may represent a compensatory response to limit the enhanced permeability that accompanies winter fasting, she observed.

Carey concluded her presentation with the observation that many intriguing questions remain, and "ultimately we would all like to . . . get beyond the species and look at the actual functional output of these hibernator microbiomes. Metagenomic analyses, coupled with mRNA, protein, and metabolite profiling, will be required to illuminate how metabolic pathways of the community as a whole, and within specific bacterial species, are modified by winter fasting and the return of dietary substrates in the spring" (Carey et al., 2013).

An Ecological View of Health and Disease

A central lesson of ecology is that perturbations often ripple through an ecosystem, leading to unexpected outcomes.

—Lemon et al. (2012)

The human body is host to many, many, highly complex microbial ecosystems. Distinct microbial populations colonize different body sites, such as the mouth, skin, vagina, and gut (Human Microbiome Project Consortium, 2012) (Figure WO-12). At the level of bacterial species and strains, the exact mix present on any given individual is as "unique as a fingerprint"—reflecting the influence of factors ranging from genetics and life history to diet (Spor et al., 2011; Human Microbiome Project Consortium, 2012; Yatsunenko et al., 2012).

The structure of our microbiota reflects our long history of evolution within a microbial world. Of the estimated 100 bacterial phyla[18] on Earth, only 4 dominate the human microbiota (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria). When compared to those found in other vertebrates, the structure of the

[18] Groups of organisms ranking above a class and below a kingdom.

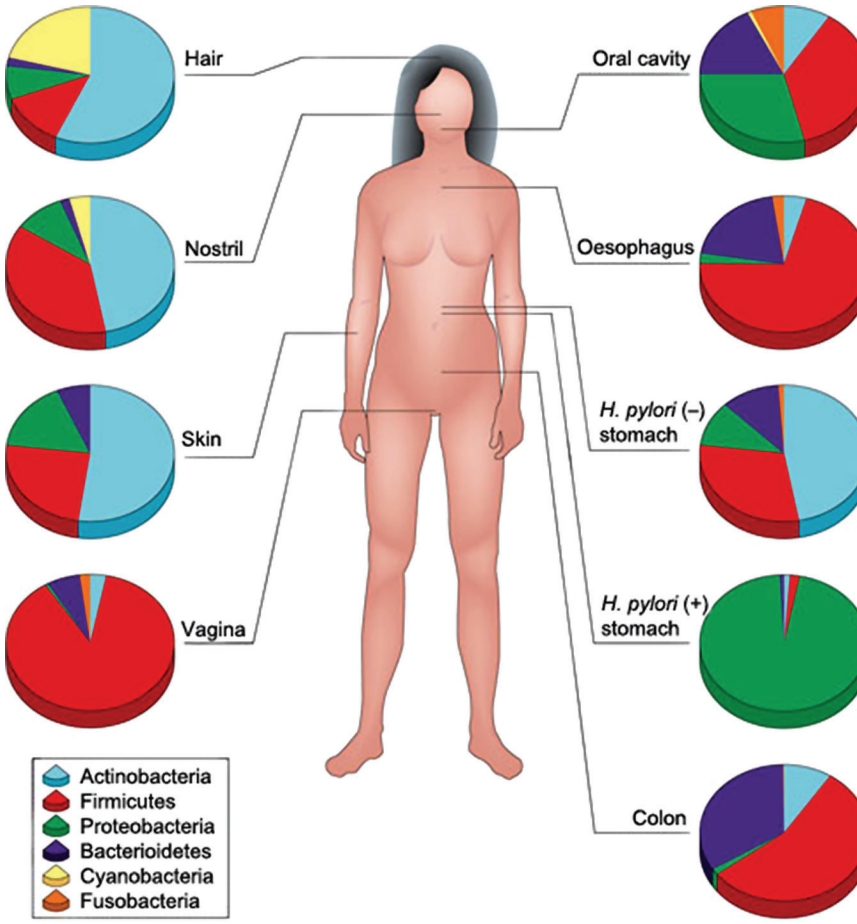


FIGURE WO-12 Compositional differences in the microbiota by anatomical site. High-throughput sequencing has revealed substantial intraindividual microbiota variation at different anatomical sites, and interindividual variation at the same anatomical sites. However, higher-level (for example at the level of phyla) taxonomic features display temporal (longitudinal) stability in individuals at specific anatomical sites. Such site-specific differences and the observed conservation between human hosts provide an important framework to determine the biological and pathological significance of a particular microbiota composition. The figure indicates the relative proportion of sequences determined at the taxonomic phylum level at eight anatomical sites. Certain features, such as the presence (+) or absence (–) of *Helicobacter pylori*, can lead to permanent and marked perturbations in community composition.
SOURCE: Cho and Blaser (2012).

human microbiota is similar but distinct, suggesting that host species coevolve with, and adapt to, their microbial inhabitants (Ley et al., 2006; Dethlefsen et al., 2007).

Despite the variation of community structure between individuals, each human microbiome—the collection of genes encoded by members of a microbiota—exhibits shared functional attributes that influence host physiology, immunity, and metabolism (Human Microbiome Project Consortium, 2012). A recent study of the human gut revealed more than 3.3 million microbial genes—about 150 times as many genes than are carried in the human genome (Qin et al., 2010).[19] Many of these genes encode biosynthetic and metabolic enzymes that the host is lacking, "thereby greatly expanding the host's own biochemical and metabolic ability" (Sommer and Bäckhed, 2013). In addition to nutrition and digestion, services provided by our microbiota include detoxification of xenobiotics;[20] promotion of growth and differentiation of mucosal tissues; stimulation and shaping of innate and adaptive immune systems; and defense against pathogen invasion through inhibition and competition (Round and Mazmanian, 2009; Hooper et al., 2012; Nicholson et al., 2012).

The human microbiome may have systemic as well as local effects on host physiology. According to McFall-Ngai et al. (2013), our microbiota contribute to as much as one-third of the metabolites[21] found in our bloodstream (McFall-Ngai, 2013). Distributed throughout the body by the circulatory system, microbial metabolites have the potential to influence distant microbiota and organs (Nicholson et al., 2012). Gut bacteria, for example, play a key role in the conversion of choline—a nutrient found in high-fat foods—to TMAO (trimethylamine N-oxide), and elevated blood levels of this metabolite have been linked to increased plaque development in arteries and an elevated risk of heart disease (Tang et al., 2013).

"Transplantation" of entire microbial communities from one host to another has also revealed tantalizing links between the microbial ecology of the gut and states of health and disease. Germ-free mice[22] that receive, via fecal transplantation,[23] the gut microbiota of obese mice gain twice as much body fat

[19] These microbial genes were detected in fecal samples obtained from 124 individuals and suggest the presence of 1,000–1,150 prevalent bacterial species. Each individual gut harbored at least 160 species (Qin et al., 2012).

[20] A chemical compound (such as a drug, pesticide, or carcinogen) that is foreign to a living organism.

[21] Metabolites are small molecules produced during the metabolism of food or other compounds. Microbial metabolites such as short chain fatty acids (SCFAs), bile acids, choline metabolites, vitamins, and lipids (including liposaccharide [LPS], peptidoglycan [PG], and triglycerides) play critical roles in shuttling information between host and microbial cells (Nicholson et al., 2012).

[22] Germ-free animals are animals that have no microorganisms living in or on them. Such animals are raised within germ-free isolators in order to control their exposure to viral, bacterial, and parasitic agents.

[23] The engraftment of gut microbiota (derived from feces) of a healthy human donor into a recipient (Borody and Khoruts, 2012).

as those that receive communities from lean mice—a phenotype thought to result from an enhanced capacity of the "obese" microbiota to extract energy from the host's diet (Turnbaugh et al., 2006, 2007). In humans, fecal transplantations have resolved otherwise recurrent and potentially fatal cases of severe diarrhea caused by *Clostridium difficile* infections. The success of this approach has been attributed to the "restoration" of a healthy gut microbial ecosystem that can resist displacement by pathogens such as *C. difficile* (Borody and Khoruts, 2012). The possibility that health may be the product of community-level microbial processes and traits—including stability, resistance, and resilience[24]—only underscores the importance of adopting an ecological perspective to illuminate the complex interactions and interrelationships between a host and its microbiome (Relman, 2012).

Current Studies of the Human Microbiome

Researchers have long suspected that microbes "indigenous" to the human body may be helpful or harmful to the host depending upon the ecological context (see Dubos et al., 1965; Savage, 1977; Mackowiak, 1982). Over the past 7 years, two high-profile projects—the National Institutes of Health's (NIH's) Human Microbiome Project and the European Union's Metagenomics of the Human Intestinal Tract (metaHIT) (see Box WO-1)—have largely focused on identifying the types, genetic potential, and functions of bacteria associated with specific sites on the human body (Bäckhed et al., 2012). These efforts have produced a variety of important data sets, computational tools, and study methods and significantly advanced our understanding of the microbiome of "healthy"[25] adult humans from developed countries (Duskko Ehrlich et al., 2010; Procter, 2011). However, "the composition and functional characteristics of a healthy microbiome remain to be precisely defined" (Bäckhed et al., 2012).

As noted by many of this workshop's participants, investigators are just beginning to explore the ecological contexts in states of health and disease. As summarized in this section, researchers at the workshop described efforts to build upon initial characterizations of the structure and function of the microbiome by mapping the spatial and temporal dynamics of host-associated microbiota; elucidating the means by which host genetics, life history, and environmental exposures may influence community structure and function; and expanding our view of our microbiota to include fungi and viruses. Workshop presentations on the inflammatory bowel diseases (IBDs) underscore the complexity of interactions between the host and microbiome, and environmental factors that may underlie complex and chronic disease states.

[24] The capacity of a system to recover from disruption.

[25] The Human Microbiome Project studied adult subjects lacking evidence of disease (including the absence of inflammatory diseases). For an extensive list of exclusion criteria see the Human Microbiome Project Consortium (2012).

BOX WO-1
Human Microbiome Research Projects

NIH: The Human Microbiome Project (HMP) is a 5-year, $157 million undertaking launched by the NIH in 2007 (Buchen, 2010) to sequence the microbial communities of several hundred people in order to define commonalities and patterns, and to determine a core microbiome if one exists. The first stage of the project was focused on the metagenomes of the human skin, nose, mouth, gut, and vagina of 300 healthy volunteers and has since expanded, sampling additional body sites. Beyond describing the human microbiota, the HMP seeks to understand aspects of communities such as function, including whether alterations to the microbiome can be correlated to changes in human health. Another project goal is to sequence 3,000 genomes from both cultured and uncultured bacteria, plus viral and small eukaryotic microbes isolated from human body sites.

Metagenomics of the Human Intestinal Tract (MetaHIT) is a project financed by the European Commission that seeks to establish associations between the genes of the human intestinal microbiota and health and disease. Launched in 2008, this 5-year, 21.2M€ project gathers 13 partners from academia and industry, from a total of eight countries (China, Denmark, France, Germany, Italy, Netherlands, Spain, and the United Kingdom). Focused on two disorders of increasing importance in Europe, inflammatory bowel disease (IBD) and obesity, MetaHIT has (1) established an extensive reference catalog of microbial genes present in the human intestine and bioinformatics tools to store, organize, and interpret this information; (2) developed tools to determine which genes of the reference catalog are present in different individuals and at what frequency; (3) gathered cohorts of individuals, some sick and some healthy; (4) determined for most which genes they carry; and (5) developed methods to study the function of bacterial genes associated with disease aiming to understand the underlying mechanisms and host–microbe interactions.

SOURCES: http://commonfund.nih.gov/index.aspx; http://www.hmpdacc.org/reference_genomes/reference_genomes.php; The Human Microbiome Jumpstart Reference Strains Consortium, 2010; Robinson et al., 2010; and http://www.metahit.eu. (all URLs accessed January 9, 2014).

Formation and Maintenance of the Human Gut Microbiome

In a dynamic process that appears to be strongly influenced by their environment and genetic makeup, humans—like other organisms studied and reported on in this workshop—develop a resident gut microbiome shortly after birth (Figure WO-13) (Domínguez-Bello et al., 2011; Clemente et al., 2012). As previously noted, the primary microbial community inoculum at birth is the mother's microbiome.[26] Following vaginal delivery, an infant's microbiome

[26] Recent research suggests that the microbial communities of an infant's mecomium may also play a role in microbial colonization during the first month of life (Moles et al., 2013).

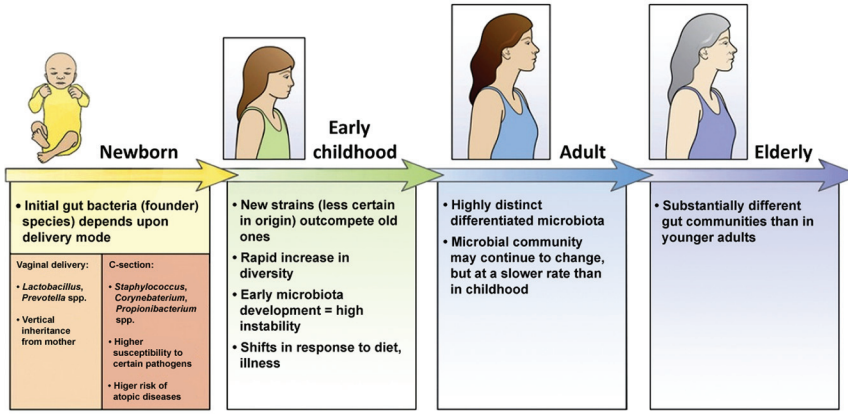


FIGURE WO-13 The development of the microbiota from the first inoculum as an infant through continued change, modified by diet, genetics, and environment, throughout life. SOURCE: Domínguez-Bello et al. (2011).

initially resembles its mother's vaginal microbiome, with Lactobacilli dominating in the infant gut. Babies born by Caesarean section are initially colonized by species associated with skin and dominated by taxa including *Staphylococcus* and *Propionibacterium spp.*—a composition that persists for several months following birth (Domínguez-Bello et al., 2010). Initial assembly of the infant gut microbiome may have implications for the early and long-term health of the host. The infant microbiome provides resistance to pathogen invasion, furnishes developmental cues, and influences immunological functions. Recent research has demonstrated that colonization during the neonatal period strongly influences mucosal immune development (reviewed in Costello et al., 2012; Lozupone et al., 2012).

Just how differences in microbial community composition in the early stages of life may influence disease susceptibility at later stages of life is not well understood. Investigators have recently described intriguing associations between the composition of the microbiota of children born by Caesarean section and their subsequent development of asthma (Couzin-Frankel, 2010). These results suggest that early colonization events might be deterministic—imparting substantial and lasting effects on the immune system—irrespective of the composition of the mature microbiome. The ways in which factors such as the environment (including diet) and life events (illness, puberty, and pregnancy) influence the community composition of the human gut from birth to old age is an area of active investigation (Domínguez-Bello et al., 2011; Lozupone et al., 2012; Maynard et al., 2012).

Following initial colonization events, the gut microbiota of human adults undergoes consecutive changes in composition and function, increasing in diversity and stability until a relatively constant adult gut community is established (Lozupone et al., 2012). The microbiota of "healthy" adults exists in dynamic

equilibrium; that is, these communities of microorganisms are generally stable, but not static, over space and time. Time series data suggest indigenous microbial communities, like other ecosystems, progress toward ecological climax,[27] after which their composition rarely varies in the absence of disturbances like changes in diet, antibiotic therapy, or pathogen invasion (Clemente et al., 2012). The host maintains this equilibrium—or homeostasis—through its mechanical, chemical, and immunological control of the microbiota (Eberl, 2010).

Certain life events, such as pregnancy, are known to dramatically shift the composition and function of an individual's microbiota. By the third trimester, a healthy pregnant woman's gut microbiota has changed markedly, perhaps in response to immunological changes known to inhibit rejection of the fetus. In its structure and function, the late-pregnancy microbiome resembles that of an individual who exhibits weight gain and inflammation associated with type 2 diabetes. In the context of late pregnancy, what might otherwise be termed dysbiosis is, in reality, "healthy," because it promotes storage of extra calories in adipose tissues, and thereby, contributes to the late growth spurt of the fetus in the 6–8 weeks before birth (Koren et al., 2012).

The workshop presentations summarized below provide insights on the interactive course of host–microbiota establishment in mammals and the ensuing dynamic equilibrium in which each partner influences the other—a process that may profoundly influence human health.

Mother's Milk and the Infant Microbiome

"Human milk is the only food that evolved to make humans healthy," according to speaker David Mills of the University of California, Davis, as he introduced the workshop to this "very complex fluid that is remarkably understudied in our opinion" (Dr. Mills' contribution may be found on pages 356-382 in Appendix A). Carbohydrates, protein, lipids, and macro- and micronutrients contained in human milk nourish the infant, he noted, while other elements, including immunoglobulins,[28] lysozyme, lactoferrin, and free fatty acids, are known to shape the microbiota of the infant gut, mostly by killing or removing organisms (Sela and Mills, 2010).

After lactose and lipids, the most prominent component of human milk is a collection of large oligosaccharides—free soluble carbohydrates consisting mostly of 3 to 15 monosaccharide units (although larger structures exist) linked through a variety of glycosidic bonds[29] (Garrido et al., 2012). Human milk also

[27] Community in an equilibrium composition (Gonzales et al., 2011).

[28] Also known as antibodies, immunoglobulins are any of numerous proteins produced by B lymphocytes in response to the presence of foreign molecules.

[29] Although these linkages could result in a vast array of oligosaccharide species, only about 200 different species are present in human breast milk. According to Mills, this suggests that "nature selected the smaller amount of oligosaccharides that are present."

contains glycoconjugates of oligosaccharides, attached to lipid and protein molecules, Mills reported. Because infants do not possess the metabolic or enzymatic capacity to degrade oligosaccharides, he said, investigators have long sought to understand its contribution to human fitness. Various hypotheses, including contributions to immune system and neurological development, and "pathogen deflection" by providing pathogen binding sites that resemble the intestinal epithelium, have been advanced to explain the presence of these oligosaccharides in human milk. Mills and coworkers have explored the possibility that the oligosaccharides in human breast milk enrich specific populations of microorganisms in the infant gut leading to early colonization events in the newborn infant that are beneficial to the infant.

Mills' group focuses on bifidobacteria, a universal member of the infant microbiome and a common probiotic in dairy foods. "Complex milk glycans enhance bifidobacteria colonization," he stated. "All of the data that we have generated so far seems to support a rationale for why that would be." For example, the researchers determined that one of the bifidobacteria species and subspecies routinely found in infants, *Bifidobacterium longum* subspecies *infantis* (henceforth *B. infantis*), preferentially consumes the specific small-mass oligosaccharides that are most abundant in human milk, while its close relative in the adult gut, *B. longum* subspecies *longum*, does not—a preference that can be traced to unique genomic features of *B. infantis* (Sela and Mills, 2010; Zivkovic et al., 2011; Garrido et al., 2012). This preferential dietary requirement apparently provides a competitive advantage to *B. infantis* in successfully colonizing the infant gut in the days to weeks after birth.

Mills and coworkers hope to apply their understanding of this host–microbe interaction to improve nutrition for preterm infants, who frequently suffer dysbiosis. According to Mills, providing preterm infants with human milk (instead of formula lacking oligosaccharides) and *B. infantis* as a probiotic[30] enabled the bacteria to become established in the gut. The group is now trying to "mine" similar oligosaccharides from bovine milk—in the form of whey, a waste product of cheese production—that might be used to supplement formula in combination with *B. infantis*.

Role of the Mammalian Immune System in Host–Microbe Equilibria

An ecological understanding of host–microbe relationships invites a reconsideration of the role of the host immune system in states of health and disease. Speaker Gérard Eberl, of the Institut Pasteur, has challenged the long-accepted view of the immune system as a discriminating killer of pathogens and proposed instead that it "does not react to combat evil, but merely shapes the microbial

[30] Live microorganisms that confer a health benefit on the host.

environment to allow the organism to live with the microbes" (Eberl, 2010) (Dr. Eberl's contribution may be found on pages 224-248 in Appendix A).

In his presentation to the workshop, Eberl characterized the mammalian immune system as an important tool, shaped over the course of coevolution with microbial communities, over many thousands of years, to maintain equilibrium in their shared ecosystem, the "superorganism." Microbial species interact with the host along a continuum that ranges from mutualism to pathogenicity, he argued, and their position on this spectrum can shift, depending on the context in which the interaction takes place (see Figure WO-14). For example, he said, "a mutualist in the gut can be good one day, but if you go into surgery . . . [and] it goes systemic, it can kill you."

A healthy immune response is both plastic and diverse, Eberl observed, reflecting the complexity of the microbial world and the necessity to establish a dynamic equilibrium through adaptation; if it is too weak, the microbiota overgrows and invades host tissues; if it is too strong, the host suffers inflammatory "collateral damage." An example of the latter has been observed in a *Drosophila* mutant lacking regulation of a key inflammatory pathway such that it kills most species of endogenous microbes—except for a single toxic strain that eventually kills the fly (Ryu et al., 2008). This same pathway is also associated with inflammatory bowel disease in man.

A growing body of evidence suggests that the gut microbiota plays a key role in establishing this adaptive ability in the mammalian immune system, according to Eberl (Ohnmacht et al., 2011). However, his group has found evidence to suggest that in mammals, the immune system also acts early in development to influence the composition of the microbiota. They discovered that a key initiator of inflammation known as RORγt[31] is first expressed in the fetal gut, prior to any encounter with microbes (Eberl, 2012). Eberl observed that while many animals develop immune cells in response to microbial exposure, mammals are unique in preparing for these colonization events during fetal development. "Only mammals have lymph nodes," he said, an adaptation that has enabled them to anticipate their inevitable colonization by microorganisms.

The developmental program that leads to the formation of lymph nodes—as well as specialized tissues called Peyer's patches in the fetal gut—replicates the inflammatory process of adults. The cells that induce the development of lymph nodes, so called Lymphoid Tissue inducer (LTi) cells, produce high levels of the inflammatory cytokines interleukin (IL)-17 and IL-22 and are also found scattered in the intestine before and after birth, Eberl reported. Although the intestine has yet to be exposed to microbes, he said, "we think that it starts at very high levels of IL-17 and IL-22 because again it is going to be colonized, and we think

[31] The nuclear hormone receptor retinoid-related orphan receptor gamma-t (RORγt) induces a pro-inflammatory program in lymphoid cells, culminating in the expression of interleukin-6 (IL-6), IL-17, IL-22, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor (Ohnmacht et al., 2011).

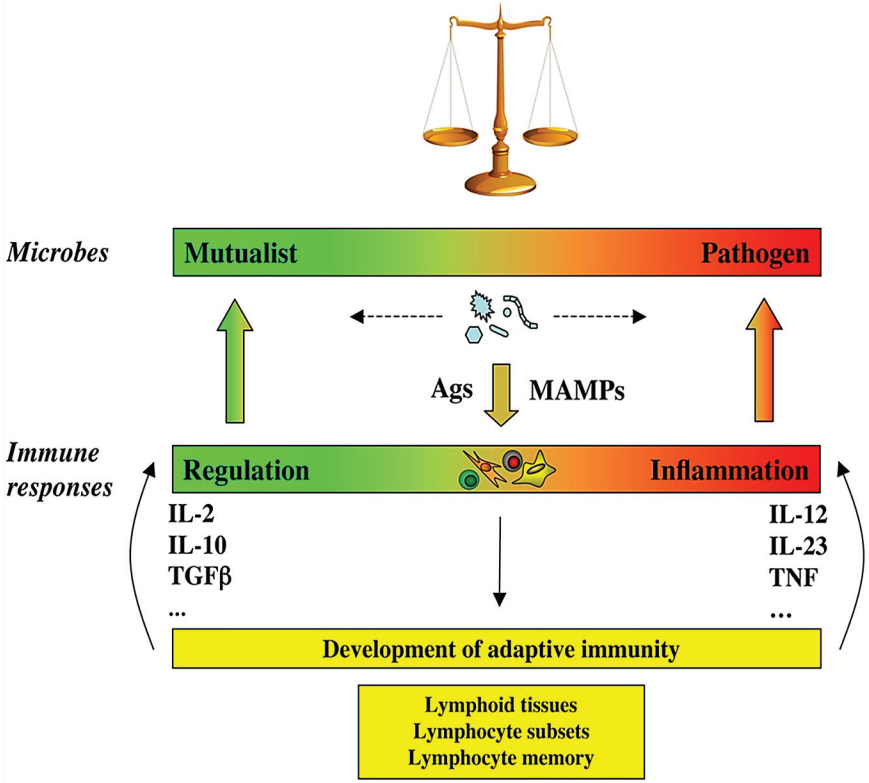


FIGURE WO-14 The continuum of microbial states and immune responses: a dynamic equilibrium. Microbes, including members of the symbiotic microbiota, are not inherently mutualistic or pathogen, but navigate between shades of mutualism and parasitism. Facing the microbes, the immune system is not designed to discriminate between mutualists or pathogens, but merely to react to signals, including MAMPs and antigens (Ags). The nature of the immune response is not purely regulatory or inflammatory, but more generally adjusts to the nature of the trigger it faces, like a spring that is pulled by the intensity of the microbial challenge. Furthermore, the immune system has the capacity to evolve when challenged, through generation of different types of lymphocyte subsets such as Th1, Th2, Th17, Treg, Th22 cells, and follicular T helper cells, and generation of memory lymphocytes and lymphoid tissues. This adds another level of adaptability to the immune system and provides it with the necessary flexibility to maintain homeostasis of the superorganism.
NOTE: MAMPs, microbe-associated molecular patterns.
SOURCE: Eberl (2010).

this is a very strong selective pressure on those microbes, which will be able to productively colonize the intestine." Shortly before weaning, IL-17 and IL-22 levels drop dramatically in response to negative feedback from the newly established microbiota, only to rise in the event of infection or trauma. He noted that the period of weaning appears "extremely important, as it is a period in which the immune system is still developing, being shaped by the microbiota, with consequences that can be measured later during adult life."

The intestinal mucosal immune system Mechanisms of host–microbe homeostasis have been extensively studied at the mucosal surfaces of the gut, which are constantly exposed to a diverse and dynamic community of microorganisms and form an interactive barrier that defends the body against invasion by commensal and pathogenic microorganisms through a variety of physical, chemical, and immune-mediated mechanisms (Sansonetti, 2004). At equilibrium, commensal bacteria contribute to barrier function directly, by competitively excluding pathogens from the host-secreted and nutrient-rich mucus. Commensals may also operate indirectly, by expressing antimicrobial peptides (AMPs) and pro-inflammatory molecules that modulate the hosts' immune response, as well as by inducing regulatory immune responses (Sansonetti, 2004; Kamada et al., 2013). These highly specialized barrier defenses restrict these microbial communities to the intestinal lumen, where nutrient uptake occurs. Immune system activity also minimizes the incidence of systemic inflammation that would normally occur in the presence of so many bacterial products. Should these tiered host defenses fail, microorganisms might be able to exploit the host for additional resources or trigger damaging systemic inflammatory responses (see Dethlefsen et al., 2007; Costello et al., 2012; Hooper et al., 2012; Maynard et al., 2012). Environmental or genetic factors may also disrupt homeostasis, leading to dysbiosis and a resulting loss of the protective functions of commensal microbes (Figure WO-15).

Indigenous Microbes and the Ecology of Chronic Diseases

In his keynote remarks, Martin Blaser, of New York University, acknowledged the "world of diversity" that has been revealed by early studies of our microbiomes and emphasized the ancient origins of our resident microbial communities (Dr. Blaser's contribution may be found on pages 110-153 in Appendix A). As a result of our long, shared, evolutionary history, he observed, these microbial associations are largely benign or beneficial (Blaser, 2006). Many of our indigenous microbes are commonly observed in the majority of human populations that have been studied to date and persist throughout our lifetimes. These enduring relationships are mediated by a variety of signaling mechanisms—through which, according to Blaser, our microbiota "know how to talk to the host and to receive signals back, to engage in a very lively conversation" that fosters homeostasis and community stability. Although robust and resilient, the host–microbiota

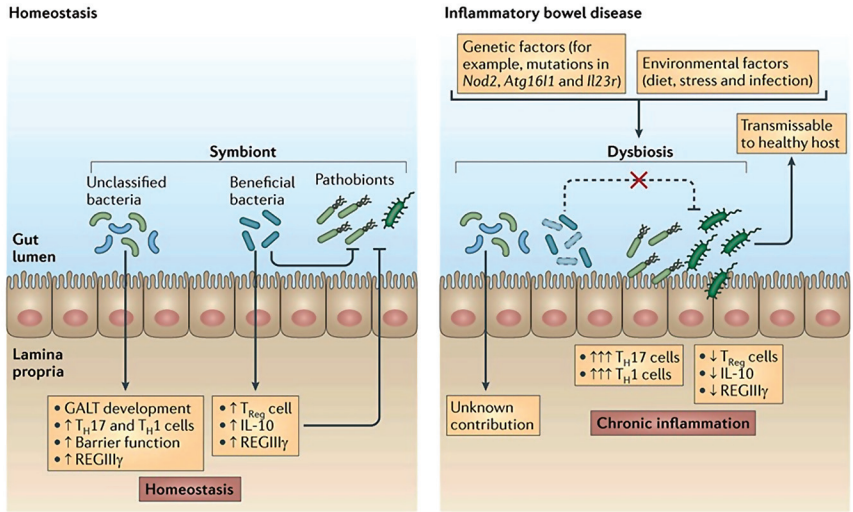


FIGURE WO-15 The intestinal mucosa in states of health and disease. During homeostasis (left), the gut microbiome has important roles in the development of intestinal immunity. Beneficial subsets of commensal bacteria tend to have anti-inflammatory activities. Pathogens can be directly suppressed by beneficial commensal bacteria, partly through the induction of regulatory immune responses, involving regulatory T (T_{reg}) cells, interleukin-10 (IL-10) and regenerating islet-derived protein 3-gamma (REGIIIγ). In inflammatory bowel disease (IBD) (right), a combination of genetic factors and environmental factors are thought to result in disruption of the microbial community structure (dysbiosis), leading to chronic inflammation.
SOURCE: Figure and adapted text from Kamada et al. (2013).

equilibrium can be perturbed, he said. Such perturbations, and their apparent relationship with a range of chronic diseases, were the focus of his presentation.

Helicobacter pylori

Blaser began his presentation with the evolving story of *Helicobacter pylori*—a bacterium indigenous to the human stomach. In 1984, Dr. Barry Marshall, an Australian physician, demonstrated that the presence of *H. pylori* in the human stomach could be associated with chronic and acute stomach ailments (in this case, acute but self-limited gastritis). In a demonstration of the causal link between bacterial infection of the stomach and the development of gastritis, Marshall ingested a solution teeming with *H. pylori* organisms and later developed acute symptomatic gastritis. This act attracted substantial attention and was one factor that overturned decades of medical dogma, asserting that

microorganisms could not survive in the stomach and attributing the chronic, "noncommunicable" condition of peptic ulcer disease to stress and lifestyle factors. Marshall and his collaborator Robin Warren were jointly awarded the 2005 Nobel Prize in Physiology or Medicine for the discovery of *H. pylori*, its linkage to chronic gastritis, and effective treatments for peptic ulcers through the treatment of *H. pylori* infection (Pincock, 2005). Marshall and Warren demonstrated that patients with ulcers could be successfully treated with antibiotics to eliminate *H. pylori* and markedly reduce the recurrence rates of ulcers in these patients.

Blaser noted that for greater than 90 percent of individuals, *H. pylori* causes a persistent, asymptomatic infection that is acquired early in life and remains "a 'silent' infection" throughout the individual's lifetime. Underlying this largely peaceful coexistence is an ancient and coevolved relationship—extending back at least 100,000 years—during which time *H. pylori* has become intertwined with the physiology of a healthy host and able to survive in the highly acidic and turbulent gastric environment (Atherton and Blaser, 2009).

H. pylori has been a ubiquitous colonist present in the stomachs of people in every part of the world. Over the course of the 20th century, and coincident with the rise of practices that may reduce *H. pylori* acquisition and persistence, including smaller family size, better nutrition, cleaner water, and widespread use of antibiotics (Blaser et al., 2008), the bacterium all but disappeared from the stomachs of people in developed countries, stated Blaser. Within the same time period and populations, gastric cancer rates have declined dramatically (Howson et al., 1986). The strength of this association is such that *H. pylori* infection in adulthood is considered to be the leading risk factor for gastric cancer worldwide (Forman et al., 1994; Peek and Blaser, 2002). Concurrently, rates for other types of cancer—including cancers of the gastroesophageal junction (GEJ)[32]—have steadily increased over the past half-century. The incidence of esophageal adenocarcinoma, the fastest-rising cancer in the United States and other developed countries, has increased six-fold over the course of the past quarter century. The inverse association between the presence of *H. pylori* and the development of this cancer and its premalignant lesions—as now shown in multiple studies—suggests that stomach colonization with *H. pylori* early in life may confer a protective effect (Chow et al., 1998; Corley et al., 2008; Islami and Kamangar, 2008).

Comparisons of the *H. pylori*–colonized "ancient" stomach to that of the "postmodern" *H. pylori*–free stomach have identified several key physiological differences with potential health consequences (Figure WO-16) (Blaser and Atherton, 2004; Blaser et al., 2008; Blaser and Falkow, 2009). Blaser observed that individuals lacking *H. pylori* were at increased risk of developing asthma in childhood (Chen and Blaser, 2007, 2008; Reibman et al., 2008; Arnold et al., 2011). This is but one of many examples of the influences of the gut microbiome. He also noted inverse correlations between the presence of *H. pylori* and risks for

[32] The lower part of the esophagus that connects to the stomach.

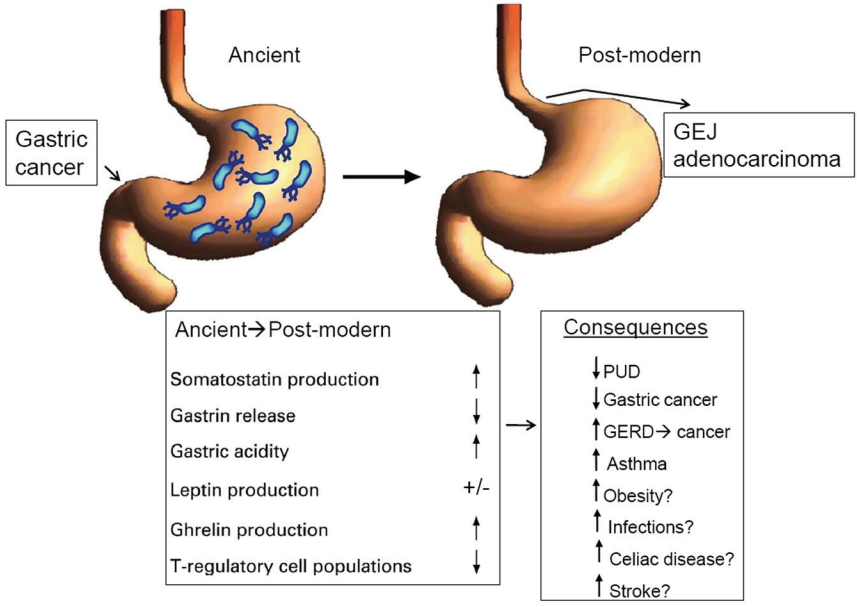


FIGURE WO-16 Physiological differences observed between the ancient (*H. pylori*–colonized) stomach and the postmodern (*H. pylori*–free) stomach. Leptin and ghrelin are gastric hormones that have a role in energy homeostasis; in the stomach, the hormone somatostatin inhibits the secretion of gastrin.
NOTE: GEJ, gastroesophageal junction; GERD, gastric esophageal reflux disease; PUD, peptic ulcer disease.
SOURCE: Adapted from Blaser et al. (2008).

obesity, celiac disease, stroke, and even tuberculosis reactivation (Blaser et al., 2008; Perry et al., 2010; Cox and Blaser, 2013).

The apparent influence of *H. pylori* on human physiology may be associated with a protein called CagA. The bacterium injects CagA into the epithelial cells of the human stomach, where it is phosphorylated and subsequently interacts with several major signal-transduction pathways that affect host cell morphology, growth, and programmed cell death (apoptosis) (Blaser and Atherton, 2004). Blaser observed that, given these wide-ranging effects, the relationship between *H. pylori* and disease is not likely to be simple. Just as most people infected with *H. pylori* never develop gastric cancer, "there are obviously other risk factors [for gastric cancer] . . . involving host responses, cofactors, and aging as well," he said. "It's a complex biology." Nevertheless, Blaser stated, "I believe that the evidence shows that *Helicobacter* is mostly beneficial early in life and is mostly harmful late in life. . . . The consequence of that is that some years from now doctors . . . [could] be giving *Helicobacter pylori* back to children to replace their ancient microbe that

has been lost, and then at some age—30, 40, 50—eradicate it, so as to have the early-life benefit and not the late-in-life cost."

Disappearing Microbiota

According to the "disappearing microbiota" hypothesis, *H. pylori* may be an indicator organism for disruptions to other ancient host–microbe associations (Blaser, 2006; Blaser and Falkow, 2009). "Changing human ecology, beginning in the late 19th century, has dramatically altered the transmission and maintenance of our indigenous microbiota," Blaser observed. The resulting shifts in microbial community composition may influence human physiology, which in turn affects risk for various diseases. "The loss of ancestral bacteria that usually are acquired early in life is especially important, because it affects a developmentally critical stage," he added.

Accumulating evidence suggests that the human gut microbiota becomes established during the first 3 years of life and is shaped by a variety of factors, including host genetics, diet, and other environmental exposures (Lozupone et al., 2012; Yatsunenko et al., 2012). Blaser highlighted recent research that suggests that this pattern of community development is consistent across distinct cultural traditions—Amerindians in Venezuela, Malawians in Africa, and Americans in the United States (Figure WO-17). Compared to adults in Venezuela and Africa, the gut microbiota of American adults, have less phylogenetic diversity. Blaser noted that this difference may reflect the different diets of these populations and has led some to speculate that factors related to "Westernization" may be altering the bacterial diversity landscape of humans in developed nations (Yatsunenko et al., 2012).

"Maternal status is very important for the composition of the resident microbiota of the next generation," Blaser observed. Mothers transmit their microbiota to their children in a variety of ways, beginning with organisms acquired during passage through the birth canal and continuing through close contact and breast-feeding (Cho and Blaser, 2012). Several factors associated with modern life are likely to have altered this process—including maternal exposure to antiseptics and antibiotics, changes in the maternal diet, cesarean section, bathing, and bottle-feeding—but none, perhaps, as profoundly as antibiotic treatment during the child's early life, observed Blaser (Figure WO-18). In the United States, five out of the eight most frequently prescribed medications for children are antibiotics (Chai et al., 2012). Furthermore, according to Blaser's estimates, the average American child under 2 years of age currently receives more than one course of antibiotics per year.

Antibiotic therapy and obesity Suspecting that the use of "drugs as powerful as antibiotics must have a cost," Blaser is exploring the connections between early-life exposure to antibiotics and the rising epidemic of human obesity. He

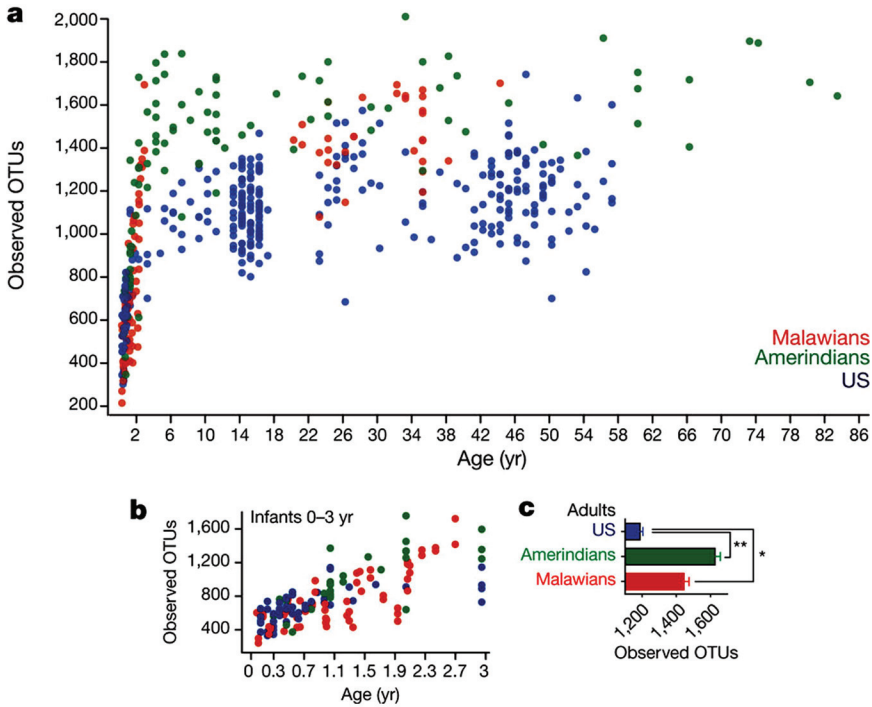


FIGURE WO-17 Bacterial diversity increases with age in populations from distinct cultural traditions. Panel A shows the number of observed operational taxonomic units (OTUs) sharing ≥97 percent nucleotide sequence identity plotted against age for all subjects; Panel B shows the number of OTUs during the first 3 years of life; and Panel C shows the number of OTUs for adults. Mean ± s.e.m. are shown in Panel C. *$P<0.05$, **$P<0.005$ (ANOVA with Bonferroni post-hoc test).
NOTE: Taxonomic level of sampling that is similar to the species level, which is more difficult to calibrate. OTU is used to describe variations in 16S ribosomal RNA sequences. Dissimilarity of <1 percent has commonly been used to define an OTU, but <3 percent (similarity ≥97 percent) and <5 percent have also been used (Cho and Blaser, 2012).
SOURCE: Yatsunenko et al. (2012).

noted that over the past two decades obesity rates have risen worldwide, including a doubling of the percentage of obese adults in the United States. Based on the observation that sub-therapeutic antibiotic treatment (STAT) of livestock stimulates weight gain and increases feeding efficiency (particularly among animals treated at young ages), Blaser and colleagues modeled these phenomena in experiments on mice (Cox and Blaser, 2013).

When continuously exposed to STAT since birth, mice gained weight at the same rate as controls, but developed a higher percentage of body fat (Cho et al., 2012). Such phenotypic effects are preceded, by several weeks, by significant

changes in the composition of the fecal microbiota of the STAT mice. Coincident with these changes, key genes involved in fat metabolism were up-regulated in the livers of STAT mice, Blaser reported. This research suggests that in STAT mice, the liver receives increased quantities of short-chain fatty acids produced by microbes in the intestine and converts them into fat; these fat calories are then transported to adipose tissue for storage. Current studies suggest that early-life antibiotic exposures in the mouse model are sufficient for lifelong physiological changes (Cho et al., 2012).

These results led Blaser's group to investigate whether a series of short, therapeutic-dose pulses of antibiotics administered in early life—an experience shared by many U.S. children—could produce sufficient change in the mouse gut microbiome to alter body composition; as demonstrated in the mouse model, preliminary results suggests lifelong effects. Blaser observed that studies in other fields may help to illuminate these results. Similar patterns have been observed in forest ecosystems, he noted, which tend to recover from infrequent disturbances unless a second disturbance follows before stability is restored (Paine et al., 1998).

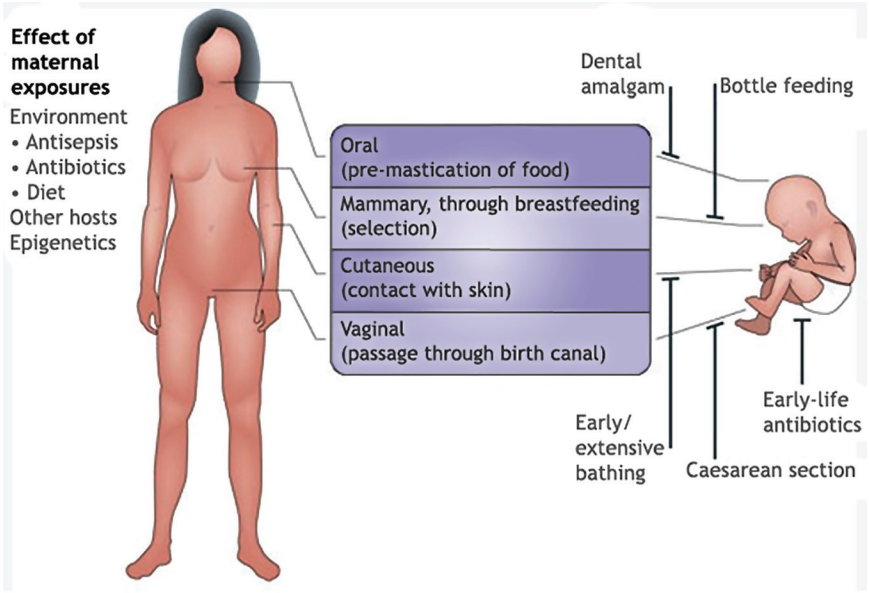


FIGURE WO-18 Factors modifying mother-to-child microbial transmission. Through live birth, mammals have important opportunities for mother-to-child microbial transmission through direct surface contact. However, many modern practices can reduce organism and gene flow; several examples are illustrated.
SOURCE: Cho and Blaser (2012).

Blaser expressed concern that current levels of antibiotic exposure in children may cause "collateral damage" to their metabolic and immune systems that may have lifelong repercussions that include obesity, other chronic diseases, and increased susceptibility to resistant organisms and potentially to epidemic infections.[33] Forum member Jesse Goodman, of the Food and Drug Administration, observed that antimicrobial therapy in humans may be associated with socioeconomic, health-related, and other unmeasured factors that could potentially confound determining the etiology of obesity and other chronic conditions. Goodman wondered whether such confounders were under examination through epidemiological studies or clinical trials. Blaser noted that few such studies have been performed, but a birth cohort study of 14,000 English children born in 1991–1992 revealed a significant association between antibiotic use in the first 6 months of life and measures of adiposity at 3.8 and 7 years of age (Trasande et al., 2013).[34] He observed that the same cohort also provided evidence of a link between birth by cesarean section and increased risk of obesity (Blustein et al., 2013). Ultimately, large-scale epidemiological studies are needed to determine the meaning of such observations and of findings from model systems, Blaser observed. In the meantime, he added, "we're working on mice, because we can control many of the important risk factors [for obesity]."

Estrogen-related cancers Blaser concluded his presentation by discussing research exploring whether the human microbiome may contribute to the fact that "only a proportion of individuals exposed to environmental carcinogens or carrying a genetic predisposition to cancer develop [the] disease" (Plottel and Blaser, 2011). He and his colleague Claudia Plottel suggest that estrogen-metabolizing bacteria may contribute to the development of estrogen-related cancers in host organisms—an influence not restricted to the tissues colonized by these bacteria. Epidemiological evidence consistent with this scenario includes the finding that among women positive for breast cancer susceptibility genes *BRCA-1* and *-2*, those born before 1940 (coincident with the dawn of the antibiotic era) tended to develop breast cancer less frequently and later in life than those born after 1940 (King et al., 2003) (Figure WO-19).

Blaser observed that like breast cancer, endometrial cancer is a "disease of development," occurring at significantly higher rates in wealthy countries where antibiotic use has been prevalent for the longest period of time (Munstedt et al., 2004). Plottel and Blaser hypothesize that the "estrobolome"—the estrogen-metabolizing genes expressed by indigenous bacteria—modulates circulation of estrogens within the liver, which in turn affects estrogen levels throughout the

[33] For example, Chang et al. (2008) suggested that decreased microbial diversity due to antibiotic treatment may contribute to the development of "antibiotic-associated" *Clostridium difficile* infection and diarrhea. Disruption of the gut microbial community by antibiotics may favor colonization by the pathogen.

[34] See http://www.bristol.ac.uk/alspac (accessed January 9, 2014).

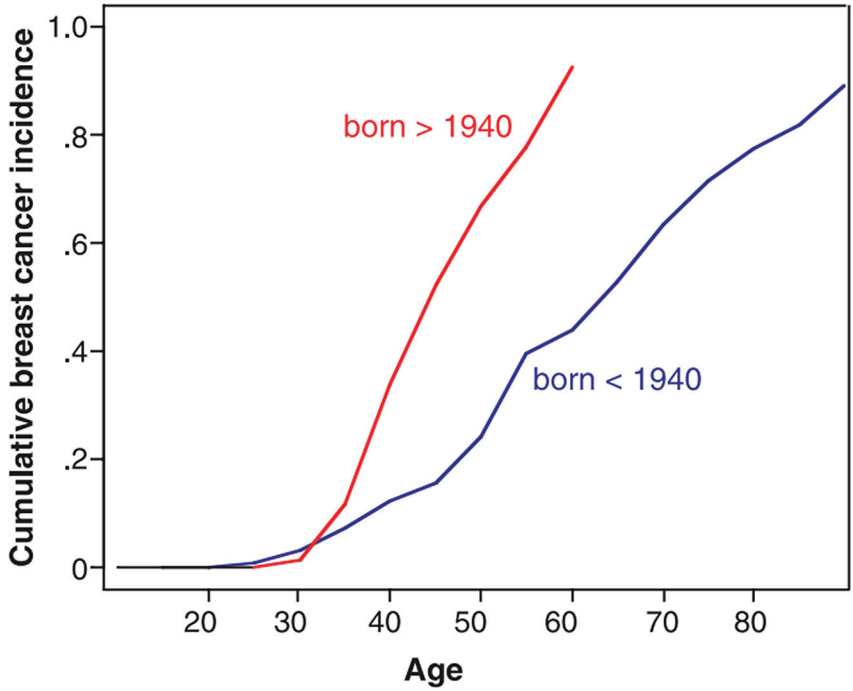


FIGURE WO-19 Influence of birth cohort on risk of breast cancer among *BRCA-1* and *BRCA-2* mutation carriers. Age by age, breast cancer risks for mutation carriers born after 1940 were significantly higher than risks for mutation carriers in the same families born before 1940 (log rank P<.0001).
SOURCE: King et al. (2003).

body[35] (Plottel and Blaser, 2011). Investigation of the estrobolome hypothesis will measure bacterial deconjugation of estrogen in the human gut and its effect on estrogen levels in the blood and other tissues and determine the risks associated with estrogen exposure at measured levels.

Expanding Perspectives on Host–Microbe Interactions

While considerable research on the role of the microbiome in human health and disease has focused on the resident bacteria of the human gut, studies of other mucosal surfaces and the skin, and of other resident microbes, have broadened our understanding of the complex interplay between the host, environment, and microbial communities. The presentations summarized below reflect expanding

[35] Estrogens leave the liver as bile conjugates and circulate to the gastrointestinal tract, from which they are excreted—unless they are deconjugated through the action of the resident bacteria, causing estrogen to be reabsorbed, Blaser explained.

knowledge of the structure and function of indigenous microbial communities, as well as nascent efforts to uncover the physiological and genetic mechanisms with which host and microbe engage each other, and the consequences of those interactions for states of human health and disease.

Community Ecology and the Human Vaginal Microbiome

Studies of the vaginal microbiome are redefining concepts such as "normal" and "healthy," noted speaker Larry Forney of the University of Idaho (Dr. Forney's contribution may be found on pages 292-323 in Appendix A). They have also provided insights into community stability, because most women are free of vaginal disease most of the time—despite frequent disturbances such as menses, sexual intercourse, and other sexual and hygienic practices. This microbial community resilience suggests coevolution of the vaginal microbiome with its host.

Forney observed that more than a century ago, *Lactobacillus* spp. was identified as a key member of the vaginal microbiome, that and its presence has been associated with vaginal health ever since. Bacterial production of lactic acid from glycogen supplied by the host is thought to prevent invasion—and therefore infection—of the vagina by nonindigenous microbes, he explained. As a result, a vaginal pH of 4.5 has long been equated with health. But as Forney and coworkers have discovered, many non-Caucasian women self-described as "healthy" have a vaginal pH significantly higher than 4.5 (Ravel et al., 2011). "This is really a call for personalized medicine," he argued. "We cannot take a simple rule like a healthy vagina is one with a pH less than 4.5, which may be true for many Caucasian women, and apply it to Hispanic and to black women equally well."

Indeed, Forney observed, much of the common wisdom regarding the vaginal microbiome is unfounded. The vast majority of studies conducted to date have focused on women of reproductive age, and have tended to advance the view that all women have "more or less the same bacteria in their vaginal microbiome," and that with the exception of menses, these bacterial communities are stable. By contrast, he and coworkers have conducted research that disputes these conclusions and raises a number of fascinating questions regarding the composition and function of the vaginal microbiome, the response of the microbiome to disturbance, and the contribution of these combined factors to states of health and disease (Hickey et al., 2012; Ma et al., 2012).

Forney's group conducted a phylogenetic examination of the vaginal microbiome in a cross-sectional survey of nearly 400 healthy, asymptomatic women equally representing Asian, Black, Caucasian, and Hispanic ethnic groups (Ravel et al., 2011). Their results reveal five distinct types of microbial communities, all of which contained significant numbers of lactic acid–producing bacteria—but not necessarily lactobacilli (Figure WO-20). According to Forney, this finding suggests that the function of the vaginal microbiome is conserved, while species

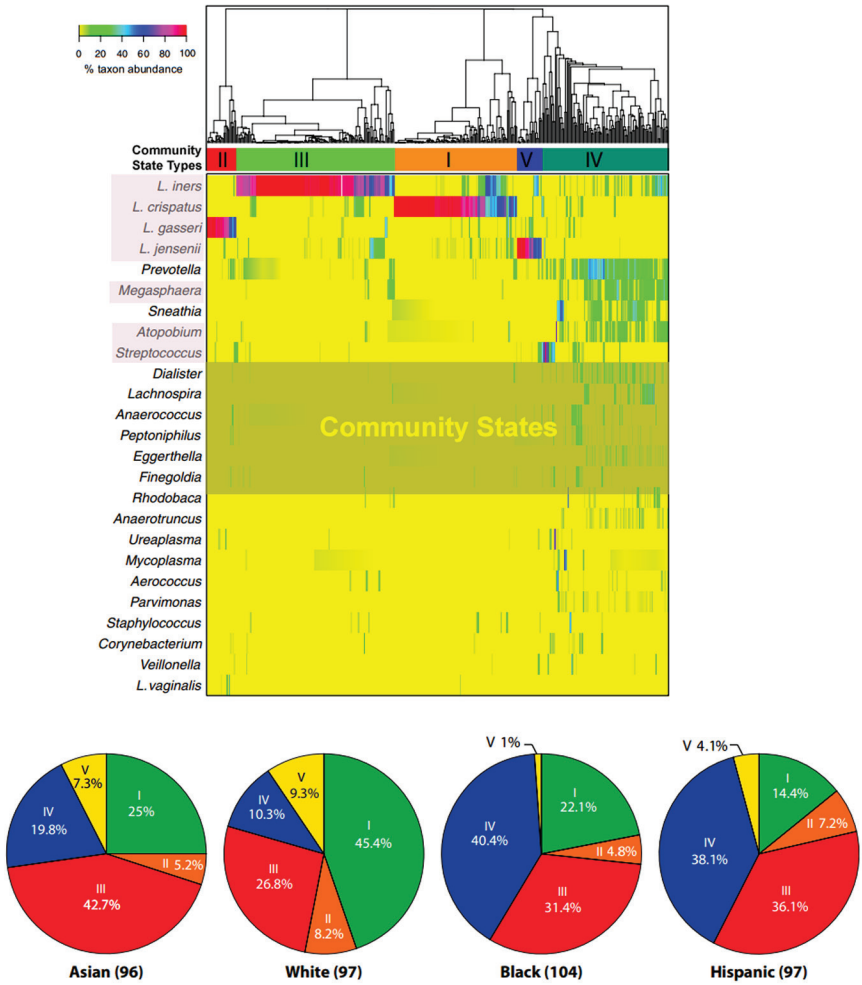


FIGURE WO-20 Phylogenetic surveys of the vaginal microbiome of healthy women identified five distinct clusters. Across community states, function may be conserved as all include significant numbers of lactic acid–producing bacteria. Panel A is a heat map of percentage abundance of microbial taxa found in the vaginal microbial communities of 394 reproductive-age women. Panel B is a representation of vaginal bacterial community groups within each ethnic group of women. The number of women in each ethnic group is in parentheses.
SOURCE: Ravel et al. (2011).

composition varies. Noting that a recent study implicated lactic acid as a signaling molecule for the immune system (Witkin et al., 2011), Forney observed that the specific role of lactic acid in maintaining vaginal health by lowering pH should be revisited.

Another study by Forney and coworkers that examined variation of vaginal bacterial communities in 32 women over the course of 16 weeks, found that each woman's vaginal microbiome was unique to her, and that no single paradigm of community ecology could explain the wide range of variation they observed both within and between subjects (Gajer et al., 2012). Ultimately, he continued, "[i]t appears as though there are different rules for different women that determine the composition of the vaginal microbiota over time, and in response to disturbance." Therefore, "we need to think critically and reevaluate what is meant by 'healthy and normal.'"

Ecosystems research suggests that stable communities resist invasion, and these findings may be applicable to vaginal health and disease, Forney observed. A disturbed community itself could elicit signs and symptoms of disease, or it could be vulnerable to invasion by infectious organisms, he explained. The former scenario seems to describe bacterial vaginosis, a condition estimated to affect more than one-quarter of American women and is associated with an increased risk of complications such as sexually transmitted diseases and preterm birth. Calling efforts to understand bacterial vaginosis "the curse of Koch's postulates,"[36] he remarked that the long history of failed efforts to find a single causative agent for this condition supports the theory that an ecological problem underlies this disease. It may also illustrate Forney's observation that differences in vaginal microbiota composition between women in different ethnic groups over short time periods may have important consequences that should be accounted for in disease diagnosis and risk assessment.

Forney noted that microbial communities that differ in species composition may also differ in community stability. If the different states identified by Forney and colleagues reflect different risks to invasion or disease, he observed, then for a particular woman disease risk will also vary with time "depending upon what day it is and what the events are that are driving the changes we are seeing," Forney observed. To better understand the relationships between community composition, stability, and disease, we need to appreciate "what functions are being performed by the microbial communities that are important to maintaining health and the ecological interactions necessary to maintain them," he concluded.

[36] Koch's postulates must be satisfied in order to state that a particular microbe causes a specific infectious disease. They include the following: (1) The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease; (2) The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite; (3) After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew (Koch, 1891; Rivers, 1937; Fredericks and Relman, 1996).

Host Defense and Immunomodulation of Mucosal Candidiasis

Continuing the discussion, begun by Eberl, of the contextual nature of host–microbe interactions related to immunity, speaker Paul Fidel, of Louisiana State University, discussed the range of human immune responses to the fungus *Candida albicans*, a commensal inhabitant of mucosal tissues (Dr. Fidel's contribution may be found on pages 249-273 in Appendix A). This member of the normal flora, in certain circumstances, can cause a range of pathogenic conditions generally known as "yeast infections." These include oropharyngeal candidiasis (OPC, commonly known as oral thrush), denture stomatitis, and vulvovaginal candidiasis (VVC), which may be acute or recurrent. As discussed by Fidel, immune responses to a particular microorganism vary by body site but also in magnitude between individuals.

In its guise as an opportunistic pathogen,[37] *C. albicans* expands beyond its commensal niche on the mucosal surface by extending hyphae[38] into the upper layers of surrounding epithelial cells. Fidel observed that it can also form biofilms that, in certain contexts, may lead to pathogenicity. Whether *C. albicans* is a commensal inhabitant or pathogen largely depends upon host environmental factors. Although OPC and denture stomatitis are primarily associated with immunocompromised individuals—people with HIV infection, those undergoing chemotherapy, transplant patients, and the elderly—Fidel noted that VVC frequently occurred in otherwise healthy women.

As Fidel and coworkers have discovered, the means by which host tissues in these different sites defend against *Candida* infection are strikingly different, with distinct consequences for the manifestation and treatment of disease (Yano et al., 2012). In the case of OPC, mucosal tissues defend against fungal invasion through an adaptive immune response involving CD4 T cells.[39] Despite considerable efforts to find a similar protective role for adaptive immunity against VVC, it was suspected—and then demonstrated—that this disease did not arise from insufficient defense by the host against invasion, but rather by an immune "overreaction" to the presence of a commensal—"collateral damage," as Eberl described it (Fidel, 2007; Yano et al., 2010, 2012).

Interactions between the vulvovaginal mucosal epithelium and *C. albicans* have evolved in the direction of maintaining a beneficial symbiosis, Fidel explained. In this context, high levels of inflammation associated with an adaptive immune response would cause far more harm than good, he said. Communication between host and microbe preempts the adaptive immune response and preserves

[37] An organism that exists harmlessly as part of the normal human body environment and does not become a health threat until it enters the body via wounds; when the body's immune system is weakened; or when there is a disturbance of a normally benign host–microbe relationship.

[38] Slender tubes that develop from germinated spores and form the structural parts of the body of a fungus.

[39] Fidel noted that HIV patients lack these cells, and in these patients CD8 cells mount the immune response.

the peace. "It turns out that epithelial cells themselves have the ability to inhibit the growth of *Candida*," he continued. "This is a very noninflammatory process. It actually enables asymptomatic colonization to remain and keep the organism in check." Details of the interaction between *Candida* and vaginal epithelial cells in conditions of "health" and "disease" are shown in Figure WO-21.

"Symptomatic infection of vaginal candidiasis is associated with an acute inflammatory response, not of the adaptive immune system, but of the innate immune system," Fidel stated. "The whole idea of protection and susceptibility to infection has gone through a paradigm shift over the past several years now, going away from some deficiency in adaptive immunity to issues of innate immunity being responsible for both protection and susceptibility." But, as his research demonstrates, both models—involving the same host and microbe—can exist simultaneously in different contexts.

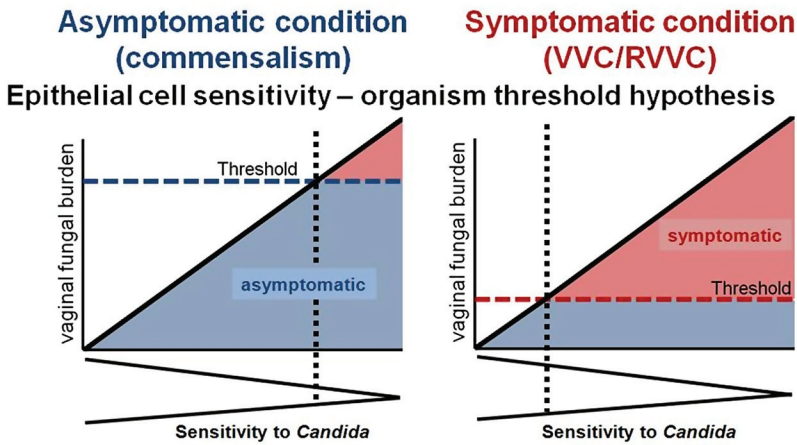


FIGURE WO-21 Epithelial cell sensitivity—organism threshold hypothesis. In women with no history of VVC (left panel), their vaginal epithelial cells are insensitive to *Candida* and fail to induce the inflammatory response. These women remain asymptomatic even in the presence of high numbers of *Candida* (threshold number to initiate pathological response rarely breached) that are held in check by noninflammatory processes. In women with RVVC (right panel), vaginal epithelial cells are extremely sensitive to *Candida* and induce the inflammatory response. These women are susceptible to symptomatic infection following exposure to even small numbers of *Candida* (low organism threshold) and with less ability to hold them in check. The thresholds represent an arbitrary organism number of the upper limit for vaginal fungal burden that would initiate symptomatic infection. SOURCE: Yano et al. (2012).

Host–commensal interactions in the presence of pathogens According to speaker Yasmine Belkaid of the National Institute of Allergy and Infectious Diseases, complex interactions between resident and infectious microbes and the host immune system are likely to occur in barrier sites such as the gut, skin, and lungs (Belkaid et al., 2013) (Dr. Belkaid's contribution may be found on pages 93-109 in Appendix A). In the gut, where most such interactions have been studied, acute infections have been shown to induce dysbiosis and lasting shifts in microbial community composition, while commensals have been shown to promote immune responses against pathogens, she noted. Little is known about similar interactions involving other barrier sites, such as the skin—the largest organ of the body and the most exposed surface—with distinct microbiota and pathogen exposures.

In order to explore how resident microbes influence immunity and inflammation in the skin, Belkaid and coworkers injected germ-free and "normal" mice with the skin parasite *Leishmania major* (Naik et al., 2012). The germ-free mice mounted a significantly weaker immune response to the pathogen and were less able to control the infection. This response, they discovered, was due to reduced production of pathogen-killing inflammatory cytokines by T cells in the skin of the germ-free mice. If the same mice were colonized with a single bacterial species from the "normal" skin microbiome before pathogen exposure; however, the germ-free mice mounted a competent immune response, as did normal mice treated with antibiotics to abolish their gut microbiome. Based on these observations, the researchers concluded that skin-colonizing microbes influenced the local response to infection. This response was driven by mechanisms that are distinct and independent from the immunity-promoting mechanisms of the gut flora. This finding should provoke further exploration of the role of niche-specific microbial communities in local immune responses and the potential for cross-talk between host sites, Belkaid added (see Belkaid and Naik, 2013).

The skin microbiome acts like a topical adjuvant, according to Belkaid, tuning the level of activation and function of T cells in their immediate environment. Further experiments have revealed that skin commensals exert this effect through an innate immune signaling pathway that has been linked to several chronic inflammatory and skin disorders, including arthritis, asthma, and psoriasis (Hand et al., 2012).

Having examined the ability of the microbiota to promote host immunity to pathogens, Belkaid then considered how an acute infection with a pathogen may alter an otherwise healthy relationship between a host and its microbiota. The gastrointenstinal (GI) tract is one of the most frequent sites of infection—there is an "average of 2 GI acute diarrheal illnesses per child [per year] in America and 12 or more in children in developing countries." When the physical barrier of the intestinal epithelium is breached by pathogens, the immune system responds not only to their presence, but also to that of commensal bacteria that gain access to otherwise inaccessible host tissues, she explained.

She and coworkers examined how acute gastrointestinal infection of mice by the protozoan parasite *Toxoplasma gondii* influenced their subsequent immune response to commensal microbes (Hand et al., 2012). Using a transgenic mouse as a source of T cells specific for a single commensal antigen (rather than the diversity of commensal antigens present in wild-type mice), they found that these T cells became activated to participate in an inflammatory immune response when transferred into *T. gondii*-infected mice, but not when transferred into uninfected mice. "In the context of [pathogen] infection, a T cell that is specific for a commensal is going to become an effector cell at the same level that the effector cells that are generated against a pathogen," she observed.

Belkaid reported that in addition to this immediate immune response, commensal microbiota-specific T cells also generate immune memory cells, much as do pathogen-specific T cells (Hand et al., 2012). This work has led to the development of a model of the potential consequences of commensal-specific memory T cells (Figure WO-22). Having T cells that recognize commensals could help control subsequent infections. However, Belkaid noted, if the host has a defect in immune regulation, or in barrier integrity, the uncontrolled immune responses to commensals could lead to inflammatory disorders such as Crohn's disease.[40] These responses could also "increase the whole inflammatory setup of the host" and potentially contribute to the etiology of other inflammatory disorders such as allergy and IBD, according to Belkaid. "This is likely to happen not only at the level of the GI tract, but [also] potentially of the lung or at the level of the skin." Every breach at these barrier sites is likely to increase the pool of commensal-specific memory cells that can be reactivated upon secondary challenge, she said. Understanding the consequences of this buildup of immunity against commensals could lead to insights into the etiology of microbiota-associated inflammatory disorders, concluded Belkaid.

Exploring the Virome

While the bacterial microbiota have been increasingly cataloged, revealing vast numbers of unculturable bacterial species, investigators are also exploring other components of our microbiota—which include all the organisms that infect and live in and on us. The virome encompasses a wide diversity of organisms—from the retroviral elements that "infest our chromosomes" to the myriad chronic infections that are a part of the normal flora of humans. As noted by speaker Herbert "Skip" Virgin, of Washington University, evidence suggests that our virome contributes to "who we are and how we respond to our environment" (Dr. Virgin's contribution may be found on pages 445-471 in Appendix A). He described recent

[40] Crohn's disease is one of the inflammatory bowel diseases (IBDs) and is characterized by chronic inflammatory disease of the intestines.

research exploring the human enteric virome that has yielded fascinating insights into its community composition, dynamics, and role in health and disease.

By way of introduction to the persistent viruses, Virgin stated that "[a]ll of us are chronically infected with multiple viruses." Each person is infected with approximately 10 different types of persistent virus, which our immune systems contain but cannot eliminate through constant surveillance (Figure WO-23)

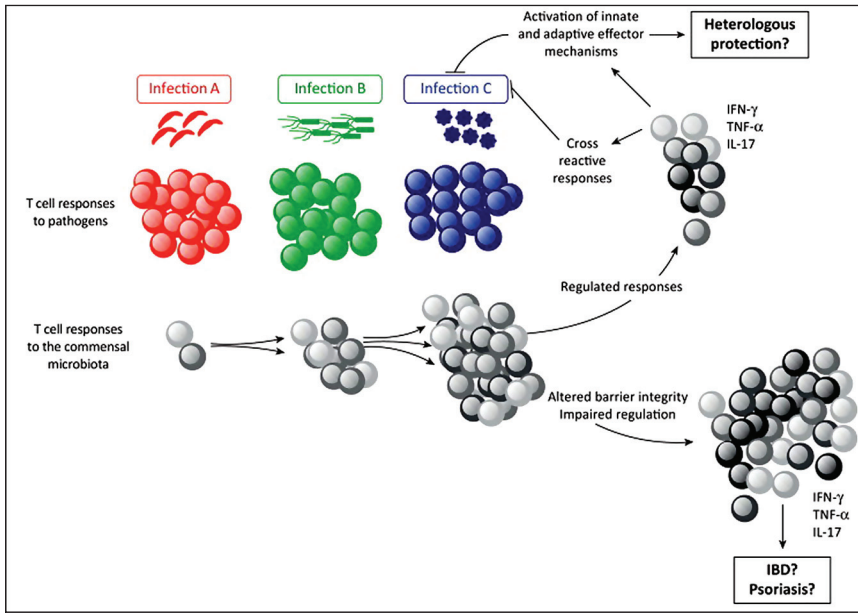


FIGURE WO-22 Potential consequences of commensal-specific memory T cells. During infection at barrier sites (gut, skin, airway, and genital mucosa), immune responses against the invading agent can be associated with specific T cell responses against a large number of coincident commensal antigens. These commensal-specific effector T cell responses can persist as memory cells that upon subsequent infection will be recalled as secondary commensal-specific effectors, alongside the priming of a novel immune response to the invasive pathogen. Therefore, each infection at barrier surfaces represents an additional opportunity for the reactivation of commensal-specific T cells. Given the extraordinary number of commensal antigens (an estimated 20 million antigens/microbiota), these responses may represent a significant proportion of memory T cells. If properly controlled, then commensal-specific T effector/memory cells could contribute to protection against infections by promoting innate and adaptive effector mechanisms assist in the clearance of the pathogen. Further, commensal memory responses could be protective because of cross reactivity with pathogen-derived antigens. By contrast, in situations where commensal-specific T cells become dysregulated because of impaired regulatory pathways and/or barrier function, these T cells could drive chronic pathology such as inflammatory bowel disease or psoriasis.
SOURCE: Belkaid et al. (2013).

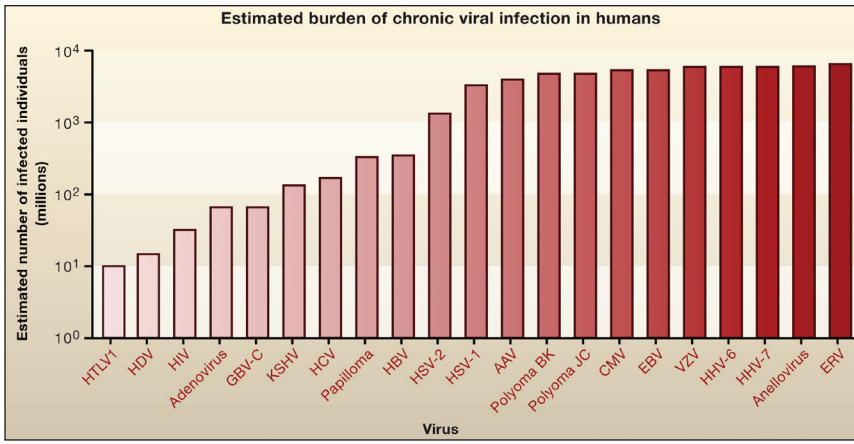


FIGURE WO-23 Chronic viral infections in humans. The number of humans infected with different chronic viruses is estimated from the percentage of humans carrying a given virus, assuming that the world population is 6.75 billion. The estimates in the graph are approximate as they apply data on prevalence in the limited populations studied to date and to the global human population. (See also Table 1 in Virgin et al. [2009]).
SOURCE: Virgin et al. (2009).

(Virgin et al., 2009). Many persistent viruses inhabit mucosal tissues, where they interact with indigenous and invasive bacteria, as well as with the host, he noted. These include the herpesviruses, which appear to have coevolved with vertebrates. "Every single species, including us, has had its own herpesviruses," he noted. "So these things have been studying us, and we have been studying them evolutionarily for a very, very long period of time—probably in the range of 100 million years or so."

Through studies of latent herpesvirus infections in mice,[41] Virgin and coworkers determined that both viruses were capable of activating their host's innate immune system, enabling the host to survive challenges with *Listeria monocytogenes* or *Yersinia pestis* that killed uninfected animals (Barton et al., 2007). Viral latency[42] was required to confer this protection, because the same bacterial pathogens killed mice infected with a mutant form of the virus that causes acute infection. In another study, infection of mice with a different latent herpesvirus "armed" the natural killer (NK) cells of the innate immune system,

[41] Murine gammaherpesvirus 68 and murine cytomegalovirus—herpesviruses that are genetically highly similar to the human pathogens Epstein–Barr virus and human cytomegalovirus (Barton et al., 2007).

[42] A type of persistent infection in which viral production ceases after initial infection but the viral genome is not fully eradicated from the host. The virus can "reactivate," emerging from latency to produce viral progeny.

to defend the host against an otherwise lethal lymphoma challenge (White et al., 2010). These research results suggest that "the fundamental nature of the immune response is determined by this latent virus, or maybe other parts of the host virome," according to Virgin.

In these examples, the combined action of virus and host genomes proved advantageous to the host, other such interactions could have negative consequences, Virgin pointed out. As he and coworkers have discovered, latent viral infection profoundly affects host gene expression (Canny et al., 2013). According to Virgin, "[T]he basic nature of the transcription in the organs of an animal is defined by whether the animal is latently infected with a herpesvirus." In particular, he and coworkers noted changes in loci that were associated with a predisposition for several chronic diseases, including celiac disease, Crohn's disease, and multiple sclerosis. "A number of these genes are changed in the latently infected animal, suggesting—but not proving—that your susceptibility to disease might also be altered by your endogenous virome," he observed (Canny et al., 2013).

Virgin speculated that "perhaps there is an immunophenotype, at least in the innate immune system, which is the individual's normal immune responsiveness, and that represents the summation of genetic, developmental, and environmental factors . . . [and] viruses are one of those environmental factors." Virgin observed that by influencing immunophenotype—whether through contributions to immunity, inflammation, or disease processes—the virome (as well as our bacterial, fungal, parasite, and phage constituents of the microbiome)—may be helping to define the field upon which pathogens or invasive species play their roles in causing disease.

To illustrate the concept of the immunophenotype, Virgin described studies his research team performed on the interaction between an enteric virus that infects mice, murine norovirus (MNV), and a hypomorphic mutation in a gene, known as Atg16L1, associated with increased susceptibility to Crohn's disease in humans (Cadwell et al., 2008, 2010). Mutation of Atg16L1 produces a pathological effect on the microbe-regulating Paneth cells of the intestinal epithelium in mice similar to what is observed in humans—but not under all circumstances. In the murine system, there was clearly an environmental trigger for the gene-associated pathology. Through both histological and gene-expression comparisons of Atg16L1-bearing tissues before and after infection with MNV, the investigators determined that the Crohn-like pathology resulted from a combination of the virus plus the susceptibility gene, in the presence of commensal bacteria. "When you put the virus and the gene together, you get a new phenotype," he said, adding that inflammatory diseases are unlikely to be exclusively attributable to the host's genetic susceptibility or to a microbial pathogen, but instead to the synergistic effects of genes being expressed (or silenced) in both the host and the bacterial microbiome and virome.

Given the great diversity of both viruses that infect humans and of alleles in the human population, one could expect a number of different outcomes related

to disease. This makes sense for many complex diseases—such as the inflammatory bowel diseases—in which genome-wide association studies suggest that the total risk conferred by the observable genotypic difference is less than 20 percent. In such cases, genome does not equal phenome,[43] unless all the components of our metagenome (including the virome) are accounted for, Virgin argued. The "lack of quantitative definition of the virome . . . could be contributing to human phenotypic variation [observed for many chronic diseases] not explained to date by variations in our own genome," he said.

Recent work from Virgin's research group illustrated the uncharacterized nature of the enteric virome. Simian immunodeficiency virus (SIV) is a close relative of HIV that, in rhesus monkeys (and under certain as yet undefined conditions), progresses to an AIDS-like disease (Handley et al., 2012). Disease progression is associated with the breakdown of the intestinal epithelial lining, which allows bacteria to invade host tissues and prompts immune activation. While "translocation" of bacteria is arguably a better predictor of progression to AIDS than other more commonly used measures (such as CD4 count and viral RNA levels), previous studies limited to bacterial 16S rRNA sequences had failed to link the intestinal bacterial microbiome to AIDS-related epithelial damage. When Virgin's group conducted similar studies of the enteric virome, using molecular methods that search for both viruses and bacteria, they discovered a significantly larger range of virus species and genera—including more than 23 previously unknown viruses from pathogenic genera—in the intestines of primates with pathogenic lentivirus infection.

"In pathogenic SIV infection there is a huge expansion of the enteric virome," Virgin reported. Some of these viruses may cause disease; others may translocate into the blood; some may damage the intestinal epithelium, enabling molecules associated with pathogens and recognized by cells of the innate immune system to move into host tissues, causing systemic inflammation that is the hallmark of AIDS. Based upon these observations, Virgin and coworkers are now studying such possible effects of AIDS-associated virome expansion in humans and primates.

Phage-Mediated Immunity at Mucosal Surfaces

Speaker Forest Rohwer of San Diego State University discussed another member of the virome: viral parasites of bacteria known as bacteriophage (Dr. Rohwer's contribution may be found on pages 383-400 in Appendix A). Some investigators have projected that there are approximately 10^{31} phages worldwide, making these viruses among the world's most numerous biological entities (Youle et al., 2012). Recent research by Rohwer and colleagues suggests that bacteriophages populating mucosal surfaces provide a form of immunity to

[43] Sum total of its phenotypic traits.

its animal host. In his presentation, Rohwer described a series of observations he and coworkers have made that led them to characterize bacteriophages as the "hired killers of the immune system."

Mucosal surfaces form the initial barrier between animals and the environment, Rohwer noted. Mucus covers the entire living surface of some organisms, such as coral, and in humans, mucus lines the lungs, sinus and oral cavities, and the gastrointestinal tract. A variety of microorganisms can be found in mucosal surfaces, which provides the microbes with structure and nutrients. After discovering that mucosal surfaces harbor unusually high concentrations of phages relative to their bacterial prey, Rohwer and colleagues conducted experiments that revealed this association to be quite specific (Barr et al., 2013). The phages stick to mucins, the large, entangled glycoproteins that form the mucus matrix, he said; this serves to concentrate the phages on mucosal surfaces where they are able to infect and kill incoming bacteria. The specificity of this interaction appears to extend to the phage itself, Rohwer continued.

About a quarter of all phages display immunoglobulin-like domains, known as "decoration proteins," on their capsid surfaces. Like immunoglobulins,[44] decoration proteins are highly variable (Minot et al., 2012). "We know that these decoration proteins, for the most part, don't matter in the lab," he added. "We can take them off, and the phages do just fine." Rohwer and his coworkers hypothesized that selection for the presence of decoration proteins might involve their adherence to mucins. As Rohwer reported, binding experiments using phage knockout mutants demonstrated that decoration proteins were required for phage-mucin adhesion, and mathematical modeling indicated that mucus-bound phages are 15 times more likely than their free-living counterparts to encounter a bacterial cell (Barr et al., 2013).

As reported by Rohwer, phages are further concentrated in the mucus layer through lysogeny of infected bacteria.[45] Through a phenomenon known as "superinfection exclusion"—the inability of a cell infected by one virus to be infected by another virus of the same type—only incoming bacteria not bearing the mucus-adherent phages will be killed, observed Rohwer. Figure WO-24 depicts these interactions at the mucosal surface, which comprise a system Rohwer and coworkers have termed the Bacteriophage Adherence to Mucus Immunity model (Barr et al., 2013). By his description, it functions as an "acquired, adaptive, and specific immune system."

[44] Also known as antibodies, immunoglobulins are any of numerous proteins produced by B lymphocytes in response to the presence of foreign molecules.

[45] The biological process in which a bacterium is infected by a bacteriophage that integrates its DNA into that of the host such that the host is not destroyed.

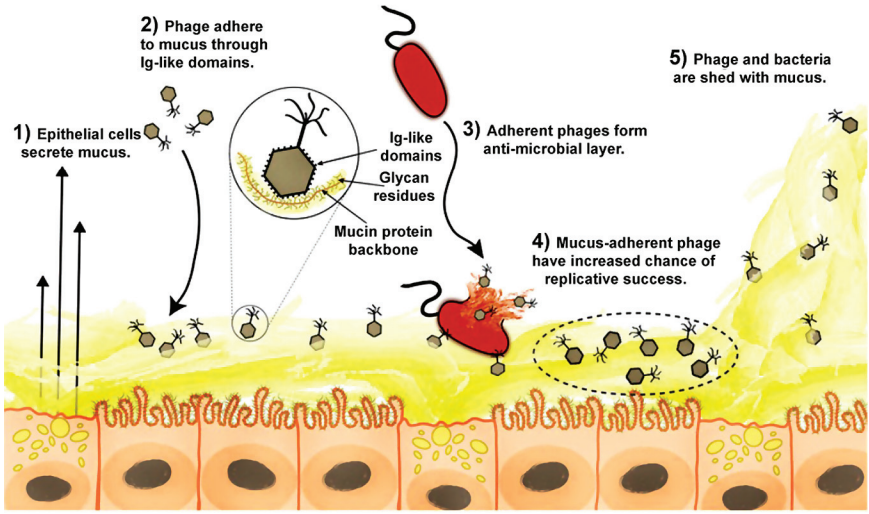


FIGURE WO-24 The Bacteriophage Adherence to Mucus Immunity model. (1) Mucus is produced and secreted by the underlying epithelium. (2) Phages bind variable glycan residues displayed on mucin glycoproteins via variable capsid proteins (e.g., Ig-like domains). (3) Phage adherence creates an antimicrobial layer that reduces bacterial attachment to and colonization of the mucus, which in turn lessens epithelial cell death. (4) Mucus-adherent phages are more likely to encounter bacterial hosts, thus are under positive selection for capsid proteins that enable them to remain in the mucus layer. (5) Continual sloughing of the outer mucus provides a dynamic mucosal environment.
SOURCE: Barr et al. (2013).

Host–Mycobiome Interactions in Gut Homeostasis and Pathogenesis

The fungal contribution to the host–microbe metagenome, like that of viruses, remains largely unknown, according to speaker David Underhill of Cedars-Sinai Medical Center, Los Angeles (Dr. Underhill's contribution may be found on pages 435-445 in Appendix A). Given their ubiquity[46] and importance as human pathogens, Underhill noted that it would seem likely that fungi are represented in the human microbiome and, in that capacity, interact with the immune system in ways that may influence human health. Indeed, he pointed out, antibodies recognizing a common fungal cell wall component, mannan, are currently used to identify cases of Crohn's disease. "This suggests that in inflammatory bowel disease, there is novel exposure to fungi," observed Underhill (Underhill and Braun, 2008).

[46] Fungi are estimated to comprise nearly one-quarter of Earth's biomass.

In order to assess the presence of fungi in the gut microbiome, Underhill's lab developed an approach to detect and characterize fungal ribosomal DNA from several sites in the mouse gastrointestinal tract; using this approach they were able to identify more than 200 species of fungi, representing more than 50 genera (Iliev et al., 2012). Employing a cell wall-binding fluorescent probe, they were able to locate fungal cells in intimate associations with bacteria in the mouse colon, and in the feces from a wide range of mammals, including humans. Treatment with fluconazole, a fungal-specific drug, was shown to abolish these populations. The researchers also observed the appearance of anti-mannan antibodies in a mouse model of chemically-induced colitis. This observation suggests that the immune system interacts with endogenous fungi when the gut is disturbed.

Dectin-1, the cell wall–binding probe that Underhill's group used to visualize endogenous fungi, is an innate immune receptor expressed by macrophages, dendritic cells, and neutrophils, he explained. Suspecting that Dectin-1 serves to recognize fungi in the gut, the researchers further explored its influence on inflammation in the presence of fungi. Dectin-1 knockout mice experienced increased levels of inflammation by every known measure, Underhill reported; in a chemically-induced mouse model of colitis, the knockout mice suffered more severe disease that featured fungal invasion of host tissue, which did not occur in wild-type mice (Iliev et al., 2012). "We think this is ultimately what contributes to the increased inflammation disease of these mice," he added. "In the absence of Dectin-1, the innate immune cells . . . in the tissue [surrounding the colon] are unable to clear these fungi as quickly." This conclusion was supported by the finding that, following induction of colitis, treating the knockout mice with fluconazole resolved their symptoms, but not those of wild-type mice, he added. Extending this observation to humans, Underhill and coworkers surveyed Dectin-1 gene sequences among patients with ulcerative colitis, and discovered a specific polymorphism associated with a two-fold increased risk for very severe disease. "The people who have the risk allele progressed to surgery much faster [than those who did not]," he reported. "There may be some predictive utility here."

Host–Microbe Interactions in the Inflammatory Bowel Diseases

The term inflammatory bowel disease is used to describe a constellation of conditions associated with chronic or recurrent inflammation of the gastrointestinal tract. Two clinically defined forms of IBD are ulcerative colitis and Crohn's disease, which "are chronic remittent or progressive inflammatory conditions that may affect the entire gastrointestinal tract and the colonic mucosa, respectively, and are associated with an increased risk for colon cancer" (Kaser et al., 2010). Extra-intestinal manifestations, including liver problems, arthritis, skin manifestations, and eye problems, also occur in Crohn's disease patients. As one of the five most prevalent gastrointestinal diseases in the United States, IBD accounts

for more than $1.7 billion in health care costs, annually.[47] According to the U.S. Centers for Disease Control and Prevention (CDC) patients often require medical care throughout their lifetime because there are no medical cures available for IBD. Once limited to the industrialized world, Crohn's disease (Figure WO-25) and ulcerative colitis are emerging as global diseases (Molodecky et al., 2012).

When compared to that of "normal" individuals IBD patients exhibit abnormalities in their gut microbiome (Figure WO-26), including

- reduced or altered levels of dominant bacteroidetes and firmicutes phyla populations;
- an overall decrease in microbes known to be protective of gut tissues; and
- increased representation by proteobacteria, some members of which are recognized by the epithelium as pathogens, leading to inflammation.

These alterations, discussed at the workshop, represent a disruption in the normal homeostatic relationship that exists between the intestinal epithelium, the immune system, and the gut microbiome—each component of which is influenced by a variety of genetic and environmental factors. The presentations summarized below explore the complex interplay of these factors in the pathogenesis of IBD.

The Influence of the Mucosal Immune System and Gut Microbiota on IBD

Recent discoveries in genetics, microbiology, and immunobiology have helped to illuminate the complex interactions between host, microbiota, and environmental factors that contribute to the development and persistence of IBD (Figure WO-27). According to speaker Richard Blumberg of Harvard Medical School, research suggests that the altered microbiota in IBD patients is largely a secondary effect of conditions leading to IBD, rather than its primary cause (Lupp et al., 2007) (Dr. Blumberg's contribution may be found on pages 153-164 in Appendix A). Indeed, various experimental regimes (including chemical treatment) can simulate the effects of IBD on the intestinal microbiota, he observed. That is not to discount the importance of dysbiosis, Blumberg continued; it may be inflammatory in and of itself, and thereby perpetuate IBD.

To identify the primary cause of IBD, Blumberg and coworkers sought to examine the coordinated development of the intestinal tract and its microbiome and ask the question, "When does IBD start?" In the course of these studies, Blumberg's research team noted that germ-free mice suffered higher rates of mortality from chemically-induced colitis, when compared to conventionally raised mice (Olszak et al., 2012). This lethal inflammatory response was associated with

[47] See http://www.cdc.gov/ibd (accessed December 5, 2013).

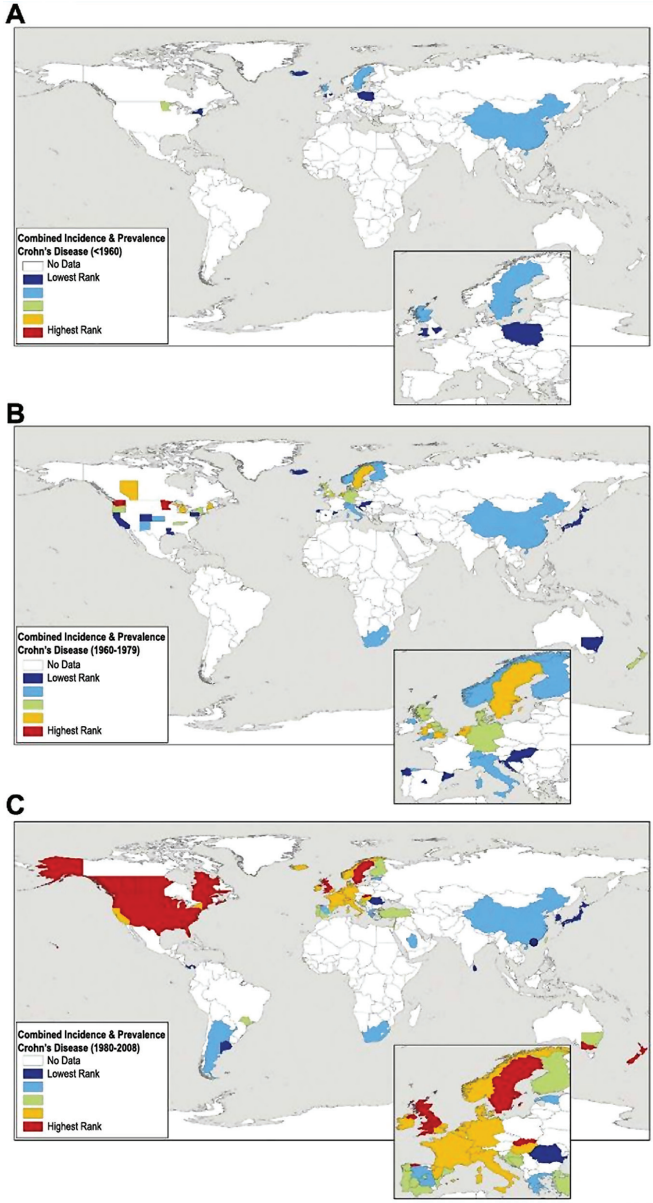


FIGURE WO-25 Worldwide Crohn's disease incidence rates and/or prevalence for countries reporting data (A) before 1960, (B) from 1960 to 1979, and (C) after 1980. Incidence and prevalence values were ranked into quintiles representing low (dark and light blue) to intermediate (green) to high (yellow and red) occurrence of disease. Maps are based on a systematic literature review of 262 studies (1950–2010).
SOURCE: Molodecky et al. (2012).

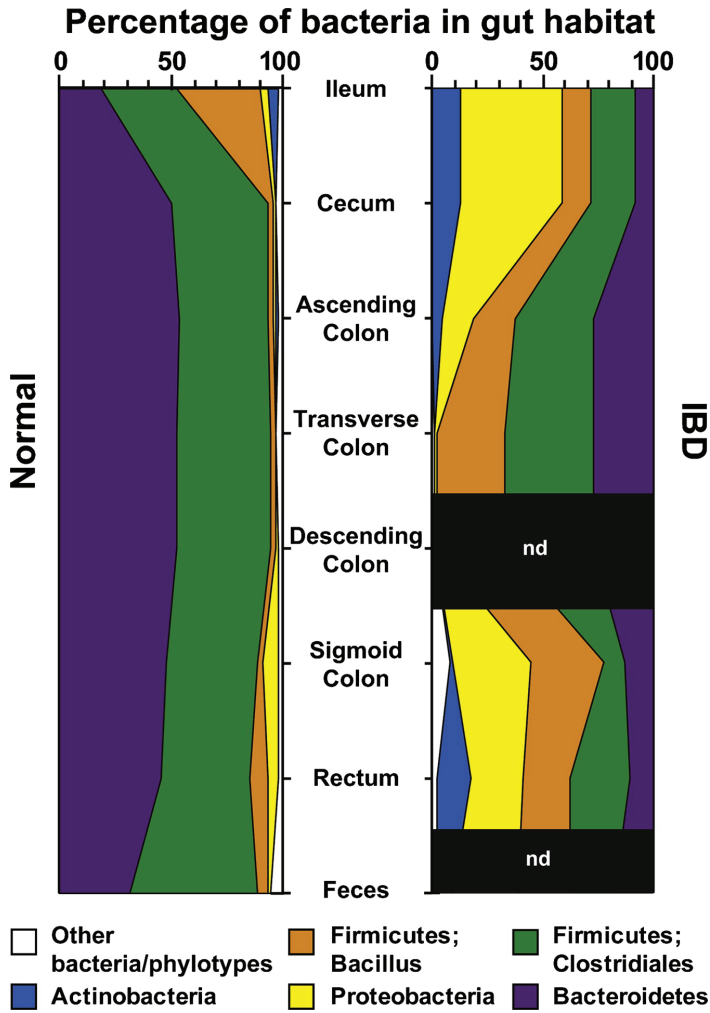


FIGURE WO-26 Bacterial phyla identified in the human gut microbiota. Distribution of predominant bacterial phylotypes in the human intestinal tract. The graphs depict relative abundance as a function of location along the cephalocaudal axis of the distal gut. DNA sequences were compiled from the studies of Eckburg et al. (2005) and Frank et al. (2007). Phylogenetic classifications were made by parsimony insertion of aligned sequences into a 16S rRNA tree provided by the Greengenes database (DeSantis et al., 2006), using ARB (Ludwig et al., 2004).

NOTES: IBD, samples from patients with either Crohn's disease or ulcerative colitis (Frank et al., 2007); Normal, data from healthy young adults from Eckburg et al. (2005) plus the non-IBD controls reported in Frank et al. (2007); n.d., not done. A total of 18,405 16S rRNA sequences were analyzed (5,405 IBD, 13,000 normal).

SOURCE: Peterson et al. (2008).

natural killer T cells[48] (NKT cells), which they found in increased concentrations in the intestinal epithelium and underlying lamina propria of germ-free mice. Other researchers (e.g., Jostins, 2012) have linked NKT cells to human IBD, and, Blumberg added, "there is a lot of reason to believe that NKT cells are a critical part of the biology of these disorders."

Blumberg observed that the natural microbiota appear to protect conventionally raised mice from the damaging inflammation typical of IBD. Seeking clues to the process by which this protection arises, the researchers discovered that germ-free mice colonized with conventional microbes within the first 2 weeks of life did not suffer from NKT cell-mediated colitis. On the other hand, germ-free mice whose guts were colonized as adults, germ-free mice, and newborn conventional mice treated with antibiotics were susceptible to colitis (Olszak et al., 2012). "What this shows is that an environmental event that degrades or diminishes or abrogates the important microbial cue in the first 2 weeks of life allows the animal to become susceptible to an environmental trigger later in life that leads to the activity of this disease," he said.

Protection against NKT-mediated colitis appears to involve a molecule called CXCL16, reported Blumberg. This molecule is produced by the cells of the intestinal epithelium and orchestrates infiltration of intestinal tissues by NKT cells. Blumberg observed that this process also takes place in the mucosal tissues of the lung. In the lung, infiltration by NKT cells has been linked to asthma in germ-free mouse models—a development that can be prevented by exposing neonates to a conventional microbiota during their first two weeks of life (Olszak et al., 2012) (Figure WO-28).

Blumberg also noted that such findings recall the hygiene hypothesis, which suggests that early-life exposures to a wide variety of microorganisms determine later-life susceptibility to immune-mediated diseases. First suggested by David Strachan to explain the relationship between household size, birth order, and hay fever in a birth cohort during 1 week in 1958 and followed for 23 years (Strachan, 1989), this hypothesis was later extended to include autoimmune diseases (Bach, 2002). Blumberg noted an increasing amount of epidemiological data that suggests that microbial exposures early in life may influence the later development of diseases such as asthma and IBD (Shaw et al., 2010; Ege et al., 2011). Blumberg concluded by noting that worldwide rates of IBD incidence and prevalence—like those of asthma—are on the rise (Benchimol et al., 2009;

[48] NKT cells are a unique group of T cells that, in contrast to classical T cells that recognize peptides, sense lipids. Lipids derived from host cells and microbes are presented to NKT cells by the CD1d molecule. "The NKT-CD1d pathway is present and operative in the intestines and very important in the management of pathogens and commensals," said Blumberg. By responding to host and pathogen-derived lipids, NKT cells are able to initiate and control a variety of different inflammatory events. NKT cells are critical because they function as the earliest phases of the immune response—at the cusp between innate and adaptive immunity.

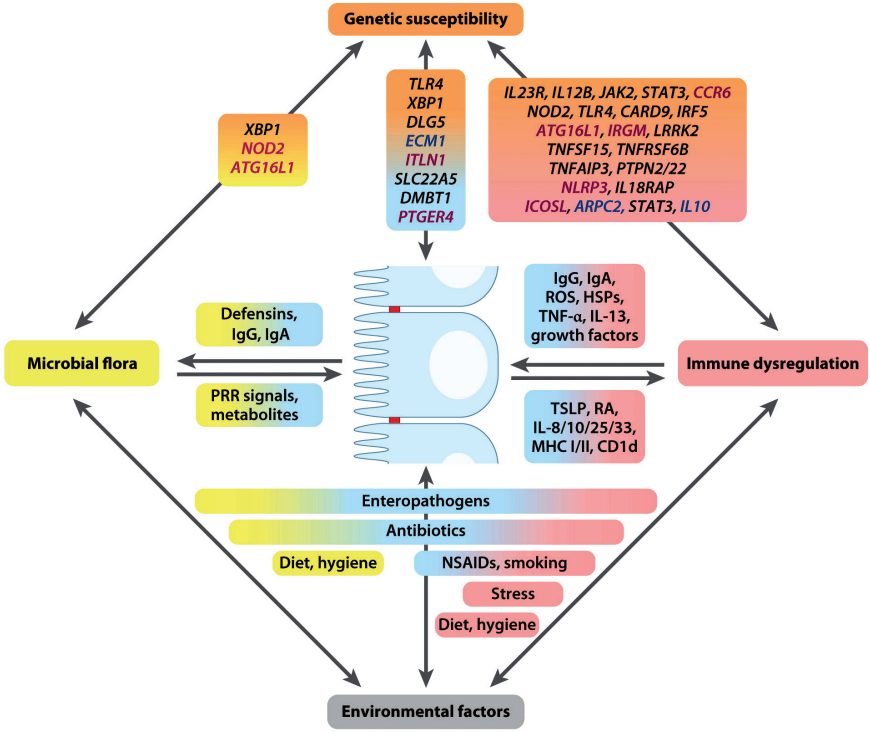


FIGURE WO-27 IBD as a multifactorial disorder. The development and course of IBD are affected by several factors including genetic susceptibility of the host, the intestinal microbiota, other environmental factors, and the host immune system. In addition, these factors cross-regulate each other in multiple ways, as shown. IBD-associated genes are summarized by molecular pathways with genes belonging to the same pathway arranged next to each other in one line. Polymorphisms in genes specific for associations shared between both diseases are shown in black text.
NOTES: HSPs, heat shock proteins; MHC, major histocompatibility complex; NSAIDs, nonsteroidal anti-inflammatory drugs; PRR, pattern-recognition receptor; RA, retinoic acid; ROS, reactive oxygen species; TSLP, thymic stromal lymphopoietin.
SOURCE: Kaser et al. (2010).

Molodecky et al., 2012). Children in the first 5 to 10 years of life appear to be the most vulnerable to environmental events associated with these diseases (Benchimol et al., 2009).

Genetics of Susceptibility in Microbiota-Associated Complex Diseases

Based on family studies, metagenomic analyses of Crohn's disease and ulcerative colitis have confirmed earlier assumptions that genetic predisposition

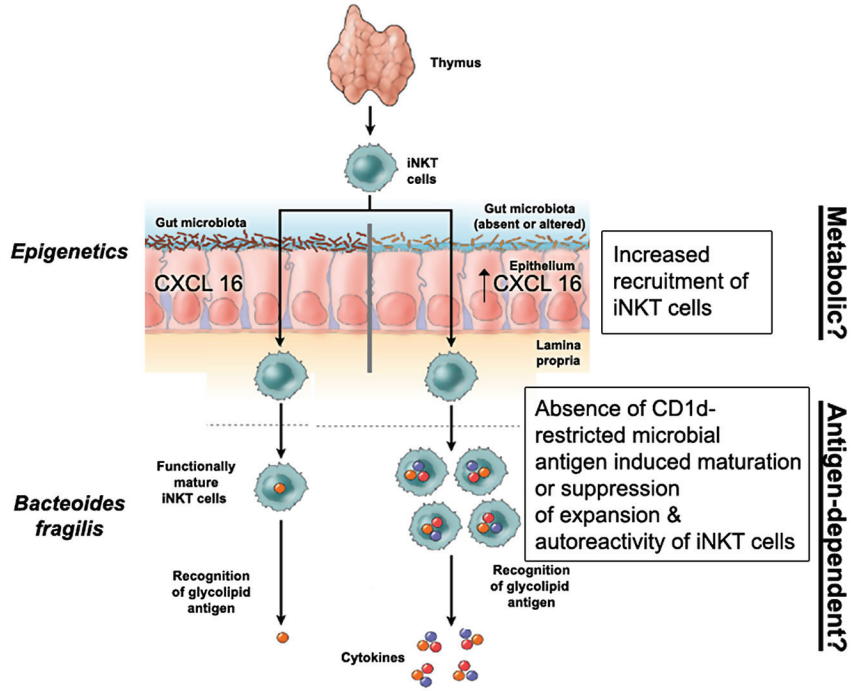


FIGURE WO-28 Potential mechanisms of NKT cell control in mucosal tissues by commensal microbiota during early life. Exposure to antigen independent (metabolic?) and antigen-dependent (bacterially-derived sphingolipids?) factors derived from the symbiotic microbiota determine the homeostatic set-point of NKT cell pathways associated with mucosal tissues in organs such as the colon and lung. These include those that affect the infiltration of these tissues with NKT cells and the degree to which they are activated by self and nonself lipid antigens. Such signals are critically received during early life and associated with durable (epigenetic) changes that provide protection from later life environmental triggers that are capable of activating NKT cells and causing inflammation and disease. In the absence of these essential microbial exposures during early life (as associated with early-life use of antimicrobials), these protective signals are not provided. SOURCE: Adapted from Coppieters and Elewaut (2012).

plays an important role in IBD, and suggest that a vast array of possible factors—directly or indirectly—contribute to risk for these complex diseases (Khor et al., 2011; Jostins et al., 2012; Graham and Xavier, 2013). Speaker Ramnik Xavier of the Harvard Medical School described his perspective on the intricate relationship between host genetics and the microbiota that he and fellow investigators are gradually unbundling (Dr. Xavier's contribution may be found on pages 471-488 in Appendix A). Following perturbation by exposure to antibiotics, early-life

events, or changes in microflora, the extent to which an individual can reverse the ensuing disease state is strongly influenced by genetic makeup, Xavier noted. By examining the functional pathways and immune responses that underlie IBD, he and his colleagues hope to reveal host genetic and microbial factors that increase or decrease risk for disease.

Reduced taxonomic diversity in the gut microbiome is now recognized as one of the hallmarks of IBD, along with characteristic increases and decreases in specific bacterial clades that may define specific subtypes of these disorders, Xavier said. Examining the functional implications of these shifts, he and colleagues discovered that they were accompanied by striking declines in carbohydrate and amino acid metabolism, short-chain fatty acid synthesis, and DNA maintenance, accompanied by increased secretory and invasive activity (Morgan et al., 2012). Xavier observed that IBD-associated microbes collectively can take up increasing amounts of host products, abrogate immune system control of inflammation (leading to further damage), and adhere more readily to the damaged epithelia. Most importantly, he continued, the functional picture of the IBD-associated microbiota is that of a community more tolerant to oxidative stress—a feature that links them to many risk loci identified in humans known to control oxidative metabolism, suggesting communication between endogenous microbes and the host genome (Figure WO-29).

To illustrate his research group's approach to understanding the many and diverse mechanisms that underlie these interactions, Xavier described his team's interest in autophagy—a constitutive and conserved process present from yeast to humans and active in every cell type—that describes the engulfment and degradation of cytoplasmic contents by lysosomes, a process associated with Crohn's disease and other inflammatory disorders (Kuballa et al., 2012). "There are an increasing number of disease associations with several of the autophagy genes," Xavier noted. "In addition to Crohn's disease, there is now emerging evidence that some of the neurodegenerative diseases are associated with polymorphisms in autophagy genes, and we and others have more recently identified that some genes might also be associated with increased risk of chronic infections such as tuberculosis." Autophagy serves a range of adaptive purposes including generating energy, healing inflammation, and controlling antigen presentation, he explained, but clearly it has the potential, if defective, to cause disease. One of the autophagy genes associated with susceptibility to Crohn's disease is Atg16L1, also discussed by Virgin as a component of an "immunophenotype" model of disease. As described in Virgin's presentation, mice expressing this gene in their epithelial tissues have abnormal Paneth cells and are defective in immune defenses in the presence of an enteric virus.

Recognizing that host gene polymorphisms can influence the host–microbe relationship opens opportunities for treating IBD and other complex microbiota-associated diseases such as psoriasis and obesity, Xavier concluded. According

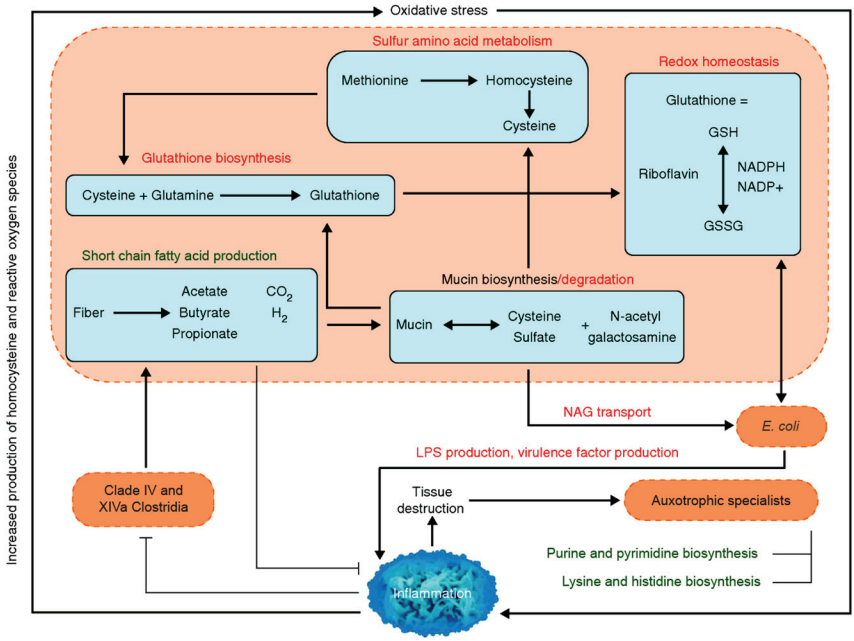


FIGURE WO-29 Proposed metabolic roles of the gut microbiome in IBD. Host-mediated processes (blue text) create an environment of oxidative stress in the intestine, which is more favorable to Enterobacteriaceae (increased abundance) than to clades IV and XIVa Clostridia (decreased abundance). This study's inferred IBD metagenomes include broadly increased oxidative metabolism, decreased SCFA production, and increased mucin degradation relative to healthy subjects. These processes all occur within microbes and rely on transport of small molecules to and from the lumen. The resulting tissue-destructive environment provides nutrients such as nucleotides and amino acids, which allow for increased growth of auxotrophic "specialists." Bacterial clades of interest are indicated in orange, bacterially mediated processes increased in IBD in red, and processes that decrease in green. Metabolic pathways differential in our IBD communities are contained in blue boxes.
NOTES: GSH and GSSG indicate reduced and oxidized forms of glutathione; LPS, lipopolysaccharide; NAG, N-acetyl galactosamine.
SOURCE: Morgan et al. (2012).

to Xavier, IBD research has progressed from identifying host gene associations, to examining the consequences of metabolites and the effects of diet, to monitoring the functional effects of gene expression, and recognizing disease-associated shifts in microbiome composition. Today, new technologies are needed to elucidate the mechanisms that connect human genetics and microbiome composition, resulting in complex diseases (Graham and Xavier, 2013).

CHALLENGES AND OPPORTUNITIES

We owe much of our biology and our individuality to the microbes that live on and in our bodies—a realization that promises to radically alter the principles and practice of medicine, public health, and basic science.
—Relman (2012)

The human metagenome is orders of magnitude more manipulatable than the human genome. This difference provides the opportunities to intercede to prevent and treat illness, if only we knew what was important!
—Blaser et al. (2013)

We have only just begun to appreciate the exquisitely complex relationships between microbial ecology and community dynamics in states of health and disease. The wealth of data and insights emerging from early studies of our resident microbes have complemented our growing knowledge of the human genome and furthered efforts to develop a holistic and dynamic view of health and disease states (Grice and Segre, 2012). The emerging picture is dauntingly complex, in which every individual is a unique host–microbial ecosystem—a dynamic assemblage of human and microbial cells that can be modulated throughout our lifetimes in ways that influence health and disease risk factors (Gonzalez et al., 2011; Relman, 2012). Many believe that this more nuanced conception of health and disease will move us closer to a vision of personalized medicine (Virgin and Todd, 2011; Holmes et al., 2012).

Exploration of the human microbiome is just one extension of the work initiated by environmental microbiologists over a half a century ago to reveal the vast diversity and complexity of the microbial world around us (Robinson et al., 2010). The same tools and techniques used to explore the microbial communities that live in and on us are also being used in a variety of fields to explore the role of microbial communities in driving biosphere processes—from the conversion of energy from the sun to the bioremediation of polluted landscapes. Research to identify factors influencing the formation, function, and stability of microbial communities in a wide range of biotic and abiotic systems will have unanticipated benefits for human, animal, plant, and ecosystem health and well-being (IOM, 2012).

Toward Ecological Therapeutics

Given the ecological parallels between assembly of the human microbiome and assembly of other ecological communities, we suggest that human medicine has more in common with park management than it does with battlefield strategy. To effectively manage a plant or animal community requires a multipronged approach of habitat restoration, promotion of native species, and targeted removal of invasives."
—Costello et al. (2012)

Unlike the host genome, our microbiome is dynamic, raising the possibility that it can be modified—with regard to its diversity, abundance, or resilience or in order to promote specific interactions between and among its microbial communities and the host—for therapeutic purposes (Lemon et al., 2012). Disease prevention and treatment would nurture the microbiome as a provider of critical services to the host. Changes in the microbiome associated with different stages of life or life events may present critical windows of opportunity for therapeutic interventions (Figure WO-30). Careful monitoring of community composition or function (via small molecules or other biomarkers) may better inform clinical decision making and help to devise strategies that favor host health (see Costello et al., 2012; Holmes et al., 2012).

Therapies designed to shift the microbiome from states associated with host disease to those that promote host health could take many forms, including

- prebiotics, which are substrates that promote targeted growth of a limited number of (beneficial) members of the microbiota;
- probiotics, which are live microorganisms that confer a health benefit on the host;
- small molecules and biological drugs targeted to the microbiota or to microbiota-responsive host factors;
- narrow-spectrum antibiotics to minimize collateral damage to the mutualist members of the microbiota, while decreasing selective pressure for the spread of antibiotic-resistant strains; and
- antibacterial conjugate vaccines that stimulate the immune system to remove only specific strains of a single species from the microbiota (see Sonnenburg and Fischbach, 2011; Lemon et al., 2012).

While noting that we have much to learn about whether and how to manipulate established microbial communities to achieve such goals, several speakers described efforts to lay a foundation for future microbiota-directed therapies—and in one case, to successfully treat potentially lethal dysbiosis through "ecological" intervention.

Therapeutic Molecules from the Human Microbiome

The chemical "conversations" that take place between host and microbe, and among members of the human microbiome, offer a promising source of molecules with therapeutic properties, according to speaker Michael Fischbach of the University of California, San Francisco (Dr. Fischbach's contribution may be found on pages 273-291 in Appendix A).

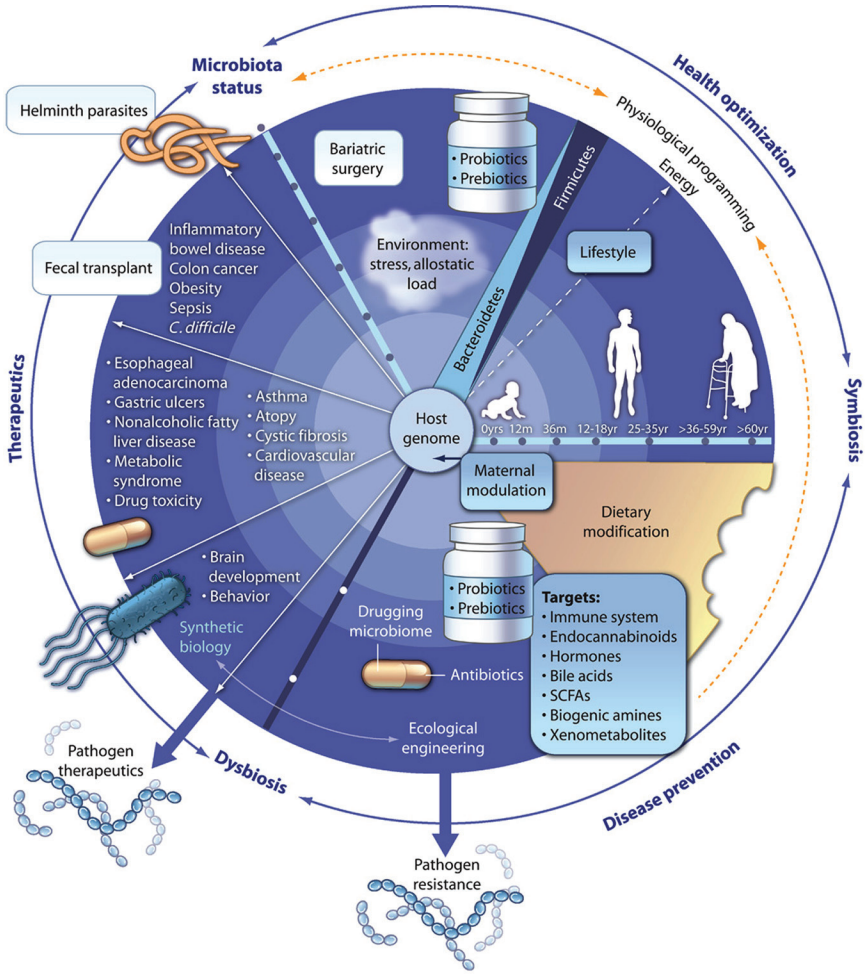


FIGURE WO-30 Therapeutic modulation of the gut microbiota: from the cradle to the grave. The changing relationship between the gut microbiota and the human host throughout life offers a series of time windows for therapeutic intervention. During infancy and early childhood, a healthy gut microbiota helps to avoid dysbiosis that can lead to disease later in life. In adulthood, corrective strategies to deal with emergent dysbiosis and associated diseases such as inflammatory bowel disease, obesity, and type 2 diabetes include modulating the microbiota using probiotics and prebiotics, antibiotics, bariatric surgery, or "drugging the microbiota." Drugs targeted to microbial enzymes could also be used to boost the efficacy and reduce the toxicity of therapeutics. For extreme cases of gut-related disease, fecal transplantation or helminth therapy may prove beneficial. In the future, it may be possible to prevent or treat abnormalities of the gut microbiota using modified organisms engineered through genetic or synthetic biology approaches.
SOURCE: Holmes et al. (2012).

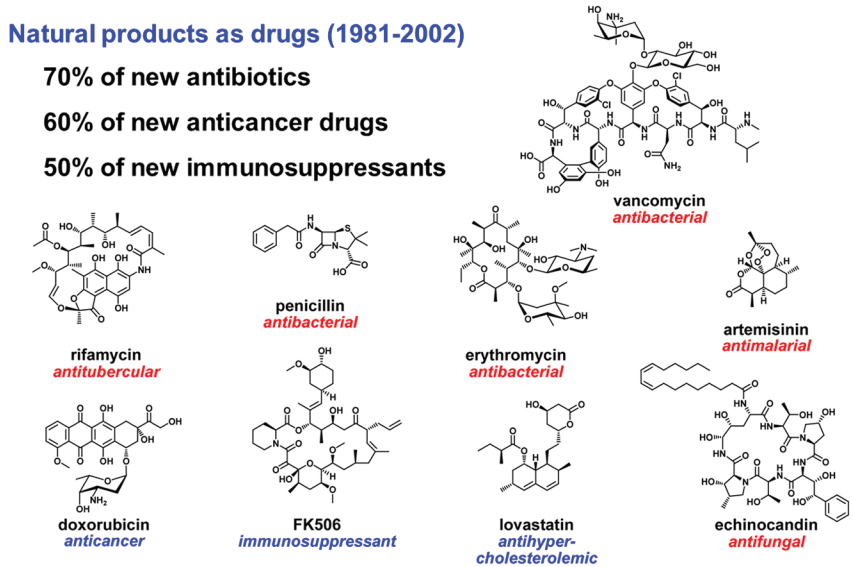


FIGURE WO-31 Natural products with medicinal relevance.
SOURCE: Newman et al. (2003).

Indeed, he noted, many current drugs derived from natural products[49]—including most antibiotics, the antihypercholesterolemic lovastatin, and the cancer drug doxorubicin, among others—are likely to have evolved to modulate interactions among microorganisms and between microbes and their hosts (Figure WO-31). Long a mainstay of drug discovery, natural products can now be identified and characterized with new technologies such as genomics and bioinformatics. These inquiries not only reveal novel and potentially useful compounds, he observed, but also provide "a new window into the biology of the organisms that produce these molecules, and maybe new ways of understanding why they make them."

The process of discovering natural products traditionally began with an environmental sample, such as soil, from which a wealth of small molecules—produced by microbes—could be isolated and characterized. Today, however, insight into the genetic architecture underlying the synthesis of these molecules presents the opportunity to rapidly identify sets of natural product genes in microbial genomes and connect them to the molecules they ultimately manufacture (Walsh and Fischbach, 2010). "If we . . . just went through all the bacterial genomes in the database and asked ourselves can we find all the sets

[49] Chemical compound or substance produced by a living organism.

of genes that probably are involved in making a small molecule . . . [then] we could make a prediction about what those molecules might be," Fischbach asserted. Such a search is feasible because of shared features of natural product gene sets that make them readily identifiable, he explained: clusters of genes—often located on mobile elements—that permit "assembly line" synthesis of small molecules.

Genomic databases provide abundant raw material for such searches, Fischbach said. "There are lots of genomes and metagenomes that have been sequenced, and the tools that we have developed are quite good at going through [these data] and finding sets of genes that encode a small molecule," he elaborated. He added that many well-characterized organisms that produce natural products such as antibiotics possess additional natural product gene sets that remain to be characterized (Figure WO-32). "This means that we probably do not need to go to the corners of the earth to find new molecules," he continued. "Maybe we should just go to our backyard or our freezer collection and find a way of tickling these organisms so they turn on the production of molecules they probably don't normally make under the artificial condition of cultivation in the lab."

In Fischbach's lab, the search for novel small molecules begins with computational analyses of genome sequences. "We have a training set of about 750 known biosynthetic gene clusters with known small molecule products, and we've devised a very simple machine-learning algorithm that has recovered an enormous set of biosynthetic gene clusters," he said. Fischbach's lab has begun to focus on the human microbiome because "many of the most interesting biosynthetic gene clusters that we found were not in exotic soil and marine microorganisms; they were in [human] gut and skin and oil bacteria." Experiments on candidate gene clusters have borne out the hypothesis that these molecules mediate host–microbe interactions and have led to the discovery of several intriguing small molecules that the Fischbach lab is currently investigating.

These initial findings represent just a tiny fraction of the untapped potential of small molecules produced by the human microbiome, noted Fischbach. "If I were to ask you, 'What are the top 10 bacterial strains by abundance in your gut?,' you would probably be able to figure the answer out by looking through some recent publications," Fischbach remarked. "But if I asked you what are the top 10 molecules in your gut and how do they compare one person to the next, you probably would not know, and I certainly don't know—but I think it's going to be just as important a question if these molecules indeed mediate signaling among microorganisms and between them and the host," he concluded. "There will be some interesting things to learn over the next few years."

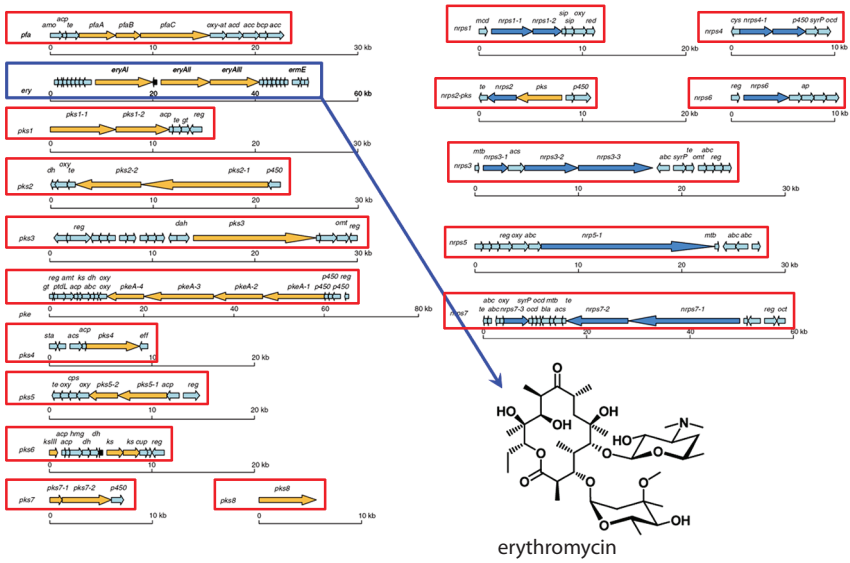


FIGURE WO-32 Genomics can reveal cryptic natural products. *Saccharopolyspora erythraea* is a well-characterized bacterial species that is cultivated on an industrial scale to produce the potent antibiotic, erythromycin. Analysis of this bacterium's genome revealed a number of additional gene clusters that potentially encode as yet unknown natural products.
SOURCE: Adapted from Oliynk et al. (2007).

Antimicrobial Peptides: Innate Defense Against Infection

The arsenal of proteins produced by the innate immune system represents another untapped resource for understanding and potentially manipulating host–microbe interactions (Figure WO-33). AMPs, an important component of innate immunity, have played a fundamental role in the evolution of multicellular life in a microbial world and represent an important paradigm for therapeutics, according to speaker Michael Zasloff of Georgetown University (Zasloff, 2002a,b) (Dr. Zasloff's contribution may be found on pages 489-496 in Appendix A).

AMPs protect the epithelial surfaces of all multicellular organisms (Zasloff, 2002a). Although diverse in composition, they share certain basic structural properties, most notably an affinity for the negatively charged outer surfaces of microbial cytoplasmic membranes. Among the more than 500 different antimicrobial peptides that have been discovered in organisms from insects to humans, most have a broad spectrum of activity and are capable of killing bacteria, fungi, and viruses. Most organisms display several different types of peptides on various epithelial surfaces, which together comprise a broad-spectrum antimicrobial

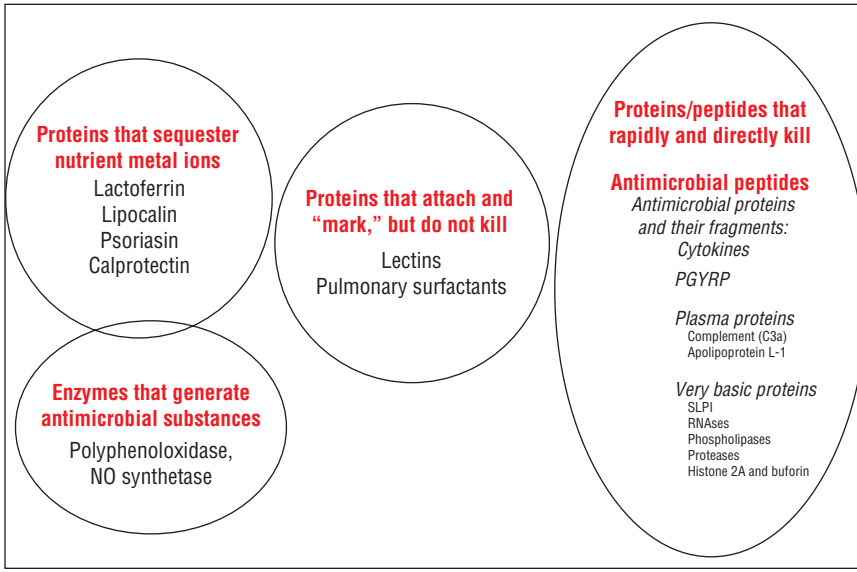


FIGURE WO-33 The secreted anti-infective protein/peptide arsenal of innate immunity. SOURCE: Zasloff (2013).

cocktail. "Anywhere on the body where a bacterial or fungal organism could creep in from the outside, we find an array of antimicrobial peptides," Zasloff observed. Zasloff also noted that AMPs appear to be highly adapted to the host organism's macroenvironment, as well as to the microenvironment of the specific tissues where they are expressed: "They are different, how they're regulated is different, what stimulates their expression is different, [and] their constitutive levels are different."

In the years since their initial discovery in the African clawed frog, *Xenopus laevis* (Zasloff, 1987), several AMPs have been developed as topical antibiotics (Zasloff, 2002b). Although AMPs are less prone to microbial resistance than conventional antibiotics and are highly active in vitro, in animal models of infection these molecules often require effective doses that are so high as to be unsafe. Nevertheless, the ubiquity, diversity, and effectiveness of AMPs continue to intrigue researchers.

AMPs have a variety of attributes through which they help mold and influence the microbiome. They are often produced on an ongoing basis, although some are induced by tissue injury and contribute to tissue defense and repair. Zasloff described these broad-ranging effects in sites such as the mammalian tongue and hair follicles. Both sites feature a complex array of both constitutive and inducible AMPs, which may contribute to the fact that although these sites seem vulnerable to pathogens, they in fact rarely become infected. He also

discussed the activation of the human AMP known as LL37 by vitamin D, as the means by which sunlight exposure stimulates antimicrobial defense of the skin and blood cells, noting that this phenomenon underlies phototherapy as a cure for tuberculosis infection of the skin (Liu et al., 2006).[50] AMPs are also of interest because of their involvement in tissue repair, Zasloff noted, for example, that LL37 interacts with receptors that in turn stimulate angiogenesis[51] (Koczulla et al., 2003).

Several diseases in humans are associated with impaired AMP function (Zasloff, 2002a). They include cystic fibrosis, in which a disturbed mucus barrier impedes the effectiveness of AMPs; shigellosis, in which the pathogen appears to suppress AMP expression, thereby increasing the likelihood of dysentery; and eczema, in which a dysfunctional inflammatory process suppresses AMP expression (Ong et al., 2002). Understanding the mechanisms by which AMPs shape the microbial ecology of their host and the processes underlying AMP-associated diseases offers another route to therapeutic discovery, Zasloff said.

To illustrate how much is left to be learned about host–microbe interactions, Zasloff concluded his presentation with his observations about the remarkable wound-healing capacity of dolphins that had suffered a severe shark bite (Figure WO-34) (Zasloff, 2011): "If one of us were bitten by a shark . . . we most certainly would die of sepsis. The shark has all sorts of *Vibrio* in its mouth and [shark bites] are among the most difficult infections to treat in man." However, continued Zasloff, dolphins in the ocean will "heal without infection or inflammation and with virtually no blood loss." Another way of isolating antimicrobial agents or identifying mechanisms for tissue repair is to study animals such as this, which appear to be "a warm blooded mammal [that has achieved] the most perfect solution to its coexistence with microbes."

Diagnostic Information from the Skin Microbiome

Human skin surfaces are complex ecosystems that provide diverse environments for our resident microorganisms, observed speaker Julie Segre, of the National Human Genome Research Institute (Dr. Segre's contribution may be found on pages 401-412 in Appendix A). In her discussion of recent phylogenetic surveys of bacterial (Grice et al., 2009) and fungal (Findley et al., 2013) diversity within and across multiple skin sites in healthy humans, Segre highlighted how these data are identifying opportunities to better diagnose and treat microbiota-related skin disorders. These findings are also providing insights into the role of endogenous microbes in disease states and the microbial interdependencies required to maintain healthy skin.

[50] Zasloff noted that Neils Finsen received the Nobel prize in 1903 for the development of phototherapy as a cure for tuberculosis infection of the skin.

[51] The formation of new blood vessels, especially blood vessels that supply oxygen and nutrients to cancerous tissues.

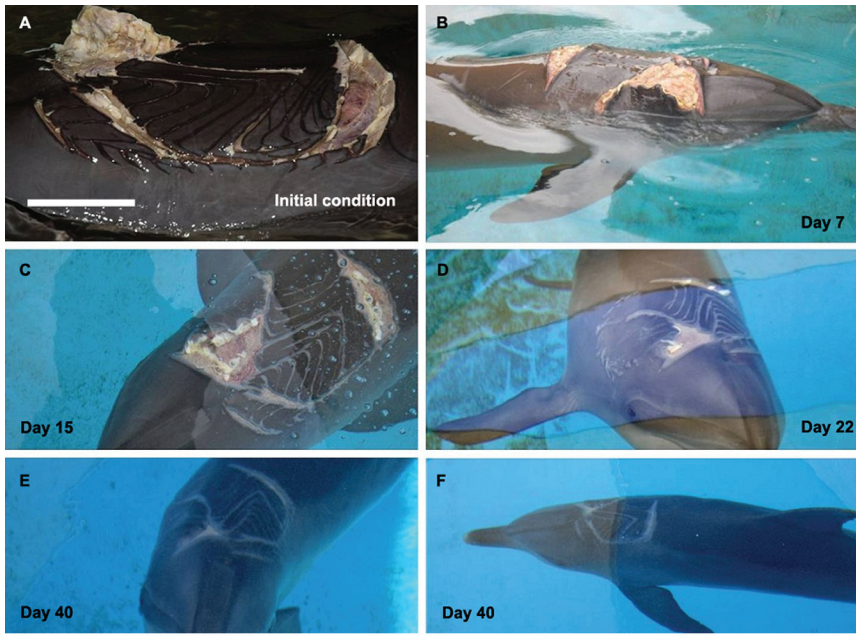


FIGURE WO-34 Wound healing in a severely wounded bottlenose dolphin. Injured animal was first observed on February 13, 2009. Photos were provided by Trevor Hassard who headed the rescue team at Tangalooma. Scale bar in Panel A corresponds to about 30 cm on the animal's surface.
SOURCE: Zasloff (2011).

Genomic surveys of the skin microbiome detected far greater microbial diversity—on the genera and species levels—than had previously been appreciated by studies limited to culturable organisms, Segre observed. Both bacterial and fungal diversity were found to be markedly site dependent—a finding that may help to explain the predilection of skin disorders for stereotypical sites. "Dermatologists have known for a long time that part of the diagnosis of a rash is about where it is on your body," she observed; for example, "eczema is always on the inside of the elbow and psoriasis is on the outside." Different forces appear to shape that diversity, she reported. While different bacterial communities were associated with oily, dry, or moist skin, fungal communities assorted themselves by body part. She also noted that they observed a dramatic shift in the skin microbiota associated with individuals before and after puberty (Oh et al., 2012), suggesting that there are transitions that we go through as humans that may be times at which we could "reset" our microbiota.

To illustrate the ways in which genomics is providing information that clinicians can use fairly rapidly, Segre discussed her lab's work on atopic dermatitis (AD), more commonly known as eczema, a condition associated with *Staphylococcus aureus* infection that affects approximately 15 percent of children in the United States (Kong et al., 2012). Over the past 30 years, the prevalence of atopic dermatitis has doubled, Segre stated, and it is widely suspected that this trend reflects changes in the skin microbiome resulting from decreased exposure to common infections in early life, in accordance with the hygiene hypothesis (Strachan, 1989). These children experience periodic "flares" of inflammation and intense itching on the insides of their elbows and behind their knees. In addition, Segre observed, approximately half of the children afflicted with severe eczema go on to develop asthma.

Through a clinical trial enrolling children with moderate to severe eczema, Segre and coworkers evaluated bacterial community diversity in the affected skin areas at baseline, during a flare, and after its resolution (Kong et al., 2012). During a flare, bacterial diversity plummets, and the amount of *S. aureus* dramatically increases, Segre reported. These observations may contribute to the generation of research hypotheses regarding potential preventable causes of flares; they may also be used as a signal to provide an early warning of incipient flares, and thus more effective management of this skin condition, she explained (Figure WO-35). Eczema, she noted, is currently treated with a variety of therapies, only some of which are effective for any given patient. According to Segre, it may eventually be possible to determine which therapies work best based on the species or genomic composition, and to treat disease earlier—possibly reducing their risk of progression to asthma. "We don't have to wait until we are all the way through these stories to come up with the mechanism if there are biomarkers we can use, based on the microbial diversity, to tailor therapies for patients or to predict who is about to have a skin flare," she concluded.

Fecal Microbiota Transplantation for Recurrent Clostridium difficile *Infection*

Over the past two decades, *Clostridium difficile* infections in humans have become among the most common hospital-acquired infections in the United States. Linked to more than 14,000 deaths (U.S.) each year, *C. difficile* infections have also become more difficult to treat (Kelly, 2013). According to speaker Josbert Keller of the University of Amsterdam, most *C. difficile* cases respond to antimicrobial therapy, but approximately one-quarter of patients experience a recurrent infection, which in turn raises their risk of subsequent relapses (Dr. Keller's contribution may be found on pages 347-355 in Appendix A). "*Clostridium difficile* [infection] can only occur in a bowel with a disturbed intestinal flora," he observed. "[Cases] typically occur after antibiotic treatment, and the longer such patients are hospitalized, the greater their risk of developing a *C. difficile* infection, with symptoms ranging from diarrhea to potentially deadly

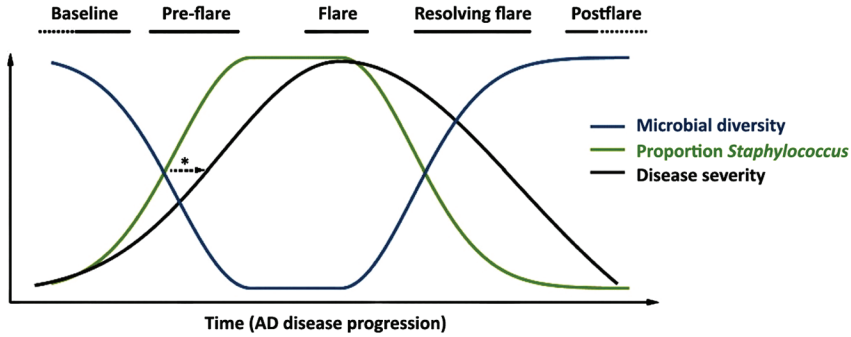


FIGURE WO-35 Atopic dermatitis (AD) progression hypothesis. Episodes of increased disease severity are accompanied by changes in microbial diversity. According to Segre, biomarkers for changes that occur during the preflare stage (*) may help patients to predict incipient flares and clinicians to better tailor therapies to individual patient needs
SOURCE: Kong et al. (2012).

fulminant colitis," he said. For patients with a second recurrence of *C. difficile* infection after antibiotic treatment, subsequent courses of antibiotics—typically vancomycin—have a success rate of less than 50 percent. In those patients, the prolonged disturbed bowel flora seems to have lost its restorative capacity (Chang et al., 2008), and the antibiotics fail to kill the pathogen's spores. In addition, an ineffective immune response may also contribute to the pathogenesis of recurrent *C. difficile* infection (Kyne et al., 2001).

Over the course of the past 50 years, more than 300 patients with recalcitrant *C. difficile* infections have been effectively treated by fecal microbiome transplantation (Figure WO-36) (Borody and Khoruts, 2012; Kelly, 2013).[52] While procedures have varied significantly, "What is consistent in all those reported patients is that there are very high success rates of about 90 percent," Keller stated. He and colleagues, having already successfully treated several patients with this technique, launched a clinical trial to compare fecal microbiome transplantation with a standard vancomycin regime (van Nood et al., 2009, 2013). The trial was halted early because of poor outcomes among patients who received vancomycin—more than two-thirds failed to resolve their infections, as compared with more than 80 percent of patients who received a single infusion of donor feces. Most of the patients in the antibiotic-treated group were subsequently treated with donor feces infusions, which cured infections in 15 out of 18 cases (Figure WO-37) (van Nood et al., 2013).

Patients who received donor feces infusions reported few side effects, Keller said, and most were cured within a day of treatment and remained free of

[52] The use of diluted mixtures of fecal matter from healthy animals has long been used by veterinarians as a treatment for stomach ailments, particularly in racehorses (McKenna, 2011).

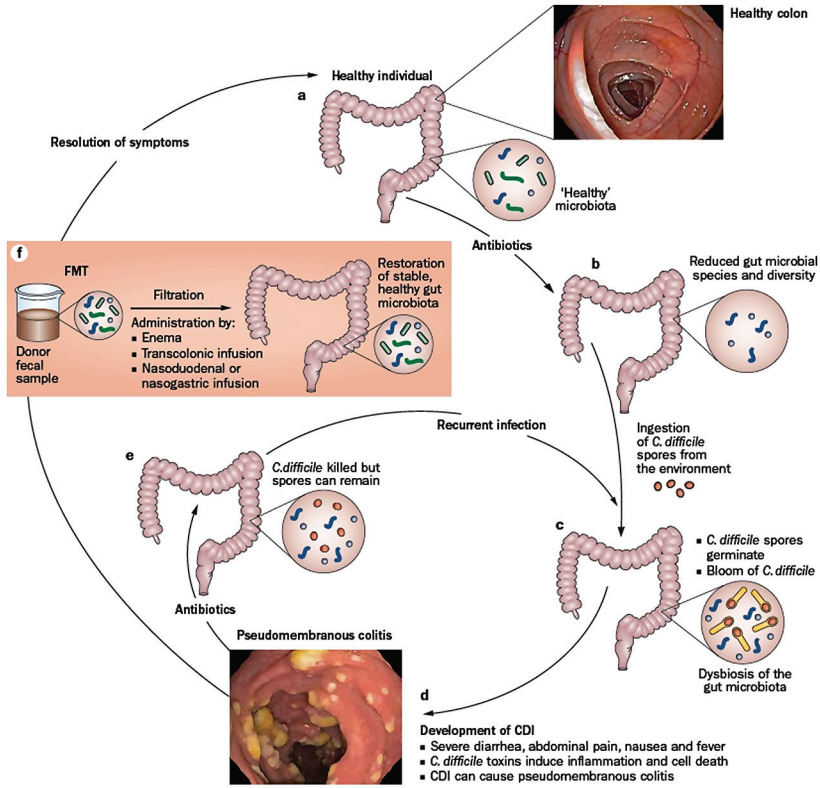


FIGURE WO-36 Fecal microbiota transplantation for patients with recalcitrant *Clostridium difficile* infection (CDI). CDI causes severe diarrhea, intestinal inflammation, and cell death as a result of toxin-mediated infection with the pathogenic bacteria. Patients with CDI are typically treated with antibiotics, which not only kill the pathogenic *C. difficile* but also exhibit activity against the dominant colonic microbiota phyla. Incomplete antibiotic eradication of *C. difficile* and persistent disturbance of healthy gut microbiota can result in recurrent CDIs. Transplantion of fecal microbiota from a healthy donor into an individual with CDI can restore the healthy gut microbiota in the patient's diseased colon, thereby probably preventing further outgrowth of *C. difficile* and leading to resolution of symptoms.
SOURCE: Borody and Khoruts (2012).

C. difficile during the 10-week follow-up period. Before-and-after comparisons of patient bowel flora revealed that the procedure increased microbiota diversity to resemble that of the donor, he reported. While Keller expressed some concern about careful screening of the donor feces to prevent the transmission of an infectious disease from a donor to recipient, he concluded that fecal microbiota transplantation was a safe (if unappealing) treatment for *C. difficile* infection.

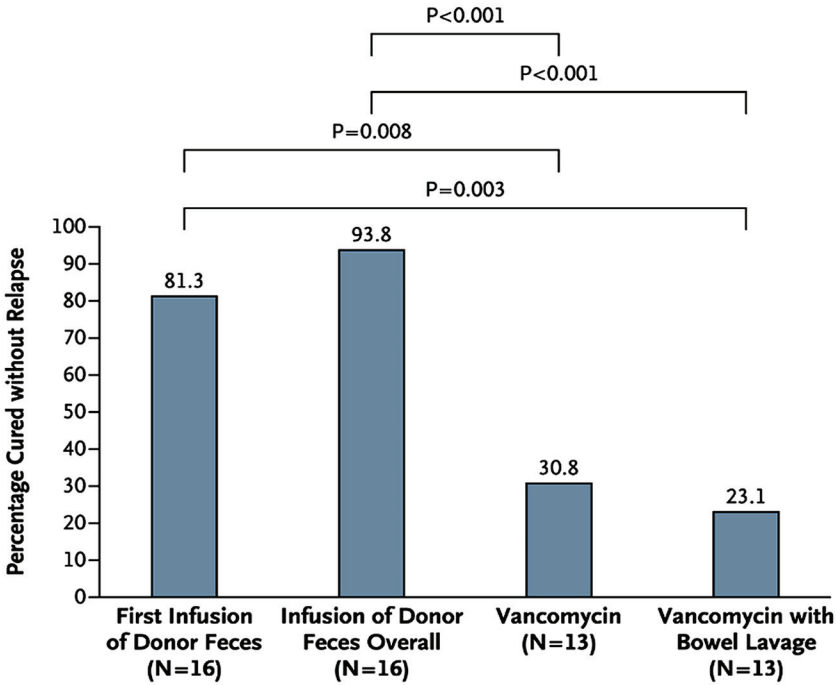


FIGURE WO-37 Rates of cure without relapse during 10 weeks of follow-up for recurrent *Clostridium difficile* infection. Shown are the proportion of patients who were cured by the infusion of donor feces (first infusion and overall results), by standard vancomycin therapy, and by vancomycin therapy plus bowel lavage.
SOURCE: van Nood (2013).

Keller also noted that researchers are investigating the potential for fecal microbiota transplants to treat other diseases, including IBD, obesity, and metabolic syndrome. However, he cautioned, for diseases other than recurrent *C. difficile* infection, fecal transplantation is a very experimental approach, and much work remains to be done before it is ready for clinical application.

Keller expressed his hope that a more appealing probiotic alternative to fecal transplantation would soon become available. A mixture of 10 different facultatively aerobic and anaerobic bacteria diluted in sterile saline has been used as a treatment for chronic relapsing *C. difficile* infection (Tvede and Rask-Madsen, 1989). He also discussed a recent proof of principle study that used a synthetic stool mixture or human probiotic called RePOOPulate. Administration of this multispecies mixture (33 purified isolates of bacteria from a healthy donor) resolved *C. difficile* infections in two patients, each of whom had failed to recover after at least three courses of antibiotics. Sequencing performed before

and 6 months post treatment suggests that the strains present in this multispecies community of bacteria had stably colonized the colon (Petrof et al., 2013).

Regulatory Considerations

The various potential and actual interventions described during the workshop, Relman noted, raise complex questions about oversight and prudent regulation. For example, efforts to alter microbiota function by introducing microbial communities, rather than a specific species, will be difficult to monitor, he said. "You are no longer saying we either did or did not have this organism, or did or did not see a fall in this particular well-understood end point," Relman explained. In these cases, biomarkers and surrogate endpoints will need to be developed to gauge the effectiveness of treatment, he concluded.

Calling fecal transplantation a "great test case," Goodman nonetheless cautioned about potential harms inherent in unrefined microbiota-based approaches to medicine, including the introduction of antibiotic-resistant bacteria through probiotic therapy. "I think that this is one of these emerging areas where the trick is going to be to get the right balance between protecting patients and letting the field . . . move forward," he observed. In the case of fecal transplantation, Goodman agreed with Keller's prior assessment that it provides a starting point for developing more precise treatments, based on an understanding of the therapeutic effects of specific microbial strains.[53]

Even with this knowledge, there are risks, as Relman pointed out, through the example of the use of "bacterial interference" therapy in the 1960s, a practice premised on the idea of using an "interfering" nonpathogenic strain of *Staphylococcus aureus* to prevent colonization of the nasal mucosa by pathogenic strains. Doctors successfully used interference therapy to curtail epidemics of disease caused by pathogenic strains of *S. aureus* in nurseries, the recurrence of boils in older persons, and persistent nasal carriage of pathogenic strains of *S. aureus* in adults (Boris et al., 1964; Light et al., 1965; Mackiowak, 1982). In 1972, Houck et al. (1972) reported that a 5.9 percent (38) of newborns treated as part of a bacterial interference program later developed disease thought to be related to the "interfering" strain of *S. aureus,* including pustules, conjunctivitis, and abscess. One newborn died from septicemia and meningitis (Houck et al., 1972).

[53] On May 2, 2013, the Food and Drug Administration, Center for Biologics Evaluation and Research, and the National Institutes of Health's National Institute of Allergy and Infectious Diseases, jointly hosted a public workshop titled "Fecal Microbiota for Transplantation" to exchange information with the medical and scientific community about the regulatory and scientific issues associated with fecal microbiota for transplantation. For more information, see http://www.fda.gov/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/ucm341643.htm (accessed January 9, 2014).

Rethinking the Antimicrobial Arsenal

Dramatic changes in human ecology over the past 40 years may have long-standing consequences for the transmission and selection of our microbiota (Table WO-2). As noted in many of the workshop presentations, correlations between changes to our microbial ecology and the recent, rapid rise of many chronic diseases may reveal the limitations of clinical medicine's "war" on microbes that has been pursued since the articulation and promotion of the germ theory of disease in the 19th century.[54] Rather than wage a war on germs, we should be conserving—and possibly replacing—our ancestral microbiota, noted Blaser. This notion was raised in presentations and discussions throughout the workshop, culminating in a lively conversation on the need to consider the collateral damage to the beneficial microbes associated with antibiotic use; whether and how we should restrict the use of antimicrobial interventions—from antibiotics and antiseptics to the widespread use of hand sanitizers—and how to convey such messages to a germ-phobic public.

Amplifying Blaser's suggestion that current levels of antibiotic exposure in children may lead to lifelong repercussions such as asthma, obesity, and increased susceptibility to infectious diseases, Belkaid speculated that "the sum of the infection and the antibiotic treatment is very likely to be [much] more severe than just antibiotic treatment." She advised that trials be devised to assess these risks over the long term. Blaser agreed, stating that "[t]he experiments have to be done, the epidemiologic studies have to be done, the clinical studies have to be done, the animal models have to be done, and they are being done." In the meantime, Blaser added, it should not be assumed that antibiotic use comes without a biological cost—that they are "innocent until proven guilty. The public is still [waging a] war against germs, and we have to educate the public better, that there are good germs as well as bad ones," he concluded.

Using antimicrobials on the skin "is an absurdity," said Belkaid. "I think there is absolutely no reason to remove what we have evolved with to be a perfectly viable way of coexisting." The skin is an important entry point for allergens and has broad inflammatory capabilities, so its natural barriers should not be disturbed, she said. "I think being exposed to commensals or microbes is a perfectly healthy way of living." The use of all antimicrobials, including preservatives, should be questioned, Zasloff added, but public fear mongering should be avoided.

Illustrating the different ways that microbes are currently perceived by the public, Segre commented on the "great divide between Activia® yogurt and Purell® hand sanitizer" noting that "in this country we want to colonize our guts

[54] The germ theory of disease, consolidated in the 19th century by Louis Pasteur and Robert Koch, understood each acute infectious disease to be caused by a single pathogen. Isolation of the pathogen via "Koch's postulates" provided standard confirmation. Once isolated, the pathogen could be targeted with antimicrobial therapies, with antibiotics becoming the weapon of choice in the 20th century. The suitable metaphor for expressing the relationship between microbes and humans as understood at the time was thus "warfare" (Lederberg, 2000; IOM, 2006).

TABLE WO-2 Changes in Human Ecology That Might Affect Microbiota Composition

Change	Consequence
Clean water	Reduced fecal transmission
Increase in Caesarean sections	Reduced vaginal transmission
Increased use of preterm antibiotics	Reduced vaginal transmission
Reduced breastfeeding	Reduced cutaneous transmission and a changed immunological environment
Smaller family size	Reduced early-life transmission
Widespread antibiotic use	Selection for a changing composition
Increased bathing, showering, and use of antibacterial soaps	Selection for a changing composition
Increased use of mercury-amalgam dental fillings	Selection for a changing composition

SOURCE: Blaser and Falkow (2009).

and sterilize our skin." The public needs to understand the difference between protecting vulnerable patients from life-threatening infection and everyday hygiene, Segre contended. Though Americans have largely manufactured their "need" for hand sanitizers, she strongly advocated hand washing. "We are concerned about oral-fecal transmission," she explained. "That is really what we need to be worried about in terms of the normal human health . . . [that] you are washing your hands after going to the bathroom, before you prepare food, or eat food."

Manipulation of Our Microbiomes

Given mounting evidence of the biological costs of antibiotic therapy—long considered a medical triumph—workshop participants were at best cautiously optimistic regarding prospects for the strategic manipulation of microbiota to improve host health. While the use of probiotics to treat IBD (as described by Keller), and perhaps the early-life replacement of *H. pylori* (as described by Blaser), or prebiotic creams to maintain the skin microbiota (as mentioned by Segre) have been proposed, some participants expressed overall skepticism with the notion of altering microbiota. "The idea that maybe we can intervene somehow and manage this system in a very effective way to sort of re-create what should have happened naturally—I hope we can," Forney stated. "But we are talking about trying to tweak something that has developed over hundreds of thousands of years, with all the unsuccessful experiments becoming extinct."

Virgin disagreed with the premise that we are "trapped by our own evolution." On the contrary, he said, "We can make cells do things that they can't do 'naturally' by manipulating signaling pathways, once we understand that, I think the idea that you can reprogram things is perfectly legitimate." McFall-Ngai

agreed, on the basis that human life histories are varied and plastic, and our relationship with our microbiota is a developmental one.

Douglas presented another view, stating that we should be careful not to be too "Panglossian"[55] about our relationship with our microbiota. "It could well be that in some instances, what we're seeing is that the animal host or human host is actually addicted to the microbiota," she said. "Perhaps we can't do without them—not because they are beneficial, but because animal signaling and metabolic systems have evolved in the context of the universal presence of these microorganisms. We should discriminate quite carefully between instances of addiction versus instances of benefit."

"We certainly are not smart enough to think that we could deliberately reprogram the entire [microbiota]," Relman said—moreover, he added, such a feat could only be achieved through a series of manipulations, akin to successional stages. Rohwer agreed, observing that science is far better at tearing things apart than putting them together. "If you really want to reestablish a complex ecosystem, it may be very complicated," he concluded.

Such hypothetical debates typify this emerging field of inquiry, during what Relman called "a lush time in the early phase of a science before the reality sets in and we come back down to some reasonable level of balance." In the meantime, he observed, "It is a pretty exciting time in this area of work, and it's made exciting by the fact that we have the means of discovering complexity that offers untold possible explanations and mechanisms. But we don't know enough about any of it to realize that much of it may be wrong."

Advancing Research

Several workshop participants emphasized the importance of assembling multidisciplinary teams and to train the next generation of scientists. Goodman advocated the training of generalist scientists, as well as specialists, who understand clinical and population science in a broad context, as well as basic research, and can coordinate members in an interdisciplinary team and evaluate the overall value of their work. Otherwise, he worried, "big science" may be done that is either irrelevant or that drains funding and talent from better opportunities.

There is great potential for collaboration—indeed, it will be essential. "Understanding this level of biological complexity will require the involvement of statisticians, computational biologists, geneticists, pathogenesis experts, virologists, bacteriologists, and parasitologists in an integrated fashion to identify mechanistically important interactions," Virgin and Todd observe (2011). As many also noted, existing organizational and funding structures fail to reward participants in multidisciplinary efforts, either in terms of promotion or grants.

[55] Blindly or naively optimistic, after *Pangloss*, an optimistic character in Voltaire's *Candide* (1759).

Xavier, who said he had been relatively successful in funding multidisciplinary studies, nonetheless observed, "When you submit large program projects, they get reviewed by individual people who read individual sections. They do not see how the entire grant comes together. You are reviewed by an individual investigator looking at his or her area of expertise without paying any attention to the interaction of the team. This often brings the grant down because the view is that you are now doing enough in that area that the reviewer is an expert in. It is very clear by reading the comments that this person paid no attention to how synergy was brought together in this application."

Exploration of our microbial ecology will also continue to benefit from the techniques, concepts, and principles developed through investigations in a wide variety of biotic and abiotic systems, and several participants noted the need to break down the arbitrary divide between clinical microbiology and the work of environmental microbiologists, ecologists, veterinary scientists, and myriad other disciplines. Relman advocated for a greater emphasis on transdisciplinarity, which occurs when "experts from one discipline adopt the perspectives of other disciplines in formulating questions and in designing experimental plans for addressing them" (Relman, 2012). Bohannan noted that transdisciplinarity may require a reconfiguration of the review process. "I think there are assumptions made about what sort of science is likely to be fruitful in terms of human health. There is a hard line drawn that separates NIH from other funding agencies. For people like me who bridge those questions, it means it is difficult to get really innovative grants reviewed."

Looking Ahead

The impact of our growing appreciation of the microbial world around us extends well beyond human health and disease. As observed by several workshop participants, new tools and approaches have inspired fresh approaches to the study of host–microbe–environment interactions in a variety of organisms and settings. These novel approaches are being exploited to investigate an increasingly broad range of topics—from the influence of the microbiota on the origins of animals to defining what constitutes an "individual" organism and exploring global principles of ecology (Gilbert et al., 2012; Shade et al., 2012; McFall-Ngai et al., 2013). The complex relationships between microbial community diversity, function, stability, and resilience are relevant to problems confronting scientists in a variety of contexts. As noted by Shade et al. (2012), "[m]icrobial communities are at the heart of all ecosystems"—thus, biomedical, environmental, agricultural, and bioenergy research all share "a common challenge: to predict how functions and composition of microbial communities respond to disturbances" (Shade et al., 2012).

REFERENCES

Arnold, I. C., N. Dehzad, S. Reuter, H. Martin, B. Becher, C. Taube, and A. Müller. 2011. *Helicobacter pylori* infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *Journal of Clinical Investigation* 121(8):3088-3093.

Atherton, J. C., and M. J. Blaser. 2009. Coadaptation of *Helicobacter pylori* and humans: Ancient history, modern implications. *Journal of Clinical Investigation* 119(9):2475-2487.

Bach, J. F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *New England Journal of Medicine* 347:911-920.

Bäckhed, C., M. Fraser, Y. Ringel, M. E. Sanders, R. B. Sartor, P. M. Sherman, J. Versalovic, V. Young, and B. B. Finlay. 2012. Defining a healthy human gut microbiome: Current concepts, future directions, and clinical applications. *Cell Host and Microbe* 12(5):611-622.

Badri, D. V., T. L. Weir, D. van der Lelie, and J. M. Vivanco. 2009. Rhizosphere chemical dialogues: Plant–microbe interactions. *Current Opinion in Biotechnology* 20(6):642-650.

Barr, J. J., R. Auro, M. Furlan, K. L. Whiteson, M. L. Erb, J. Pogliano, A. Stotland, R. Wolkowicz, A. S. Cutting, K. S. Doran, P. Salamon, M. Youle, and F. Rohwer. 2013. Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proceedings of the National Academy of Sciences of the United States of America*. DOI:10.1073/pnas.1305923110.

Barton, E. S., D. W. White, J. S. Cathelyn, K. A. Brett-McClellan, M. Engle, M. S. Diamond, V. L. Miller, and H. W. Virgin. 2007. Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447(7142):326-329.

Bates, J. M., J. Akerlund, E. Mittge, and K. Guillemin. 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host & Microbe* 2(6):371-382.

Belkaid, Y., and S. Naik. 2013. Compartmentalized and systemic control of tissue immunity by commensals. *Nature Immunology* 14:646-653.

Belkaid, Y., N. Bouladoux, T. W. Hand. 2013. Effector and memory T cell responses to commensal bacteria. *Trends in Immunology* 34(6):299-306.

Benchimol, E. I., A. Guttmann, A. M. Griffiths, L. Rabeneck, D. R. Mack, H. Brill, J. Howard, J. Guan, and T. To. 2009. Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: Evidence from health administrative data. *Gut* 58(11):1490-1497.

Berendsen, R. L., C. M. J. Pieterse, and P. A. H. M. Bakker. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* 17(8):478-486.

Blaser, M. J. 2006. Who are we? *EMBO Reports* 7(10):956-960.

Blaser, M. J., and J. C. Atherton. 2004. *Helicobacter pylori* persistence: Biology and disease. *Journal of Clinical Investigation* 113(3):321-333.

Blaser, M. J., and S. Falkow. 2009. What are the consequences of the disappearing human microbiota? *Nature Reviews Microbiology* 7(12):887-894.

Blaser, M. J., Y. Chen, and J. Reibman. 2008. Does *Helicobacter pylori* protect against asthma and allergy? *Gut* 57(5):561-567.

Blaser, M., P. Bork, C. Fraser, R. Knight, and J. Wang. 2013. The microbiome explored: Recent insights and future challenges. *Nature Reviews Microbiology* 11(3):213-217.

Blustein, J., T. Attina, M. Liu, A. M. Ryan, L. M. Cox, M. J. Blaser, and L. Trasande. 2013. Association of caesarean delivery with child adiposity from age 6 weeks to 15 years. *International Journal of Obesity.* doi: 10.1038/ijo.2013.49. [Epub ahead of print]

Bohannan, B. 2013. *Session III: The application of ecological concepts to host-associated microbial communities*. Presentation at the Forum on Microbial Threats Workshop, Microbial Ecology in States of Health and Disease, Washington, DC, Institute of Medicine, Forum on Microbial Threats. March 19.

Boris, M., T. F. Sellers, H. F. Eichenwald, J. C. Ribble, and H. R. Schinefeld. 1964. Bacterial interference: Protection of adults against nasal Staphylococcus aureus infection after colonization with a heterologous *S. aureus* strain. *American Journal of Diseases of Children* 108(3):252-261.

Borody, T. J., and A. Khoruts. 2012. Fecal microbiota transplantation and emerging applications. *Nature Reviews Gastroenterology and Hepatology* 9:88-96.

Boyle, N. R., and R. T. Gill. 2012. Tools for genome-wide strain design and construction. *Current Opinion in Biotechnology* 23(5):666-671.

Buchen, L. 2010. Microbiology: The new germ theory. *Nature* 468(7323):492-495.

Bulgarelli, D., M. Rott, K. Schlaeppi, E. Ver Loren van Themaat, N. Ahmadinejad, F. Assenza, P. Rauf, B. Huettel, R. Reinhardt, E. Schmelzer, J. Peplies, F. O. Gloeckner, R. Amann, T. Eickhorst, and P. Schulze-Lefert. 2012. Revealing structure and assembly cues for arabidopsis root-inhabiting bacterial microbiota. *Nature* 488(7409):91-95.

Cadwell, K., J. Y. Liu, S. L. Brown, H. Miyoshi, J. Loh, J. K. Lennerz, C. Kishi, W. Kc, J. A. Carrero, S. Hunt, C. D. Stone, E. M. Brunt, R. J. Xavier, B. P. Sleckman, E. Li, N. Mizushima, T. S. Stappenbeck, and H. W. Virgin IV. 2008. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal paneth cells. *Nature* 456(7219):259-263.

Cadwell, K., K. K. Patel, N. S. Maloney, T. C. Liu, A. C. Ng, C. E. Storer, R. D. Head, R. Xavier, T. S. Stappenbeck, and H. W. Virgin. 2010. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16l1 phenotypes in intestine. *Cell* 141(7):1135-1145.

Canny, S. P., G. Goel, T. A. Reese, X. Zhang, R. Xavier, and H. W. Virgin. 2013. Latent gammaherpesvirus 68 infection induces distinct transcriptional changes in different organs. *Journal of Virology*. Epub ahead of print.

Carey, H. V. 1990. Seasonal changes in mucosal structure and function in ground squirrel intestine. *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology* 259(2):R385-R392.

———. 2013. *Session I: Annual cycles of extreme dietary change shape gut microbiota and their hibernator hosts.* Presentation at the Forum on Microbial Threats Workshop, Microbial Ecology in States of Health and Disease, Washington, DC, Institute of Medicine, Forum on Microbial Threats. March 18.

Carey, H. V., W. A. Walters, and R. Knight. 1992. Effects of fasting and hibernation on ion secretion in ground squirrel intestine. *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology* 263(6):R1203-R1208.

———. 2013. Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology Regulatory, Integrative, and Comparative Physiology* 304(1):R33-R42.

Carey, H. V., M. T. Andrews, and S. L. Martin. 2003. Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews* 83:1153-1181.

Chai, G., L. Governale, A. W. McMahon, J. P. Trinidad, J. Staffa, and D. Murphy. 2012. Trends of outpatient prescription drug utilization in US children, 2002-2010. *Pediatrics* 130(1):23-31.

Chang, J. Y., D. A. Antonopoulos, A. Kalra, A. Tonelli, W. T. Khalife, T. M. Schmidt, and V. B. Young. 2008. Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. *Journal of Infectious Diseases* 197(3):435-438.

Chen, Y., and M. J. Blaser. 2007. Inverse associations of *Helicobacter pylori* with asthma and allergy. *Archives of Internal Medicine* 167(8):821-827.

———. 2008. *Helicobacter pylori* colonization is inversely associated with childhood asthma. *Journal of Infectious Diseases* 198(4):553-560.

Cho, I., and M. J. Blaser. 2012. The human microbiome: At the interface of health and disease. *Nature Reviews Genetics*13(4):260-270.

Cho, I., S. Yamanishi, L. Cox, B. A. Methe, J. Zavadil, K. Li, Z. Gao, D. Mahana, K. Raju, I. Teitler, H. Li, A. V. Alekseyenko, and M. J. Blaser. 2012. Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* 488(7413):621-626.

Chow, W. H., M. J. Blaser, W. J. Blot, M. D. Gammon, T. L. Vaughan, H. A. Risch, G. I. Perez-Perez, J. B. Schoenberg, J. L. Stanford, H. Rotterdam, A. B. West, and J. F. Fraumeni, Jr. 1998. An inverse relation between caga+ strains of *Helicobacter pylori* infection and risk of esophageal and gastric cardia adenocarcinoma. *Cancer Research* 58(4):588-590.

Clemente, J. C., L. K. Ursell, L. W. Parfrey, and R. Knight. 2012. The impact of the gut microbiota on human health: An integrative view. *Cell* 148(6):1258-1270.

Coppieters, K. T., and D. Elewaut. 2012. Natural killer T cells: Born in the thymus, raised in the gut. *Gastroenterology* 143(2):293-296.

Corley, D. A., A. Kubo, T. R. Levin, G. Block, L. Habel, W. Zhao, P. Leighton, G. Rumore, C. Quesenberry, P. Buffler, and J. Parsonnet. 2008. *Helicobacter pylori* infection and the risk of Barrett's oesophagus: A community-based study. *Gut* 57(6):727-733.

Costello, E. K., K. Stagaman, L. Dethlefsen, B. J. Bohannan, and D. A. Relman. 2012. The application of ecological theory toward an understanding of the human microbiome. *Science* 336(6086):1255-1262.

Couzin-Frankel, J. 2010. Bacteria and asthma: Untangling the links. *Science* 330(6008):1168-1169.

Cox, L., and M. A. Blaser. 2013. Pathways in microbe-induced obesity. *Cell Metabolism* 17:883-894.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16s rRNA gene database and workbench compatible with arb. *Applied and Environmental Microbiology* 72(7):5069-5072.

Desbrosses, G. J., and J. Stougaard. 2011. Root nodulation: A paradigm for how plant-microbe symbiosis influences host developmental pathways. *Cell Host Microbe* 10(4):348-358.

Dethlefsen, L., M. McFall-Ngai, and D. A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449(7164):811-818.

Domazet-Loso, T., and D. Tautz. 2008. An ancient evolutionary origin of genes associated with human genetic diseases. *Molecular Biology and Evolution* 25(12):2699-2707.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences of the United States of America* 107(26):11971-11975.

Dominguez-Bello, M. G., M. J. Blaser, R. E. Ley, and R. Knight. 2011. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* 140(6):1713-1719.

Douglas, A. E. 2011. Lessons from studying insect symbioses. *Cell Host Microbe* 10(4):359-367.

Dubos, R., R. W. Schaedler, R. Costello, and P. Hoet. 1965. Indigenous, normal, and autochthonous flora of the gastrointestinal tract. *Journal of Experimental Medicine* 122:67-76.

Dusko Ehrlich, S. 2010. [metagenomics of the intestinal microbiota: Potential applications]. *Gastroenterologie Clinique et Biologique* 34(Suppl 1:)S23-S28.

Eberl, G. 2010. A new vision of immunity: Homeostasis of the superorganism. *Mucosal Immunology* 3(5):450-460.

———. 2012. Development and evolution of RORγt+ cells in a microbe's world. *Immunological Reviews* 245(1):177-188.

Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* 308(5728):1635-1638.

Ege, M. J., M. Mayer, A. C. Normand, J. Genuneit, W. O. Cookson, C. Braun-Fahrlander, D. Heederik, R. Piarroux, and E. von Mutius. 2011. Exposure to environmental microorganisms and childhood asthma. *New England Journal of Medicine* 364(8):701-709.

Eisen, J. A. 2007. Environmental shotgun sequencing: Its potential and challenges for studying the hidden world of microbes. *PLoS Biology* 5(3).

Everard, A., C. Belzer, L. Geurts, J. P. Ouwerkerk, C. Druart, L. B. Bindels, Y. Guiot, M. Derrien, G. G. Muccioli, N. M. Delzenne, W. M. de Vos, and P. D. Cani. 2013. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 110(22):9066-9071.

Fidel, P. L., Jr. 2007. History and update on host defense against vaginal candidiasis. *American Journal of Reproductive Immunology* 57(1):2-12.

Findley, K., J. Oh, J. Yang, S. Conlan, C. Deming, J. A. Meyer, D. Schoenfeld, E. Nomicos, M. Park, H. H. Kong, and J. A. Segre. 2013. Topographic diversity of fungal and bacterial communities in human skin. *Nature* 498(7454):367-370.

Forman, D., P. Webb, and J. Parsonnet. 1994. *H. pylori* and gastric cancer. *Lancet* 343(8891):243-244.

Frank, D. N., A. L. St. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America* 104(34):13780-13785.

Fraune, S., and T. C. Bosch. 2010. Why bacteria matter in animal development and evolution. *Bioessays* 32(7):571-580.

Fredricks, D. N., and D. A. Relman. 1996. Sequence-based identification of microbial pathogens: A reconsideration of Koch's postulates. *Clinical Microbiology Reviews* 9:18-33.

Gajer, P., R. M. Brotman, G. Bai, J. Sakamoto, U. M. Schutte, X. Zhong, S. S. Koenig, L. Fu, Z. S. Ma, X. Zhou, Z. Abdo, L. J. Forney, and J. Ravel. 2012. Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine* 4(132):132ra152.

Garrido, D., D. Barile, and D. A. Mills. 2012. A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. *Advances in Nutrition* 3(3):415S-421S.

Gilbert, S. F., J. Sapp, and A. I Tauber. 2012. A symbiotic view of life: We have never been individuals. *Quarterly Review of Biology* 87(4):325-341.

Gonzalez, A., J. C. Clemente, A. Shade, J. L. Metcalf, S. Song, B. Prithiviraj, B. E. Palmer, and R. Knight. 2011. Our microbial selves: What ecology can teach us. *EMBO Reports* 12(8):775-784.

Gordon, J. I. 2012. Honor thy gut symbionts redux. *Science* 336(6086):1251-1253.

Gottel, N. R., H. F. Castro, M. Kerley, Z. Yang, D. A. Pelletier, M. Podar, T. Karpinets, E. Uberbacher, G. A. Tuskan, R. Vilgalys, M. J. Doktycz, and C. W. Schadt. 2011. Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied Environmental Microbiology* 77(17):5934-5944.

Graham, D. B., and R. J. Xavier. 2013. From genetics of inflammatory bowel disease towards mechanistic insights. *Trends in Immunology* 34(8):371-378.

Grice, E. A., and J. A. Segre. 2012. The human microbiome: Our second genome. *Annual Review of Genomics and Human Genetics* 13:151-170.

Grice, E. A., H. H. Kong, S. Conlan, C. B. Deming, J. Davis, A. C. Young, G. G. Bouffard, R. W. Blakesley, P. R. Murray, E. D. Green, M. L. Turner, and J. A. Segre. 2009. Topographical and temporal diversity of the human skin microbiome. *Science* 324(5931):1190-1192.

Guillemin, K. 2013. Session II: Interactions and functions of the gut microbiota in a model vertebrate host. Presentation at that Forum on Microbial Threats Workshop, Microbial Ecology in States of Health and Disease, Washington DC, Institute of Medicine, Forum on Microbial Threats. March 18.

Han, M. K., Y. J. Huang, J. J. Lipuma, H. A. Boushey, R. C. Boucher, W. O. Cookson, J. L. Curtis, J. Erb-Downward, S. V. Lynch, S. Sethi, G. B. Toews, V. B. Young, M. C. Wolfgang, G. B. Huffnagle, and F. J. Martinez. 2012. Significance of the microbiome in obstructive lung disease. *Thorax* 67(5):456-463.

Hand, T. W., L. M. Dos Santos, N. Bouladoux, M. J. Molloy, A. J. Pagan, M. Pepper, C. L. Maynard, C. O. Elson, 3rd, and Y. Belkaid. 2012. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science* 337(6101):1553-1556.

Handelsman, J. 2004. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* 68(4):669-685.

Handley, S. A., L. B. Thackray, G. Zhao, R. Presti, A. D. Miller, L. Droit, P. Abbink, L. F. Maxfield, A. Kambal, E. Duan, K. Stanley, J. Kramer, S. C. Macri, S. R. Permar, J. E. Schmitz, K. Mansfield, J. M. Brenchley, R. S. Veazey, T. S. Stappenbeck, D. Wang, D. H. Barouch, and H. W. Virgin. 2012. Pathogenic simian immunodeficiency virus infection is associated with expansion of the enteric virome. *Cell* 151(2):253-266.

Hickey, R. J., X. Zhou, J. D. Pierson, J. Ravel, and L. J. Forney. 2012. Understanding vaginal microbiome complexity from an ecological perspective. *Translational Research* 160(4):267-282.

Holmes, E., J. Kinross, G. R. Gibson, R. Burcelin, W. Jia, S. Pettersson, and J. K. Nicholson. 2012. Therapeutic modulation of microbiota-host metabolic interactions. *Science Translational Medicine* 4(137):137rv136.

Hooper, L. V., and J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science* 292(5519):1115-1118.

Hooper, L. V., D. R. Littman, and A. J. Macpherson. 2012. Interactions between the microbiota and the immune system. *Science* 336(6086):1268-1273.

Houck, P. W., J. D. Nelson, and J. L. Kay. 1972. Fatal septicemia due to *Staphylococcus aureus* 502a. Report of a case and review of the infectious complications of bacterial interference programs. *American Journal of Diseases of Children* 123(1):45-48.

Howson, C. P., T. Hiyama, and E. L. Wynder. 1986. The decline in gastric cancer: Epidemiology of an unplanned triumph. *Epidemiologic Reviews* 8:1-27.

Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486(7402):207-214.

Iliev, I. D., V. A. Funari, K. D. Taylor, Q. Nguyen, C. N. Reyes, S. P. Strom, J. Brown, C. A. Becker, P. R. Fleshner, M. Dubinsky, J. I. Rotter, H. L. Wang, D. P. McGovern, G. D. Brown, and D. M. Underhill. 2012. Interactions between commensal fungi and the c-type lectin receptor dectin-1 influence colitis. *Science* 336(6086):1314-1317.

IOM (Institute of Medicine). 2004. *The infectious etiology of chronic diseases: Defining the relationship, enhancing the research, and mitigating the effects: Workshop summary.* Washington, DC: The National Academies Press.

———. 2006. *Ending the war metaphor: The changing agenda for unraveling the host-microbe relationship: Workshop summary.* Washington, DC: The National Academies Press.

———. 2009. *Microbial evolution and co-adaptation: A tribute to the life and scientific legacies of Joshua Lederberg: Workshop summary.* Washington, DC: The National Academies Press.

_____. 2012. *The social biology of microbial communities: Workshop summary.* Washington, DC: The National Academies Press.

Islami, F., and F. Kamangar. 2008. *Helicobacter pylori* and esophageal cancer risk: A meta-analysis. *Cancer Prevention Research* 1(5):329-338.

Jostins, L., S. Ripke, R. K. Weersma, R. H. Duerr, D. P. McGovern, K. Y. Hui, J. C. Lee, L. P. Schumm, Y. Sharma, C. A. Anderson, J. Essers, M. Mitrovic, K. Ning, I. Cleynen, E. Theatre, S. L. Spain, S. Raychaudhuri, P. Goyette, Z. Wei, C. Abraham, J. P. Achkar, T. Ahmad, L. Amininejad, A. N. Ananthakrishnan, V. Andersen, J. M. Andrews, L. Baidoo, T. Balschun, P. A. Bampton, A. Bitton, G. Boucher, S. Brand, C. Buning, A. Cohain, S. Cichon, M. D'Amato, D. De Jong, K. L. Devaney, M. Dubinsky, C. Edwards, D. Ellinghaus, L. R. Ferguson, D. Franchimont, K. Fransen, R. Gearry, M. Georges, C. Gieger, J. Glas, T. Haritunians, A. Hart, C. Hawkey, M. Hedl, X. Hu, T. H. Karlsen, L. Kupcinskas, S. Kugathasan, A. Latiano, D. Laukens, I. C. Lawrance, C. W. Lees, E. Louis, G. Mahy, J. Mansfield, A. R. Morgan, C. Mowat, W. Newman, O. Palmieri, C. Y. Ponsioen, U. Potocnik, N. J. Prescott, M. Regueiro, J. I. Rotter, R. K. Russell, J. D. Sanderson, M. Sans, J. Satsangi, S. Schreiber, L. A. Simms, J. Sventoraityte, S. R. Targan, K. D. Taylor, M. Tremelling, H. W. Verspaget, M. De Vos, C. Wijmenga, D. C. Wilson, J. Winkelmann, R. J. Xavier, S. Zeissig, B. Zhang, C. K. Zhang, H. Zhao, M. S. Silverberg, V. Annese, H. Hakonarson, S. R. Brant, G. Radford-Smith, C. G. Mathew, J. D. Rioux, E. E. Schadt, M. J. Daly, A. Franke, M. Parkes, S. Vermeire, J. C. Barrett, and J. H. Cho. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 491(7422):119-124.

Kamada, N., S. U. Seo, G. Y. Chen, and G. Nunez. 2013. Role of the gut microbiota in immunity and inflammatory disease. *Nature Reviews: Immunology* 13(5):321-335.

Karlsson, F. H., F. Fak, I. Nookaew, V. Tremaroli, B. Fagerberg, D. Petranovic, F. Bäckhed, and J. Nielsen. 2012. Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nature Communications* 3:1245.

Karlsson, F. H., V. Tremaroli, I. Nookaew, G. Bergström, C. J. Behre, B. Fagerberg, J. Nielsen, and F. Bäckhed. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 498:99-103.

Kaser, A., S. Zeissig, and R. S. Blumberg. 2010. Inflammatory bowel disease. *Annual Review of Immunology* 28:573-621.

Kelly, C. P. 2013. Fecal microbiota transplantation—An old therapy comes of age. *New England Journal of Medicine* 368(5):474-475.

Khor, B., A. Gardet, and R. J. Xavier. 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 474(7351):307-317.

King, M. C., J. H. Marks, and J. B. Mandell. 2003. Breast and ovarian cancer risks due to inherited mutations in *BRCA*1 and *BRCA*2. *Science* 302(5645):643-646.

Koch, R. 1891. A further communication on a remedy for tuberculosis. *British Medical Journal* 1(1568):125-127.

Koczulla, R., G. von Degenfeld, C. Kupatt, F. Krotz, S. Zahler, T. Gloe, K. Issbrucker, P. Unterberger, M. Zaiou, C. Lebherz, A. Karl, P. Raake, A. Pfosser, P. Boekstegers, U. Welsch, P. S. Hiemstra, C. Vogelmeier, R. L. Gallo, M. Clauss, and R. Bals. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *Journal of Clinical Investigation* 111(11):1665-1672.

Kong, H. H., J. Oh, C. Deming, S. Conlan, E. A. Grice, M. A. Beatson, E. Nomicos, E. C. Polley, H. D. Komarow, P. R. Murray, M. L. Turner, and J. A. Segre. 2012. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Research* 22(5):850-859.

Koren, O., J. K. Goodrich, T. C. Cullender, A. Spor, K. Laitinen, H. K. Bäckhed, A. Gonzalez, J. J. Werner, L. T. Angenent, R. Knight, F. Bäckhed, E. Isolauri, S. Salminen, and R. E. Ley. 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* 150(3):470-480.

Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman, and M. J. McFall-Ngai. 2004. Microbial factor-mediated development in a host-bacterial mutualism. *Science* 306(5699):1186-1188.

Kremer, N., E. E. R. Philipp, M-C. Carpentier, C. A. Brennan, L. Kraemer, M. A. Altura, R. Augustin, R. Häsler. E. A. C. Heath-Heckman, S. M. Peyer, J. Schwartzman, B. A. Rader, E. G. Ruby, P. Rosenstiel, and M. J. McFall-Ngai. 2013. Initial symbiont contact orchestrates host-organ-wide transcriptional changes that prime tissue colonization. *Cell Host & Microbe* 14(2):183-194.

Kuballa, P., W. M. Nolte, A. B. Castoreno, and R. J. Xavier. 2012. Autophagy and the immune system. *Annual Review of Immunology* 30:611-646.

Kyne, L., M. Warny, A. Qamar, and C. P. Kelly. 2001. Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet* 357(9251):189-193.

Lederberg, J. 2000. Infectious history. *Science* 288(5464):287-293.

Lemon, K. P., G. C. Armitage, D. A. Relman, and M. A. Fischbach. 2012. Microbiota-targeted therapies: An ecological perspective. *Science Translational Medicine* 4(137):137rv135.

Ley, R. E., F. Backhed, P. Turnbaugh, C. A. Lozupone, R. D. Knight, and J. I. Gordon. 2005. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America* 102(31):11070-11075.

Light, I. J., J. M. Sutherland, and J. E. Schott. 1965. Control of a staphylococcal outbreak in a nursery, use of bacterial interference. *JAMA* 193:699-704.

Liu, P. T., S. Stenger, H. Li, L. Wenzel, B. H. Tan, S. R. Krutzik, M. T. Ochoa, J. Schauber, K. Wu, C. Meinken, D. L. Kamen, M. Wagner, R. Bals, A. Steinmeyer, U. Zugel, R. L. Gallo, D. Eisenberg, M. Hewison, B. W. Hollis, J. S. Adams, B. R. Bloom, and R. L. Modlin. 2006. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311(5768):1770-1773.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature* 489(7415):220-230.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer. 2004. ArbRB: A software environment for sequence data. *Nucleic Acids Research* 32(4):1363-1371.

Lupp, C., M. L. Robertson, M. E. Wickham, I. Sekirov, O. L. Champion, E. C. Gaynor, and B. B. Finlay. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of enterobacteriaceae. *Cell Host Microbe* 2(3):204.

Ma, B., L. J. Forney, and J. Ravel. 2012. Vaginal microbiome: Rethinking health and disease. *Annual Review of Microbiology* 66:371-389.

MacDonald, S. J., G. H. Thomas, and A. E. Douglas. 2011. Genetic and metabolic determinants of nutritional phenotype in an insect-bacterial symbiosis. *Molecular Ecology* 20(10):2073-2084.

Mackowiak, P. A. 1982. The normal microbial flora. *New England Journal of Medicine* 307:83-93.

Mandel, M. J., A. L. Schaefer, C. A. Brennan, E. A. C. Heath-Heckman, C. R. Deloney-Marino, M. J. McFall-Ngai, and E. G. Ruby. 2012. Squid-derived chitin oligosaccharides are a chemotactic signal during colonization by *Vibrio fischeri*. *Applied and Environmental Microbiology* 78:4620-4626.

Maynard, C. L., C. O. Elson, R. D. Hatton, and C. T. Weaver. 2012. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489(7415):231-241.

McFall-Ngai, M., M. G. Hadfield, T. C. Bosch, H. V. Carey, T. Domazet-Loso, A. E. Douglas, N. Dubilier, G. Eberl, T. Fukami, S. F. Gilbert, U. Hentschel, N. King, S. Kjelleberg, A. H. Knoll, N. Kremer, S. K. Mazmanian, J. L. Metcalf, K. Nealson, N. E. Pierce, J. F. Rawls, A. Reid, E. G. Ruby, M. Rumpho, J. G. Sanders, D. Tautz, and J. J. Wernegreen. 2013. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America* 110(9):3229-3236.

McKenna, M. 2011. Swapping germs. *Scientific American* 305(6):34-36.

Minot, S., S. Grunberg, G. D. Wu, J. D. Lewis, and F. D. Bushman. 2012. Hypervariable loci in the human gut virome. *Proceedings of the National Academy of Sciences of the United States of America* 109(10):3962-3966.

Moles, L., M. Gómez, H. Heilig, G. Bustos, S. Fuentes, W. de Vos, L. Fernández, J. Rodríguez, and E. Jiménez. (2013) Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. *PLoS ONE* 8(6):e66986. doi:10.1371/journal.pone.0066986

Molodecky, N. A., I. S. Soon, D. M. Rabi, W. A. Ghali, M. Ferris, G. Chernoff, E. I. Benchimol, R. Panaccione, S. Ghosh, H. W. Barkema, and G. G. Kaplan. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142(1):46-54 e42.

Morgan, X. C., T. L. Tickle, H. Sokol, D. Gevers, K. L. Devaney, D. V. Ward, J. A. Reyes, S. A. Shah, N. LeLeiko, S. B. Snapper, A. Bousvaros, J. Korzenik, B. E. Sands, R. J. Xavier, and C. Huttenhower. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol*ogy 13(9):R79.

Munstedt, K., P. Grant, J. Woenckhaus, G. Roth, and H. R. Tinneberg. 2004. Cancer of the endometrium: Current aspects of diagnostics and treatment. *World Journal of Surgical Oncology* 2:24.

Naik, S., N. Bouladoux, C. Wilhelm, M. J. Molloy, R. Salcedo, W. Kastenmuller, C. Deming, M. Quinones, L. Koo, S. Conlan, S. Spencer, J. A. Hall, A. Dzutsev, H. Kong, D. J. Campbell, G. Trinchieri, J. A. Segre, and Y. Belkaid. 2012. Compartmentalized control of skin immunity by resident commensals. *Science* 337(6098):1115-1119.

Newman, D. J., G. M. Cragg, and K. M. Snader. 2003. Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products* 66(7):1022-1037.

Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson. 2012. Host-gut microbiota metabolic interactions. *Science* 336(6086):1262-1267.

Nyholm, S. V., and M. J. McFall-Ngai. 2004. The winnowing: Establishing the squid-*Vibrio* symbiosis. *Nature Reviews Microbiology* 2(8):632-642.

Oh, J., S. Conlan, E. C. Polley, J. A. Segre, and H. H. Kong. 2012. Shifts in human skin and nares microbiota of healthy children and adults. *Genomic Medicine* 4(10):77.

Ohnmacht, C., R. Marques, L. Presley, S. Sawa, M. Lochner, and G. Eberl. 2011. Intestinal microbiota, evolution of the immune system and the bad reputation of pro-inflammatory immunity. *Cell Microbiology* 13(5):653-659.

Oliynyk, M., M. Samborskyy, J. B. Lester, T. Mironenko, N. Scott, S. Dickens, S. F. Haydock, and P. F. Leadlay. 2007. Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* nrrl23338. *Nature Biotechnology* 25(4):447-453.

Olszak, T., D. An, S. Zeissig, M. P. Vera, J. Richter, A. Franke, J. N. Glickman, R. Siebert, R. M. Baron, D. L. Kasper, and R. S. Blumberg. 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336(6080):489-493.

Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *New England Journal of Medicine* 347(15):1151-1160.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* 276(5313):734-740.

Paine, R. T., M. J. Tegner, and E. A. Johnson. 1998. Compounded perturbations yield ecological surprises. *Ecosystems* 1(6):535-545.

Peek, R. M., Jr., and M. J. Blaser. 2002. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nature Reviews Cancer* 2(1):28-37.

Perry, S., B. C. de Jong, J. V. Solnick, M. de la Luz Sanchez, S. Yang, P. L. Lin, L. M. Hansen, N. Talat, P. C. Hill, R. Hussain, R. A. Adegbola, J. Flynn, D. Canfield, and J. Parsonnet. 2010. Infection with *Helicobacter pylori* is associated with protection against tuberculosis. *PloS ONE* 5(1):e8804.

Peterson, D. A., D. N. Frank, N. R. Pace, and J. I. Gordon. 2008. Metagenomic approaches for defining the pathogenesis of inflammatory bowel disease. *Cell Host Microbe* 3(6):417-427.

Petrof, E. O., G. B. Gloor, S. J. Vanner, J. S. Weese, D. Carter, M. C. Daigneault, E. M. Brown, K. Schroeder, and E. Allen-Vercoe. 2013. Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: "Repoopulating" the gut. *Microbiome* 1(1):1-12.

Pincock, S. 2005. Nobel prize winners Robin Warren and Barry Marshall. *Lancet* 366(9495):1429.

Plottel, C. S., and M. J. Blaser. 2011. Microbiome and malignancy. *Cell Host Microbe* 10(4):324-335.

Poliakov, A., C. W. Russell, L. Ponnala, H. J. Hoops, Q. Sun, A. E. Douglas, and K. J. van Wijk. 2011. Large-scale label-free quantitative proteomics of the pea aphid-buchnera symbiosis. *Molecular & Cellular Proteomics* 10(6):M110 007039.

Qin, J., R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J. M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, M. Jian, Y. Zhou, Y. Li, X. Zhang, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, P. Bork, and S. D. Ehrlich. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464(7285):59-65.

Qin, J., Y. Li, Z. Cai, S. Li, J. Zhu, F. Zhang, S. Liang, W. Zhang, Y. Guan, D. Shen, Y. Peng, D. Zhang, Z. Jie, W. Wu, Y. Qin, W. Xue, J. Li, L. Han, D. Lu, P. Wu, Y. Dai, X. Sun, Z. Li, A. Tang, S. Zhong, X. Li, W. Chen, R. Xu, M. Wang, Q. Feng, M. Gong, J. Yu, Y. Zhang, M. Zhang, T. Hansen, G. Sanchez, J. Raes, G. Falony, S. Okuda, M. Almeida, E. LeChatelier, P. Renault, N. Pons, J. M. Batto, Z. Zhang, H. Chen, R. Yang, W. Zheng, S. Li, H. Yang, J. Wang, S. D. Ehrlich, R. Nielsen, O. Pedersen, K. Kristiansen, and J. Wang. 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490(7418):55-60.

Ramirez-Puebla, S. T., L. E. Servin-Garciduenas, B. Jimenez-Marin, L. M. Bolanos, M. Rosenblueth, J. Martinez, M. A. Rogel, E. Ormeno-Orrillo, and E. Martinez-Romero. 2013. Gut and root microbiota commonalities. *Applied and Environmental Microbiology* 79(1):2-9.

Ravel, J., P. Gajer, Z. Abdo, G. M. Schneider, S. S. Koenig, S. L. McCulle, S. Karlebach, R. Gorle, J. Russell, C. O. Tacket, R. M. Brotman, C. C. Davis, K. Ault, L. Peralta, and L. J. Forney. 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America* 108(Suppl 1):4680-4687.

Reibman, J., M. Marmor, J. Filner, M. E. Fernandez-Beros, L. Rogers, G. I. Perez-Perez, and M. J. Blaser. 2008. Asthma is inversely associated with *Helicobacter pylori* status in an urban population. *PLoS ONE* 3(12):e4060.

Relman, D. A. 2012. The human microbiome: Ecosystem resilience and health. *Nutrition Reviews* 70(Suppl 1):S2-S9.

Ridaura, V. K., J. J. Faith, F. E. Rey, J. Cheng, A. E. Duncan, A. L. Kau, N. W. Griffin, V. Lombard, B. Henrissat, J. R. Bain, M. J. Muehlbauer, O. Ilkayeva, C. F. Semenkovich, K. Funai, D. K. Hayashi, B. J. Lyle, M. C. Martini, L. K. Ursell, J. C. Clemente, W. Van Treuren, W. A. Walters, R. Knight, C. B. Newgard, A. C. Heath, and J. I. Gordon. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341(6150):1241214.

Ridley, E. V., A. C. Wong, S. Westmiller, and A. E. Douglas. 2012. Impact of the resident microbiota on the nutritional phenotype of drosophila melanogaster. *PLoS ONE* 7(5):e36765.

Ridley, E. V., A. C. Wong, and A. E. Douglas. 2013. Microbe-dependent and nonspecific effects of procedures to eliminate the resident microbiota from *Drosophila melanogaster*. *Applied and Environmental Microbiology* 79(10):3209-3214.

Rivers, T. M. 1937. Viruses and Koch's postulates. *Journal of Bacteriology* 33:1-12.

Robinson, C. J., B. J. Bohannan, and V. B. Young. 2010. From structure to function: The ecology of host-associated microbial communities. *Microbiology and Molecular Biology Reviews* 74(3):453-476.

Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology* 9(5):313-323.

Ryu, J. H., S. H. Kim, H. Y. Lee, J. Y. Bai, Y. D. Nam, J. W. Bae, D. G. Lee, S. C. Shin, E. M. Ha, and W. J. Lee. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in drosophila. *Science* 319(5864):777-782.

Sansonetti, P. J. 2004. War and peace at mucosal surfaces. *Nature Reviews Immunology* 4(12):953-964.

Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annual Review of Microbiology* 31:107-133.

Sela, D. A., and D. A. Mills. 2010. Nursing our microbiota: Molecular linkages between bifidobacteria and milk oligosaccharides. *Trends in Microbiology* 18(7):298-307.

Shade, A., H. Peter, S. D. Allison, D. L. Baho, M. Berga, H. Burgmann, D. H. Huber, S. Langenheder, J. T. Lennon, J. B. Martiny, K. L. Matulich, T. M. Schmidt, and J. Handelsman. 2012. Fundamentals of microbial community resistance and resilience. *Frontiers in Microbiology* 3:417.

Shaw, S. Y., J. F. Blanchard, and C. N. Bernstein. 2010. Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease. *American Journal of Gastroenterology* 105(12):2687-2692.

Smith, M. I., T. Yatsunenko, M. J. Manary, I. Trehan, R. Mkakosya, J. Cheng, A. L. Kau, S. S. Rich, P. Concannon, J. C. Mychaleckyj, J. Liu, E. Houpt, J. V. Li, E. Holmes, J. Nicholson, D. Knights, L. K. Ursell, R. Knight, and J. I. Gordon. 2013. Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science* 339(6119):548-554.

Sommer, F., and F. Bäckhed. 2013. The gut microbiota—Masters of host development and physiology. *Nature Reviews Microbiology*11(4):227-382.

Sonnenburg, J. L., and M. A. Fischbach. 2011. Community health care: Therapeutic opportunities in the human microbiome. *Science Translational Medicine* 3(78):78ps12.

Spor, A., O. Koren, and R. Ley. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews Microbiology* 9:279-290.

Strachan, D. P. 1989. Hay fever, hygiene, and household size. *British Medical Journal* 299(6710): 1259-1260.

Tang, W. H., Z. Wang, B. S. Levison, R. A. Koeth, E. B. Britt, X. Fu, Y. Wu, and S. L. Hazen. 2013. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *New England Journal of Medicine* 368(17):1575-1584.

Taormina, M. J., M. Jemielita, W. Z. Stephens, A. R. Burns, J. V. Troll, R. Parthasarathy, and K. Guillemin. 2012. Investigating bacterial-animal symbioses with light sheet microscopy. *Biological Bulletin* 223(1):7-20.

Thomas, G. H., J. Zucker, S. J. Macdonald, A. Sorokin, I. Goryanin, and A. E. Douglas. 2009. A fragile metabolic network adapted for cooperation in the symbiotic bacterium *Buchnera aphidicola*. *BMC Systems Biology* 3:24.

Trasande, L., J. Blustein, M. Liu, E. Corwin, L. M. Cox, and M. J. Blaser. 2013. Infant antibiotic exposures and early-life body mass. *International Journal of Obesity* 37(1):16-23.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444: 1027-1031.

Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon. 2007. The human microbiome project. *Nature* 449(7164):804-810.

Tuskan, G. 2013. *Session I: Plant-microbe interactions in root endophyte and rhizosphere communities of* Populus. Presentation at the Forum on Microbial Threats Workshop, Microbial Ecology in States of Health and Disease, Washington, DC, Institute of Medicine, Forum on Microbial Threats. March 18.

Tuskan, G. A., S. Difazio, S. Jansson, J. Bohlmann, I. Grigoriev, U. Hellsten, N. Putnam, S. Ralph, S. Rombauts, A. Salamov, J. Schein, L. Sterck, A. Aerts, R. R. Bhalerao, R. P. Bhalerao, D. Blaudez, W. Boerjan, A. Brun, A. Brunner, V. Busov, M. Campbell, J. Carlson, M. Chalot, J. Chapman, G. L. Chen, D. Cooper, P. M. Coutinho, J. Couturier, S. Covert, Q. Cronk, R. Cunningham, J. Davis, S. Degroeve, A. Dejardin, C. Depamphilis, J. Detter, B. Dirks, I. Dubchak, S. Duplessis, J. Ehlting, B. Ellis, K. Gendler, D. Goodstein, M. Gribskov, J. Grimwood, A. Groover, L. Gunter, B. Hamberger, B. Heinze, Y. Helariutta, B. Henrissat, D. Holligan, R. Holt, W. Huang, N. Islam-Faridi, S. Jones, M. Jones-Rhoades, R. Jorgensen, C. Joshi, J. Kangasjarvi, J. Karlsson, C. Kelleher, R. Kirkpatrick, M. Kirst, A. Kohler, U. Kalluri, F. Larimer, J. Leebens-Mack, J. C. Leple, P. Locascio, Y. Lou, S. Lucas, F. Martin, B. Montanini, C. Napoli, D. R. Nelson, C. Nelson, K. Nieminen, O. Nilsson, V. Pereda, G. Peter, R. Philippe, G. Pilate, A. Poliakov, J. Razumovskaya, P. Richardson, C. Rinaldi, K. Ritland, P. Rouze, D. Ryaboy, J. Schmutz, J. Schrader, B. Segerman, H. Shin, A. Siddiqui, F. Sterky, A. Terry, C. J. Tsai, E. Uberbacher, P. Unneberg, J. Vahala, K. Wall, S. Wessler, G. Yang, T. Yin, C. Douglas, M. Marra, G. Sandberg, Y. Van de Peer, and D. Rokhsar. 2006. The genome of black cottonwood, *Populus trichocarpa* (torr. & gray). *Science* 313(5793):1596-1604.

Tvede, M., and J. Rask-Madsen. 1989. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet* 1(8648):1156-1160.

Underhill, D., and J. Braun. 2008. Current understanding of fungal microflora in inflammatory bowel disease pathogenesis. *Inflammatory Bowel Diseases* 14(8):1147-1153.

van Nood, E., P. Speelman, E. J. Kuijper, and J. J. Keller. 2009. Struggling with recurrent *Clostridium difficile* infections: Is donor faeces the solution? *Eurosurveillance* 14(34).

van Nood, E., A. Vrieze, M. Nieuwdorp, S. Fuentes, E. G. Zoetendal, W. M. de Vos, C. E. Visser, E. J. Kuijper, J. F. Bartelsman, J. G. Tijssen, P. Speelman, M. G. Dijkgraaf, and J. J. Keller. 2013. Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *New England Journal of Medicine* 368(5):407-415.

Vellend, M. 2010. Conceptual synthesis in community ecology. *Quarterly Review of Biology* 85(2):183-206.

Virgin, H. W., and J. A. Todd. 2011. Metagenomics and personalized medicine. *Cell* 147(1):44-56.

Virgin, H. W., E. J. Wherry, and R. Ahmed. 2009. Redefining chronic viral infection. *Cell* 138(1):30-50.

Walsh, C. T., and M. A. Fischbach. 2010. Natural products version 2.0: Connecting genes to molecules. *Journal of the American Chemical Society* 132(8):2469-2493.

Weston, D. J., D. A. Pelletier, J. L. Morrell-Falvey, T. J. Tschaplinski, S. S. Jawdy, T. Y. Lu, S. M. Allen, S. J. Melton, M. Z. Martin, C. W. Schadt, A. A. Karve, J. G. Chen, X. Yang, M. J. Doktycz, and G. A. Tuskan. 2012. *Pseudomonas fluorescens* induces strain-dependent and strain-independent host plant responses in defense networks, primary metabolism, photosynthesis, and fitness. *Molecular Plant-Microbe Interactions* 25(6):765-778.

White, D. W., C. R. Keppel, S. E. Schneider, T. A. Reese, J. Coder, J. E. Payton, T. J. Ley, H. W. Virgin, and T. A. Fehniger. 2010. Latent herpesvirus infection arms NK cells. *Blood* 115(22):4377-4383.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences of the United States of America* 95(12):6578-6583.

Wilson, A. C., P. D. Ashton, F. Calevro, H. Charles, S. Colella, G. Febvay, G. Jander, P. F. Kushlan, S. J. Macdonald, J. F. Schwartz, G. H. Thomas, and A. E. Douglas. 2010. Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Molecular Biology* 19(Suppl 2:)249-258.

Witkin, S. S., S. Alvi, A. M. Bongiovanni, I. M. Linhares, and W. J. Ledger. 2011. Lactic acid stimulates interleukin-23 production by peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide. *FEMS Immunology and Medical Microbiology* 61(2):153-158.

Wong, A. C., J. M. Chaston, and A. E. Douglas. 2013. The inconstant gut microbiota of Drosophila species revealed by 16s rRNA gene analysis. *ISME Journal.* doi: 10.1038/ismej.2013.86.

Wong, C. N., P. Ng, and A. E. Douglas. 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environmental Microbiology* 13(7):1889-1900.

Xu, J., and J. I. Gordon. 2003. Honor thy symbionts. *Proceedings of the National Academy of Sciences of the United States of America* 100(18):10452-10459.

Yano, J., E. Lilly, M. Barousse, and P. L. Fidel, Jr. 2010. Epithelial cell-derived s100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during *Candida vaginitis*. *Infection and Immunity* 78(12):5126-5137.

Yano, J., M. C. Noverr, and P. L. Fidel, Jr. 2012. Cytokines in the host response to candida vaginitis: Identifying a role for non-classical immune mediators, s100 alarmins. *Cytokine* 58(1):118-128.

Yatsunenko, T., F. E. Rey, M. J. Manary, I. Trehan, M. G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R. N. Baldassano, A. P. Anokhin, A. C. Heath, B. Warner, J. Reeder, J. Kuczynski, J. G. Caporaso, C. A. Lozupone, C. Lauber, J. C. Clemente, D. Knights, R. Knight, and J. I. Gordon. 2012. Human gut microbiome viewed across age and geography. *Nature* 486(7402):222-227.

Youle, M., M. Haynes, and F. Rohwer. 2012. Scratching the surface of biology's dark matter. In *Viruses: Essential agents of life*, edited by G. Witzany. Springer.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two active forms, and partial cdnaDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America* 84(15):5449-5453.

———. 2002a. Antimicrobial peptides in health and disease. *New England Journal of Medicine* 347(15):1199-1200.

———. 2002b. Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389-395.

———. 2011. Observations on the remarkable (and mysterious) wound-healing process of the bottlenose dolphin. *Journal of Investigative Dermatology* 131(12):2503-2505.

———. 2013. *Session IV: Innate antimicrobial mechanisms in disease prevention and treatment.* Presentation at the Forum on Microbial Threats Workshop, Microbial Ecology in States of Health and Disease, Washington, DC, Institute of Medicine, Forum on Microbial Threats. March 19.

Zivkovic, A. M., J. B. German, C. B. Lebrilla, and D. A. Mills. 2011. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America* 108(Suppl 1):4653-4658.

Appendix A

Contributed Manuscripts

A1

EFFECTOR AND MEMORY T CELL RESPONSES TO COMMENSAL BACTERIA[1]

*Yasmine **Belkaid**,[2] Nicolas Bouladoux,[2] and Timothy W. Hand[2]*

Barrier surfaces are home to a vast population of commensal organisms that together encode millions of proteins, each of them possessing several potential foreign antigens. Regulation of immune responses to this enormous antigenic load represents a tremendous challenge for the immune system. Tissues exposed to commensals have developed elaborate systems of regulation including specialized populations of resident lymphocytes that maintain barrier function and limit potential responses to commensal antigens. However, in settings of infection and inflammation these regulatory mechanisms are compromised and specific effector responses against commensal bacteria can develop. This review discusses the circumstances controlling the fate of commensal specific T cells and how dysregulation of these responses could lead to severe pathological outcomes.

[1] Reprinted with permission from Yasmine Belkaid. Originally published as Belkaid, Y. et al. 2013. Effector and memory T cell responses to commensal bacteria. *Trends in Immunology* 34:6. doi: 10.1016/j.it.2013.03.003.0.

[2] Mucosal Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda 20892, USA.

Commensal Microbiota Shape T Cell Resident Homeostasis

The epithelial surfaces in the body act as a scaffold to sustain diverse communities of commensal organisms that include bacteria, archaea, fungi, protozoa, and viruses (Eckberg et al., 2003; Foulongne et al., 2012; Grice and Segre, 2012; Iliev et al., 2012; Reyes et al., 20102). With an estimated composition of 100 trillion cells, commensals outnumber host cells by at least a factor of 10 and encode 100-fold more genes than the host genome (Ley et al., 2006). All barrier sites, including the genital mucosa, skin, airways, and gut are constitutively colonized by highly diverse and site-specific flora. The gastrointestinal (GI) tract represents the most abundant commensal niche with a population of more than 1,000 individual strains that contain some 3 million unique genes (Clemente et al., 2012; Qin et al., 2010). Although commensalism is defined in ecology as a relationship in which only one party benefits while the other is neutral, many of the bacteria of the GI tract are better described as mutualists, adding tremendous enzymatic and protective capability to the host while taking advantage of the nutrient-rich environment that the host provides (Backhed et al., 2005; Costello et al., 2012). In particular, commensal bacteria can prevent colonization by pathogenic organisms (Stecher and Hardt, 2011) and control many aspects of host physiology, not the least of which are lymphocytes of the immune system.

T cells are found in large numbers lining barrier surfaces where they are tasked with surveillance against infection while maintaining diplomatic relations with the resident commensal microbiota (Hooper et al., 2012). As a result, CD4 T cells at these surfaces can adopt multiple inter-related fates associated with the expression of characteristic cytokines and transcription factors (Murphy and Stockinger, 2010; Zhou et al., 2008). Under steady-state conditions, the GI tract and gut-associated lymphoid tissue (GALT) is dominated by interleukin (IL)-17A-producing T helper (Th)17 cells, interferon (IFN)-γ-producing Th1 cells, and forkhead box (Fox)p3$^+$ regulatory T (Treg) cells (Hall et al., 2008; Maynard and Weaver, 2009). The balance between these populations is tightly controlled by the cytokine milieu, which at barrier surfaces is in part dependent upon dietary elements and the microbiota (Bettelli et al., 2006; Hall et al., 2011; Hu et al., 2011; Konkel and Chan, 2011). Th17 cells are largely limited to barrier surfaces and have been an area of particular interest in the study of mucosal immunology. The protective role of IL-17A is associated with its capacity to induce neutrophil granulopoiesis by stimulating epithelial cells to secrete granulocyte colony-stimulating factor (G-CSF) and drive the recruitment of neutrophils by local stromal cells (Fossiez et al., 1996; Korn et al., 2009). Additionally, via their capacity to also produce both IL-22 and IL-17A, Th17 cells can bolster innate epithelial defense mechanisms and reinforce tight junctions (Ishigame et al., 2009; Sonnenberg et al., 2011, 2012; Zheng et al., 2008). The function of mucosal Th1 cells under steady-state conditions remains unclear, but we might speculate that these cells also contribute to the promotion of various aspects of innate mucosal responses. Treg cells also represent a prominent population of

resident cells at barrier sites. Treg cells are required for the maintenance of tolerance to both self-antigens and innocuous antigens derived from food, commensal bacteria, and other environmental sources (Bilate and Lafaille, 2012). Treg cells that line the GI tract can arise from the thymus or be induced locally in response to oral antigen, a process required for the acquisition of oral tolerance (Coombes et al., 2007; Sun et al., 2007).

The differentiation of T cells at barrier sites into each of these different fates has been associated with the presence of signals derived from the commensal microbiota (Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Mazmanian et al., 2008). Notably, the capacity of T cells to produce IL-17A and IFN-γ is severely compromised in the absence of commensals in both the gut and skin (Hall et al., 2008; Ivanov et al., 2009; Naik et al., 2012). Germ-free mice also tend to harbor higher frequencies of Th2 cells and this too can be reversed by colonization of the mice with a single species of commensal bacteria (Mazmanian et al., 2005). In the GI tract, microbial products such as bacterial DNA or defined group of bacteria such as segmented filamentous bacteria (SFBs) can play a dominant role in the promotion of steady-state GI-resident Th1 and Th17 cells (Hall et al., 2008; Ivanov et al., 2009). In the skin, reconstitution of germ-free mice with *Staphylococcus epidermidis* restores dermal IL-17A (Naik et al., 2012). The frequencies, origin, and activation of $Foxp3^+$ Treg cells in the skin and GI tract are also influenced by the microbiota. In the skin for instance, the absence of commensals is associated with enhanced frequencies of Treg cells (Naik et al., 2012). By contrast, in the GI tract, *Bacillus fragilis* via expression of polysaccharide A can expand IL-10-producing $CD4^+$ T cells and Treg cells at the expense of the differentiation of Th17 cells (Mazmanian et al., 2008; Ochoa-Reparaz et al., 2010; O'Mahony et al., 2008; Round et al., 2011). Bacteria of the *Clostridium* cluster XIV species also promote Treg cell accumulation in the colon, but not small intestine via an increased capacity to promote transforming growth factor (TGF)-β (Atarashi et al., 2011). The gut microbiota can also set the tone of immune responses at distal sites during infection and in some cases contribute to the induction of autoimmune disorders (Abt et al., 2012; Ichinohe et al., 2011; Ivanov et al., 2009; Molloy et al., 2012; Wu et al., 2010).

Antigen-Specific Responses to Commensal Organisms at Steady State

Independent of differentiation state and function, a critical question associated with T cells residing at barrier sites is their antigen specificity. The microbiota encodes millions of proteins, each of them expressing several potential foreign antigens directly associated with inflammatory pathogen-associated molecular patterns. This enormous antigenic load represents a tremendous challenge for barrier immunity as unwanted responses against these antigens could lead to severe pathological consequences. A central strategy utilized by the mucosal immune system to maintain its homeostatic relation with the microbiota is to limit

contact between luminal microorganisms and the epithelial cell surface. This is accomplished by the establishment of a structural and immunological barrier resulting from the combined action of mucus, IgA, and antimicrobial proteins (Hooper et al., 2012) (Figure A1-1A). Further, commensals can promote their own containment and thus mucosal tissue homeostasis by enhancing various aspects of this physical and immunological shield (Brandl et al., 2008; Cong et al., 2009; Vaishnava et al., 2011).

The physical segregation between the host and microbiota is not absolute, and the GI mucosal barrier are prevented from systemic traffic and confined to the GALT by the mucosal firewall (Macpherson et al., 2009; Macpherson and Uhr, 2004). In the GALT, active mechanisms of tolerance regulate T cell responses, and as a result, the GI tract represents a privileged site for the induction of tolerance (Figure A1-1A). The regulatory nature of the GI tract is perhaps best demonstrated by experiments examining oral tolerance to food (Weiner et al., 2011). This phenomenon has long been recognized in both rodent models and humans and is clinically important, because dysregulated T cell responses against food-derived antigens are associated with both celiac disease and GI allergies. Experiments have shown that under steady-state conditions, the predominant antigen-presenting cells of food-derived antigens are dendritic cells (DCs) that express the integrin CD103 (Coombes et al., 2007; Sun et al., 2007). $CD103^+$ DCs drive the differentiation of $Foxp3^+$ Treg cells via their capacity to produce TGF-β and to convert retinol to retinoic acid (RA) (Benson et al., 2007; Coombes et al., 2007; Denning et al., 2011; Mucida et al., 2007, 2009; Sun et al., 2007). Further $CD103^+$ DCs can also induce the expression of gut homing receptors on immune cells, including Treg cells, favoring their migration to the GI tract (Mora et al., 2006). Commensal antigens are likely encountered in the context of these tolerogenic responses, thus, it has been proposed that the GI tract also promotes the induction of Treg cells specific for commensal antigens (Figure A1-1A). Indeed, some types of commensal bacteria seem to support the differentiation and maintenance of colonic Treg cells, although whether these Treg cells are specific to commensal-derived antigens is not known (Atarashi et al., 2011; Round and Mazmanian, 2010). T cell receptor transgenic mice made specific to commensal-derived antigens can differentiate to become Treg cells after transfer to lymphopenic hosts (Feng et al., 2011; Lathrop et al., 2011), and $Foxp3^+$Treg cells specific for commensals have been identified in the GI tract of healthy mice (Lathrop et al., 2011). Aside from the direct maintenance of T cell tolerance, commensal-specific Treg cells may reinforce the mucosal firewall. Treg cells present in the Peyer's patches of the small intestine have been shown to promote class-switching to IgA in an antigen-specific manner (Cong et al., 2009; Tsuji et al., 2009). IgA can directly modulate expression of commensal antigens and mucosal association, therefore, this implies that Treg cells may play multiple and complementary roles in controlling the host relation with the microbiota (Peterson et al., 2007; Suzuki et al., 2004). Commensal-specific

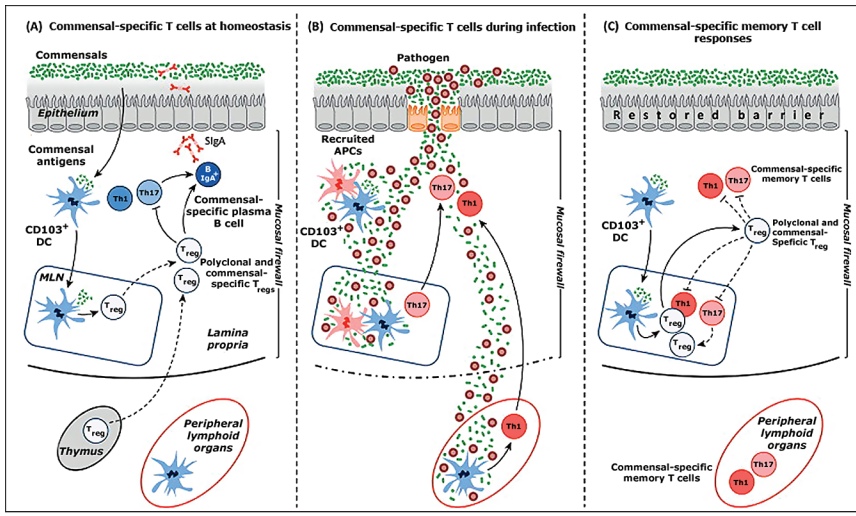


FIGURE A1-1 Commensal-specific T cells at homeostasis and during infection. **(A)** At homeostasis. Commensal and food-derived antigens are presented to T cells by CD103$^+$ T cells that have trafficked to the mesenteric lymph node (MLN) from the lamina propria. Presentation by these dendritic cells (DCs) to commensal-specific T cells may lead to the differentiation of commensal-specific T regulatory (Treg) cells. Commensal-specific Treg cells traffic to the lamina propria and Peyer's patches, where they, along with polyclonal Treg cells, can regulate effector T cell responses and induce class switching and IgA production from resident commensal-specific B cells, reinforcing the commensal barrier. Critically, the combination of the epithelial barrier, mucus layer, IgA and regulatory DCs and T cells comprises the "mucosal firewall," which limits the passage of commensal and food-derived antigens to the gut-associated lymphoid tissue (GALT), preventing untoward activation and pathology. **(B)** During GI infection. Infection via the intestinal barrier can cause inflammation and tissue damage. This pathology can disrupt the mucosal firewall and allows for systemic translocation of commensal organisms and their associated antigens. During times of translocation, the host immune system becomes unable to discriminate between commensal and pathogen-derived antigens and therefore commensal-specific T cell responses mimic responses to the invasive pathogen. **(C)** After clearance of infection, the intestinal barrier reforms and the mucosal firewall is restored perhaps preventing the chronic activation of commensal-specific memory T cells. Treg cells may regulate commensal-specific responses either directly or via the modulation of resident DCs. Abbreviation: APCs, antigen-presenting cells.

T cells transferred into lymphopenic hosts can also differentiate into Th1 and Th17 cells (Feng et al., 2011), but Th1, Th17, and Treg cells resident in the GI tract are not necessarily specific to commensal-derived antigens (Geuking et al., 2011; Lochner et al., 2011). For example, Th17 cells develop in the absence of cognate antigen in mice expressing a single T cell receptor (TCR) (Geuking et al., 2011; Lochner et al., 2011). Furthermore, Treg cells can be found in the GI tract of germ-free mice, and albeit significantly reduced, Th17 and Th1 cells are also present in the absence of live commensals (Ivanov et al., 2008). It is worth noting that the diet of germ-free mice contains microbial products, including antigens, that can provide surrogate signals to the ones normally provided by the flora and may be responsible for the presence of these cells without live commensals (Hill et al., 2010). Alternatively, T cell differentiation and gut homing may occur at a low level independently of signals derived from the commensal flora. Although these data support the notion that the flora promotes the induction and/or maintenance of T cells in the GI tract independent of antigen specificity, they do not exclude that a significant portion of mucosal T cells recognize commensal antigens and the specificity of Th1, Th17, or Treg cells that reside at barrier sites under physiological settings remains an important open question.

Environmentally Induced Shifts in the Microbiota

Active regulation of immune tolerance to the commensal microbiota is a lifelong process, because, in contrast to the host genome, the commensal microbiota is not fixed. Recent studies have shown that, although the core metagenome of the gut microbiota is stable, species composition and consequently antigenic composition of the flora vary over time in response to a variety of factors such as diet, sanitation, infection, and antibiotic use (Dethlefsen and Relman, 2011; Lozupone et al., 2012). Therefore, the adaptive immune system of the GI mucosa must be flexible and constantly reset itself to take into account novel commensal antigens. Changes in the microbiota are particularly pervasive during GI infection and inflammation where the commensal microbiota becomes dominated by commensals with enhanced invasive properties (Chow et al., 2011). For instance, the γ-proteobacteria class of bacteria is particularly selected for growth during a diverse set of inflammatory conditions that ranges from Crohn's disease (CD), colon cancer, and type 2 diabetes in humans to *Toxoplasma gondii* infection and inflammatory bowel disease (IBD) induction in mice (Arthur et al., 2012; Craven et al., 2012; Garrett et al., 2010; Heimesaat et al., 2006; Lupp et al., 2007; Rolhion and Darfeuille-Michaud, 2007; Qin et al., 2012). Because the γ-proteobacteria family is mostly composed of genera that are opportunistic or obligate pathogens, this effect could be due to the unique ability of these organisms to colonize the inflamed GI tract. Indeed, *Escherichia coli* that dominates the GI tract in *T. gondii* infection is skewed toward inflammatory and invasive strains that can contribute to the pathological process during inflammatory responses and

infection (Benson et al., 2009; Craven et al., 2012; Egan et al., 2011; Heimesaat et al., 2006). This phenomenon is not limited to γ-proteobacteria because many other clinically relevant species, such as *Enterococcus faecalis* and *Clostridium difficile*, can bloom during bouts of GI dysbiosis (Britton and Young, 2012; Ubeda et al., 2010). How the immune system deals with these opportunistic residents and in particular whether tolerance to antigen associated with these bacteria is maintained during inflammation are of central importance to our understanding of mucosal inflammatory disorders.

Environmentally Induced Breach of the Mucosal Firewall

At homeostasis and in highly controlled experimental settings, the mucosal firewall ensures that bacterial translocation is strictly limited to the intestinal tissue and associated lymphoid structures (Macpherson and Uhr, 2004). In mouse models, this system has been proven extraordinarily robust because only complete deficiency of key innate and adaptive mucosal immune mechanisms leads to systemic commensal specific antibody responses (Slack et al., 2009). Accordingly, in mice raised under pathogen-free conditions, commensal-specific T cells present outside the mucosa remain naïve despite the presence of commensal antigens in the GI tract (Cong et al., 2009; Hand et al., 2012). However, controlled environments that lack pathogens completely represent a highly artificial setting to understand tissue homeostasis and local immune responses. Indeed, we are now beginning to appreciate that under physiological conditions, systemic translocation of commensals and microbial products beyond the mucosa and GALT is a more common occurrence than initially postulated. Translocation of bacterial products to the systemic circulation has been associated clinically with a diverse set of circumstances such as alcohol abuse and cirrhosis, chronic nonsteroidal anti-inflammatory drug (NSAID) use, malnutrition, chronic inflammation, extreme exercise regimens, and in particular infections (Arakawa et al., 2012; Berklery et al., 2005; Hughes et al., 2009; Purohit et al., 2008; Nieman et al., 2006; Wong, 2012; Zimmermann et al., 2012). In several murine models of GI infections, such as *T. gondii* and *Yersinia pseudotuberculosis*, immunopathology can induce the translocation of commensal bacteria (Hand et al., 2012; Heimesaat et al., 2006; Meinzer et al., 2012). Chronic GI barrier dysfunction is also observed during HIV infection in humans and in simian immunodeficiency virus (SIV) infection of rhesus macaques (Brenchley et al., 2006; Brenchley and Douek, 2012; Estes et al., 2010). Barrier sites including the gut, skin, and airways are primary sites for infections. It is estimated that a child will suffer 10–15 diarrheal episodes on average before the age of 5 years; all of them potentially associated with transient commensal translocation (Kosek et al., 2003; Vernacchio et al., 2006), which if added together with common skin and lung infections, provides ample opportunity for exposure of the immune system to commensal antigens under inflammatory conditions.

Commensal-Specific T Cell Responses During Infection and Inflammation

Infections represent highly volatile situations for the mucosal immune system, because pathogens and commensals share the same inflamed environment. The potential danger of this situation is illustrated by studies that suggest that oral tolerance to newly introduced food antigens breaks down during acute GI infection (Oldenhove et al., 2009; Severance et al., 2012). Recent evidence suggests that in a similar manner, tolerance to commensal-derived antigens may be lost during acute infections. In a mouse model of *T. gondii* infection, Treg cells are lost, commensals translocate, and the immune system becomes unable to discriminate between commensals and pathogen-derived antigens (Hand et al., 2012) (Figure A1-1B). During this highly Th1 polarized infection, commensal-specific T cells also develop as Th1 cells according to cues provided by the inflammatory milieu, rather than Th17 cells or Treg cells, as previous studies have associated with commensal-driven responses (Atarashi et al., 2011; Ivanov et al., 2009). The idea that innate inflammatory cues drive the fate of commensal-specific T cells rather than their specificity is further supported by the observation that physical breakdown of the intestinal barrier via the administration of dextran sodium sulfate (DSS) resulted in the activation of commensal-specific T cells that differentiated toward the Th17 fate (Hand et al., 2012). Thus, the immune response to GI pathogens is associated with parallel responses against commensal-derived antigens that develop according to the inflammatory milieu. The commensal antigen followed in that study is expressed by the *Clostridium* cluster XIV class of bacteria that is known to live in the mucus layer of the intestinal mucosa and is a dominant antigen in CD (Atarashi et al., 2011; Lodes et al., 2004). It will be important to determine with future studies whether these responses are directed against all commensal antigens or are limited to the most prevalent or accessible antigens.

Commensal-Specific Memory T Cell Responses

One remarkable feature of all barrier sites is their ability to repair efficiently after inflammation or breach. In the GI tract, this implies that after acute tissue damage and transiently increased exposure to commensals, physical segregation between the flora and the immune system is rapidly restored. In the absence of chronic exposure to antigen, activated lymphocytes can survive long term as memory populations capable of rapid reactivation and proliferation. Indeed, following GI infection with *T. gondii*, commensal-specific CD4 T cells can persist long term in both the GI tract and secondary lymphoid tissue and maintain the ability to become activated, express Th1 inflammatory cytokines, and proliferate upon secondary encounter with their cognate antigen (Hand et al., 2012).

Much like pathogen-specific CD4 T cells and in contrast to virus-specific CD8 memory T cells, commensal-specific memory T cells declined steadily over time (Hand et al., 2012; Homann et al., 2001; Pepper et al., 2009). CD4 T cells

carry out the complex task of discriminating pathogenic organisms from benign organisms in the face of a constantly changing environment; therefore, perhaps development of CD4 memory reflects this necessity for flexibility. An evolving pool of specificities within the Treg and CD4 effector compartment may allow for the maintenance of tolerance and barrier function in the context of fluctuating commensal populations and intermittent infection. Such flexible repertoire has been proposed for mucosal IgA responses that lack the memory characteristics associated with CD8 T cells and are able to respond to flux in the commensal microbiota composition. Indeed, established IgA-producing clones are outcompeted by novel antibacterial responses, allowing the mucosal immune system to respond to a constantly changing microbiota (Hapfelmeier et al., 2010).

The physiological consequence of long-term $CD4^+$ T cell memory against commensals remains to be addressed. Due to the extraordinary antigenic diversity of the host microbiota at all body surfaces and the prevalence of infections, a significant fraction of memory cells are expected to be commensal specific and could develop over time in response to successive infections and/or various barrier breaches (Figure A1-2). In support of this hypothesis, healthy human serum contains antibodies specific to skin and intestinal microbiota (Zimmermann et al., 2012). Thus, primary exposure to a pathogen in the skin, lung and GI tract is likely to occur in the context of a much broader recall response against commensal bacteria. One possible consequence of these responses may be the induction of heterologous memory wherein antigen-specific responses against previously encountered commensal bacteria could drive the rapid production of inflammatory cytokines, leading to increased protection against secondary infection and associated translocation of commensal bacteria (Figure A1-2). Colon-resident Th17 cells have been shown to contribute to early protection against enteric pathogens, although whether these cells are specific to commensal antigens is not clear (Geddes et al., 2011). Further contributing to the possibility of heterologous protection against infection by commensal-specific T cells, a recent study has suggested that CD4 T cell clones that are cross-reactive to commensals and viruses are common in healthy patients (Su et al., 2013). By contrast, aberrant accumulation of commensal-specific T cells under defined settings may lead to several pathogenic consequences, such as IBD and psoriasis (Sartor, 2006). Exploration of the antigen specificity of the memory cell compartment of lymphocytes residing at all barrier sites would inform us of the potential impact of these commensal-specific T cell responses on tissue physiology and subsequent pathologies. It would be of particular interest to address how responses to conserved bacterial antigens across barrier surfaces affect local and systemic tissue responses over time.

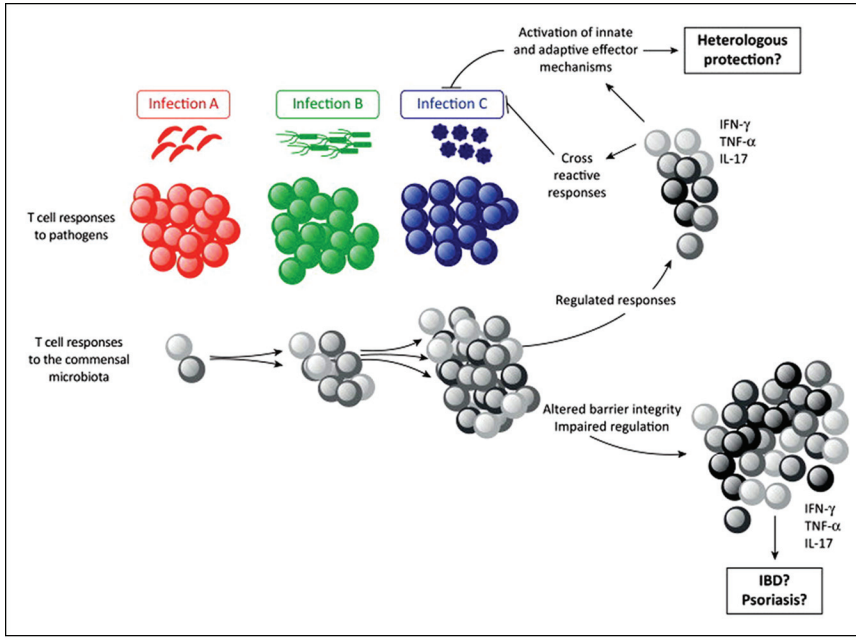


FIGURE A1-2 Potential consequences of commensal-specific memory T cells. During infection at barrier sites (gut, skin, airway, and genital mucosa), immune responses against the invading agent can be associated with specific T cell responses against a large number of coincident commensal antigens. These commensal-specific effector T cell responses can persist as memory cells that upon subsequent infection will be recalled as secondary commensal-specific effectors, alongside the priming of a novel immune response to the invasive pathogen. Therefore, each infection at barrier surfaces represents an additional opportunity for the reactivation of commensal-specific T cells. Given the extraordinary number of commensal antigens, these responses may represent a significant proportion of memory T cells. If properly controlled, commensal-specific effector/memory cells could contribute to protection against infections by promoting innate and adaptive effector mechanisms that assist in the clearance of the pathogen. Further, commensal memory responses could be protective due to cross-reactivity with pathogen-derived antigens. By contrast, in situations where commensal-specific T cells become dysregulated due to impaired regulatory pathways and/or barrier function, these T cells could drive chronic pathology such as inflammatory bowel disease or psoriasis.

Commensal-Specific T Cells and GI Disorders

IBD refers to a group of chronic inflammatory disorders affecting the GI tract (Kaser et al., 2010). There are two main clinical forms of IBD: CD, which can affect any part of the GI tract, and ulcerative colitis (UC), in which pathology is restricted to the colonic mucosa (Kaser et al., 2010). The etiology of these

disorders is complex and believed to be the consequence of genetic factors, the host immune system, and environmental factors such as the microbiota (Maloy and Powrie, 2011). Individual genomewide association studies have revealed that a large number of risk factors are associated with active immune responses and altered barrier function (Anderson et al., 2011; Barrett et al., 2008; Franke et al., 2010; Jostins et al., 2012; McGovern et al., 2010). In light of recent findings, commensal-specific T cells may represent an important component of the disease. In mouse models of colitis, it is well known that the commensal microbiota is necessary for the induction of disease via activation of both innate and adaptive immune mechanisms. Commensal-specific CD4 T cells have been identified in murine models of colitis and in some models are required to drive disease (Kim et al., 2007; Kullberg et al., 2002; Yoshida et al., 2002). For example, immortalized commensal-specific CD4 T cell clones derived from colitis-prone mice are capable of transferring disease to wild type mice (Cong et al., 1998). Activated T cells can also induce colitis in nucleotide-binding oligomerization domain-containing protein 2 NOD2-deficient mice in response to antigens carried by commensal bacteria (Watanabe et al., 2006). Studies comparing TCR transgenic cells in the recombination activating gene/severe combined immunodeficiency (Rag/SCID) model of colitis indicate that although commensal specificity is unnecessary for the proliferation and accumulation of CD4 T cells at mucosal sites, cognate antigen responses are required for the induction of colitis (Feng et al., 2010). Additionally, germ-free IL-10 knockout mice monocolonized with either *E. coli* or *Ent. faecalis* have been shown to develop CD4 T cell responses against the colonizing bacteria (Kim et al., 2007). Furthermore, feeding IL-10 knockout mice a diet high in milk fat is associated with increased Th1 T cell responses to a particular bacteria, *Bilophila wadsworthia*, although whether these are strictly antigen specific remains unclear (Devkota et al., 2012). A single genus of bacteria, *Helicobacter*, is sufficient for the induction of colitis in both the IL-10 and Rag/SCID models of colitis, and T cells specific to *Helicobacter* are present and sufficient for disease in animal models of IBD (Kullberg et al., 2001, 2003; Powrie and Uhlig, 2004). Finally, in mice in which both IL-10 and TGFβ signaling is deficient in T cells, spontaneous colitis occurs that is directly dependent upon the presence of bacteria of the genus *Bacteroides* (Bloom et al., 2011). One key point taken from these studies is that multiple bacterial types comprising several distantly related bacterial phyla can promote colitis, possibly via the induction of specific CD4 T cell responses, supporting the idea that IBD may develop as a consequence of a broad loss of tolerance to the commensal microbiota (Sartor, 2006). In support of this hypothesis, higher titers of commensal-specific antibodies are found in the serum of CD patients compared to healthy donors, and the measurement of antibody responses to a panel of seemingly unrelated commensal-derived antigens is commonly used as a diagnostic method for IBD (Iskandar and Ciorba, 2012; Zimmermann et al., 2012). However, commensal-specific responses are observed in healthy individuals, suggesting that on its own, immunity to commensals is not

sufficient for the induction of diseases (Haas et al., 2011; Sartor, 2006). Thus, under normal settings, effector and memory responses against commensals that have been induced by infections or injuries are likely held in check by the combined effect of the mucosal firewall and active mechanisms of tolerance. IBD, however, could be the result of the environmental activation of commensal-specific T cells in the context of a genetic predisposition for intestinal pathology and in particular defects in repair and immune regulation. This multiple hit mechanism of disease induction is supported by data that show mucosal viral infection, the commensal microbiota, and diminished Paneth cell function due to reduced expression of autophagy-related protein 16 L1 (ATG16L1), converge to increase disease severity in experimental colitis (Cadwell et al., 2010). However, despite clear connections between commensal-specific T cell responses and IBD, more remains to be done to understand how immunity to commensals could be causative in disease and how commensal-specific effector T cells are regulated under homeostatic conditions (Figure A1-1C).

Concluding Remarks

Adaptive immunity, as defined by the presence of lymphocytes with rearranged antigen receptors of near infinite specificity, is a characteristic of organisms that carry complex populations of microbial symbionts upon their mucosal surfaces. One might speculate that the coevolution between the adaptive immune system and commensal microbiota was primarily driven by the difficulty of maintaining and controlling such a complex relationship. However, barrier surfaces are not static and are often perturbed by environmental or infectious challenges, causing changes to the commensal microbiota and increasing tissue permeability. In Westernized countries, increased use of antibiotics, reduced worm infections, and drastic changes in nutrition have imposed massive changes in our relations with these organisms. Our understanding of commensal–immune interactions under these highly fluctuating circumstances is still in its infancy and much remains to be understood about commensal-specific responses and their consequences for human health.

Acknowledgments

This work was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. We would like to thank the members of the Belkaid laboratory for helpful discussions and in particular Drs. A. Poholek, E. Wohlfert, and S. Naik for critical reading.

References

Abt, M.C. et al. (2012) Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 37, 158–170

Anderson, C.A. et al. (2011) Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat. Genet. 43, 246–252

Arakawa, T. et al. (2012) Small intestinal injury caused by NSAIDs/ aspirin: finding new from old. Curr. Med. Chem. 19, 77–81

Arthur, J.C. et al. (2012) Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338, 120–123

Atarashi, K. et al. (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331, 337–341

Backhed, F. et al. (2005) Host-bacterial mutualism in the human intestine. Science 307, 1915–1920

Barrett, J.C. et al. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat. Genet. 40, 955–962

Benson, A. et al. (2009) Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. Cell Host Microbe 6, 187–196

Benson, M.J. et al. (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J. Exp. Med. 204, 1765–1774

Berkley, J.A. et al. (2005) Bacteremia among children admitted to a rural hospital in Kenya. N. Engl. J. Med. 352, 39–47

Bettelli, E. et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238

Bilate, A.M. and Lafaille, J.J. (2012) Induced CD4+Foxp3+ regulatory T cells in immune tolerance. Annu. Rev. Immunol. 30, 733–758

Bloom, S.M. et al. (2011) Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe 9, 390–403

Brandl, K. et al. (2008) Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 455, 804–807

Brenchley, J.M. and Douek, D.C. (2012) Microbial translocation across the GI tract. Annu. Rev. Immunol. 30, 149–173

Brenchley, J.M. et al. (2006) Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat. Med. 12, 1365–1371

Britton, R.A. and Young, V.B. (2012) Interaction between the intestinal microbiota and host in Clostridium difficile colonization resistance. Trends Microbiol. 20, 313–319

Cadwell, K. et al. (2010) Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 141, 1135–1145

Chow, J. et al. (2011) Pathobionts of the gastrointestinal microbiota and inflammatory disease. Curr. Opin. Immunol. 23, 473–480

Clemente, J.C. et al. (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148, 1258–1270

Cong, Y. et al. (1998) CD4+ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H/HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. J. Exp. Med. 187, 855–864

Cong, Y. et al. (2009) A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc. Natl. Acad. Sci. U.S.A. 106, 19256–19261

Coombes, J.L. et al. (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF- {beta}- and retinoic acid-dependent mechanism. J. Exp. Med. 204, 1757–1764

Costello, E.K. et al. (2012) The application of ecological theory toward an understanding of the human microbiome. Science 336, 1255–1262

Craven, M. et al. (2012) Inflammation drives dysbiosis and bacterial invasion in murine models of ileal Crohn's disease. PLoS ONE 7, e41594

Denning, T.L. et al. (2011) Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. J. Immunol. 187, 733–747

Dethlefsen, L. and Relman, D.A. (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. U.S.A. 108 (Suppl. 1), 4554–4561

Devkota, S. et al. (2012) Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 487, 104–108

Eckburg, P.B. et al. (2003) Archaea and their potential role in human disease. Infect. Immun. 71, 591–596

Egan, C.E. et al. (2011) Synergy between intraepithelial lymphocytes and lamina propria T cells drives intestinal inflammation during infection. Mucosal Immunol. 4, 658–670

Estes, J.D. et al. (2010) Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog. 6, e1001052

Feng, T. et al. (2010) Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. J. Exp. Med. 207, 1321–1332

Feng, T. et al. (2011) Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis. Gastroenterology 140, 2031–2043

Fossiez, F. et al. (1996) T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J. Exp. Med. 183, 2593–2603

Foulongne, V. et al. (2012) Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. PLoS ONE 7, e38499

Franke, A. et al. (2010) Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat. Genet. 42, 1118–1125

Gaboriau-Routhiau, V. et al. (2009) The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 31, 677–689

Garrett, W.S. et al. (2010) Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. Cell Host Microbe 8, 292–300

Geddes, K. et al. (2011) Identification of an innate T helper type 17 response to intestinal bacterial pathogens. Nat. Med. 17, 837–844

Geuking, M.B. et al. (2011) Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity 34, 794–806

Grice, E.A. and Segre, J.A. (2012) The human microbiome: our second genome. Annu. Rev. Genomics Hum. Genet. 13, 151–170

Haas, A. et al. (2011) Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection. Gut 60, 1506–1519

Hall, J.A. et al. (2008) Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity 29, 637–649

Hall, J.A. et al. (2011) Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity 34, 435–447

Hand, T.W. et al. (2012) Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. Science 337, 1553–1556

Hapfelmeier, S. et al. (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 328, 1705–1709

Heimesaat, M.M. et al. (2006) Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. J. Immunol. 177, 8785–8795

Hill, D.A. et al. (2010) Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 3, 148–158

Homann, D. et al. (2001) Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat. Med. 7, 913–919

Hooper, L.V. et al. (2012) Interactions between the microbiota and the immune system. Science 336, 1268–1273

Hu, W. et al. (2011) Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. Immunity 35, 1010–1022

Hughes, S.M. et al. (2009) Dendritic cell anergy results from endotoxemia in severe malnutrition. J. Immunol. 183, 2818–2826

Ichinohe, T. et al. (2011) Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. U.S.A. 108, 5354–5359

Iliev, I.D. et al. (2012) Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 336, 1314–1317

Ishigame, H. et al. (2009) Differential roles of interleukin-17A and - 17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 30, 108–119

Iskandar, H.N. and Ciorba, M.A. (2012) Biomarkers in inflammatory bowel disease: current practices and recent advances. Transl. Res. 159, 313–325

Ivanov, I.I. et al. (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349

Ivanov, I.I. et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139, 485–498

Jostins, L. et al. (2012) Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 491, 119–124

Kaser, A. et al. (2010) Inflammatory bowel disease. Annu. Rev. Immunol. 28, 573–621

Kim, S.C. et al. (2007) Dual-association of gnotobiotic IL-10-/- mice with 2 nonpathogenic commensal bacteria induces aggressive pancolitis. Inflamm. Bowel Dis. 13, 1457–1466

Konkel, J.E. and Chen, W. (2011) Balancing acts: the role of TGF-beta in the mucosal immune system. Trends Mol. Med. 17, 668–676

Korn, T. et al. (2009) IL-17 and Th17 Cells. Annu. Rev. Immunol. 27, 485–517

Kosek, M. et al. (2003) The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. World Health Organ. 81, 197–204

Kullberg, M.C. et al. (2001) Helicobacter hepaticus-induced colitis in interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation. Infect. Immun. 69, 4232–4241

Kullberg, M.C. et al. (2002) Bacteria-triggered CD4(+) T regulatory cells suppress Helicobacter hepaticus-induced colitis. J. Exp. Med. 196, 505–515

Kullberg, M.C. et al. (2003) Induction of colitis by a CD4+ T cell clone specific for a bacterial epitope. Proc. Natl. Acad. Sci. U.S.A. 100, 15830–15835

Lathrop, S.K. et al. (2011) Peripheral education of the immune system by colonic commensal microbiota. Nature 478, 250–254

Ley, R.E. et al. (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837–848

Lochner, M. et al. (2011) Restricted microbiota and absence of cognate TCR antigen leads to an unbalanced generation of Th17 cells. J. Immunol. 186, 1531–1537

Lodes, M.J. et al. (2004) Bacterial flagellin is a dominant antigen in Crohn disease. J. Clin. Invest. 113, 1296–1306

Lozupone, C.A. et al. (2012) Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230

Lupp, C. et al. (2007) Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2, 119–129

Macpherson, A.J. and Uhr, T. (2004) Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science 303, 1662–1665

Macpherson, A.J. et al. (2009) The mucosal firewalls against commensal intestinal microbes. Semin. Immunopathol. 31, 145–149

Maloy, K.J. and Powrie, F. (2011) Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474, 298–306

Maynard, C.L. and Weaver, C.T. (2009) Intestinal effector T cells in health and disease. Immunity 31, 389–400

Mazmanian, S.K. et al. (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122, 107–118

Mazmanian, S.K. et al. (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453, 620–625

McGovern, D.P. et al. (2010) Genome-wide association identifies multiple ulcerative colitis susceptibility loci. Nat. Genet. 42, 332–337

Meinzer, U. et al. (2012) Yersinia pseudotuberculosis effector YopJ subverts the Nod2/RICK/TAK1 pathway and activates caspase-1 to induce intestinal barrier dysfunction. Cell Host Microbe 11, 337–351

Molloy, M.J. et al. (2012) Intestinal microbiota: shaping local and systemic immune responses. Semin. Immunol. 24, 58–66

Mora, J.R. et al. (2006) Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 314, 1157–1160

Mucida, D. et al. (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 317, 256–260

Mucida, D. et al. (2009) Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. Immunity 30, 471–473

Murphy, K.M. and Stockinger, B. (2010) Effector T cell plasticity: flexibility in the face of changing circumstances. Nat. Immunol. 11, 674–680

Naik, S. et al. (2012) Compartmentalized control of skin immunity by resident commensals. Science 337, 1115–1119

Nieman, D.C. et al. (2006) Ibuprofen use, endotoxemia, inflammation, and plasma cytokines during ultramarathon competition. Brain Behav. Immun. 20, 578–584

O'Mahony, C. et al. (2008) Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. PLoS Pathog. 4, e1000112

Ochoa-Reparaz, J. et al. (2010) Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. J. Immunol. 185, 4101–4108

Oldenhove, G. et al. (2009) Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity 31, 772–786

Pepper, M. et al. (2009) Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nat. Immunol. 11, 83–89

Peterson, D.A. et al. (2007) IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe 2, 328–339

Powrie, F. and Uhlig, H. (2004) Animal models of intestinal inflammation: clues to the pathogenesis of inflammatory bowel disease. Novartis Found. Symp. 263, 164–174

Purohit, V. et al. (2008) Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. Alcohol 42, 349–361

Qin, J. et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65

Qin, J. et al. (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490, 55–60

Reyes, A. et al. (2010) Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466, 334–338

Rolhion, N. and Darfeuille-Michaud, A. (2007) Adherent-invasive Escherichia coli in inflammatory bowel disease. Inflamm. Bowel Dis. 13, 1277–1283

Round, J.L. and Mazmanian, S.K. (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc. Natl. Acad. Sci. U.S.A. 107, 12204–12209

Round, J.L. et al. (2011) The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science 332, 974–977

Sartor, R.B. (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat. Clin. Pract. Gastroenterol. Hepatol. 3, 390–407

Severance, E.G. et al. (2012) Anti-gluten immune response following Toxoplasma gondii infection in mice. PLoS ONE 7, e50991

Slack, E. et al. (2009) Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science 325, 617–620

Sonnenberg, G.F. et al. (2011) Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat. Immunol. 12, 383–390

Sonnenberg, G.F. et al. (2012) Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science 336, 1321–1325

Stecher, B. and Hardt, W.D. (2011) Mechanisms controlling pathogen colonization of the gut. Curr. Opin. Microbiol. 14, 82–91

Su, L.F. et al. (2013) Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults. Immunity 38, 373–383

Sun, C.M. et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J. Exp. Med. 204, 1775–1785

Suzuki, K. et al. (2004) Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc. Natl. Acad. Sci. U.S.A. 101, 1981–1986

Tsuji, M. et al. (2009) Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science 323, 1488–1492

Ubeda, C. et al. (2010) Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J. Clin. Invest. 120, 4332–4341

Vaishnava, S. et al. (2011) The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 334, 255–258

Vernacchio, L. et al. (2006) Diarrhea in American infants and young children in the community setting: incidence, clinical presentation and microbiology. Pediatr. Infect. Dis. J. 25, 2–7

Watanabe, T. et al. (2006) Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. Immunity 25, 473–485

Weiner, H.L. et al. (2011) Oral tolerance. Immunol. Rev. 241, 241–259

Wong, F. (2012) Recent advances in our understanding of hepatorenal syndrome. Nat. Rev. Gastroenterol. Hepatol. 9, 382–391

Wu, H.J. et al. (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 32, 815–827

Yoshida, M. et al. (2002) Differential localization of colitogenic Th1 and Th2 cells monospecific to a microflora-associated antigen in mice. Gastroenterology 123, 1949–1961

Zheng, Y. et al. (2008) Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat. Med. 14, 282–289

Zhou, L. et al. (2008) TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature 453, 236–240

Zimmermann, K. et al. (2012) Systemic antibody responses to gut microbes in health and disease. Gut Microbes 3, 42–47

A2

WHAT ARE THE CONSEQUENCES OF THE DISAPPEARING HUMAN MICROBIOTA?[3]

*Martin J. **Blaser**[4] and Stanley Falkow*[5]

Humans and our ancestors have evolved since the most ancient times with a commensal microbiota. The conservation of indicator species in a niche-specific manner across all of the studied human population groups suggests that the microbiota confer conserved benefits on humans. Nevertheless, certain of these organisms have pathogenic properties and, through medical practices and lifestyle changes, their prevalence in human populations is changing, often to an extreme degree. In this essay, we propose that the disappearance of these ancestral indigenous organisms, which are intimately involved in human physiology, is not entirely beneficial and has consequences that might include post-modern conditions such as obesity and asthma.

We have met the enemy, and he is us.
—Walt Kelly, 1953

The enemy of my enemy is my friend.
—Arabic Proverb

This is an era of great change. We are exploring the outer reaches of the universe and can travel across continents in hours, but we are also warming the Earth and depleting its oceans. These major changes in our macroecology represent the price for the past century's technological progress. We have also begun to explore the microscopic universe within us. Is this smaller-scale biosphere affected by similar changes? In particular, has the progress that has lowered infant mortality and prolonged lifespan also caused unanticipated alterations in our microecology and, consequently, our health?

In this essay, we focus on our intimate interface with microorganisms: residents and transients, and symbionts and pathogens. We will explore whether and how our microecology is changing, in analogy to our altered macroecology.

---

[3] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Blaser, Martin and Stanley Falkow. 2009. What are the consequences of the disappearing human microbiota? *Nature Reviews Microbiology* 7(12): 887-894), copyright 2009.

[4] Department of Medicine and the Department of Microbiology, New York University Langone Medical Center, New York, New York 10017, USA.

[5] Department of Microbiology and Immunology and the Department of Medicine, Stanford University School of Medicine, Stanford, California 94307, USA.

Correspondence to M.J.B. E-mail: Martin.Blaser@med.nyu.edu

What Are Our Microbiota?

We live in a microbial world (Whitman et al., 1998). When animals emerged, microorganisms had long (> 3 billion years) been part of a vast community of life that included complex endosymbiotic relationships, and it has been calculated that animals have carried resident microorganisms since at least the emergence of sponges (Hoffmeister and Martin, 2003; Iyer et al., 2004). Humans have become host to a myriad of microorganisms that assemble into complex, largely beneficial communities that outnumber human cells by tenfold. The dominant forms of human–microorganism interactions are those in which microorganisms benefit the host without causing harm (commensal relationships) and those in which both host and microorganism benefit (symbiotic or mutualistic relationships). Co-evolution, co-adaptation and codependency are all features of our relationships with our indigenous microbiota (Dethlefsen et al., 2007; Hickman, 2005).

The vertebrate microbiota can be characterized as: ancient, with deep ancestries; conserved in their host species; often present for defined life cycle events or persisting for life; and host-niche specific. These properties imply that the microbiota have been selected, but it is becoming increasingly clear that although they are bounded by these rules they are also highly individual.

The human microbiome is the subject of intensive studies, including the international Human Microbiome Project (Turnbaugh et al., 2007) (Box A2-1). Although possibly germ-free (gnotobiotic) before birth, humans develop a resident microbiota shortly after birth. In the neonatal period, the community assembly process is dynamic and is influenced by early environmental (in particular, maternal) exposure and stochastic effects (Palmer et al., 2007). The composition of the indigenous microbiota evolves in a generally orderly manner in response to diet and other environmental factors and is also influenced by diverse human genetic backgrounds. The bacterial diversity in the human body is striking in its richness of distinct species and strains, but it is noteworthy that a limited number of phyla are commonly found in indigenous microbial communities. Only 4 of the more than 50 bacterial phyla that have been identified in the environment (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria) dominate human mucosal and cutaneous habitats, which suggests that strong selective forces have limited diversity over at least hundreds of thousands of years of co-evolution (Aas et al., 2005; Bik et al., 2006; Eckberg et al., 2005; Flerer et al., 2008; Gao et al., 2007; Gill et al., 2006; Ley et al., 2008; Lepp et al., 2004; Pei et al., 2004). Despite this stereotypical assembly process, each individual in a single mammalian species, including *Homo sapiens*, has a virtually unique microbiota (Ley et al., 2008; Pei et al., 2004). The composition of the microbiota and the phenotypes that are expressed affect, as well as reflect, the overall biological diversity of humans.

Most research has focused on defining the core elements of the microbiota that are shared by all, or at least most, humans (Eckburg et al., 2005). To date, the rules that govern life on and in humans are poorly understood, and we are still in the earliest stages of discerning the functions of the microbiota in health

BOX A2-1
The Human Microbiome Project

In recent years, there has been a growing appreciation of the extent of the commensal microbial populations in humans. Collectively, these populations have been termed "the human microbiome," originally by Joshua Lederberg (Lederberg and McCray, 2001), and also the "human microbiota." Owing to advances in DNA sequencing technologies and improvements in bioinformatics, it has become possible to characterize the great diversity in the human microbiota. In 2007, the U.S. National Institutes of Health (NIH) launched the Human Microbiome Project (see Further Information) as one of its major roadmap initiatives, earmarking ~US$140 million for its completion (Peterson, 2009). This major scientific endeavour has the following aims:

- To determine whether individuals share a core human microbiome;
- To understand whether changes in the human microbiome can be correlated with changes in human health;
- To develop the technological tools to support these goals;
- To address the ethical, legal, and social implications of human microbiome research.

One value of the Human Microbiome Project is that it can help us to ascertain how much the microbiota have been changing. Work is now ongoing, and the European Union and other countries are committed to similar projects, all working under the umbrella of the International Human Microbiome Consortium (see Further Information).

and disease (Dethlefsen et al., 2007). The human microbiota facilitate the extraction of energy from food, provide accessory growth factors, promote post-natal terminal differentiation of mucosal structure and function, stimulate both the innate and adaptive immune systems, and provide "colonization resistance" against pathogen invasion (Crowe et al., 1973; Mackowiak, 1982; Rosebury, 1962; Smith et al., 2007). However, studies of gnotobiotic animals have shown that microorganisms (or at least bacteria) are not necessary for life or for the completion of an animal life cycle (Gordon and Pesti, 1971; Mackowiak, 1982; Smith et al., 2007). Paradoxically, therefore, there seems to be no absolute requirement for a functional resident microbiota, but nor does gnotobiosis occur in nature.

Although the relationship between animals and their resident microbiota is thought to be largely beneficial, it can also become detrimental (Mackowiak, 1982; Pamer, 2007). In humans, commensal α-haemolytic streptococci colonize the mouth and intestinal tract and, in part, protect us against incursions by pathogenic streptococci (Crowe et al., 1973; Roos et al., 1996). However, these commensal organisms are often transiently present in the bloodstream after we chew food, brush our teeth or defecate (Durack, 1995; Roberts, 1999). If there

is damaged tissue present, such as an ageing heart valve, these long-term residents can cause a fatal infection. Thus, our generally beneficial microbiota have the capacity to cause our demise. They thin the herd, particularly if the normal defences that hold microorganisms in check are compromised in some manner.

It is clear that we share a complex biological relationship with our microscopic companions. Are these organisms symbionts or parasites? (Box A2-2). The ecologist Theodor Rosebury created the term "amphibiosis" to describe relationships that can be symbiotic or parasitic, depending on the biological context (Rosebury, 1962). This concept is the guiding principle for the remainder of this essay.

When influenza sweeps across the globe, infecting millions and causing disease and sometimes death, we agree that the virus is a pathogen, as is *Yersinia pestis*, the cause of bubonic plague. However, the distinction between a commensal resident microorganism and a pathogen can be blurred at times, in part because some commensals cause disease, albeit often in immunocompromised hosts, whereas some of the most feared pathogens can persist in humans for a lifetime without causing any symptoms (Falkow, 2006). For example, several members of the nasopharyngeal microbiota, including *Streptococcus pneumoniae* and *Neisseria meningitidis*, regularly cause disease. Such "commensal pathogens" colonize a substantial proportion of the human population, most of whom are asymptomatic carriers (Murphy et al., 1993). Are these organisms pathogens,

BOX A2-2
What Is a Pathogen?

Microbial pathogens can range from bacteria to metazoa to viruses or prions. All pathogens are excellent cell biologists, taking advantage of chinks in the armour of their hosts to propagate.

There is no intrinsic difference between a pathogen and a symbiont, only context. However, there are fingerprints that are sufficiently characteristic of pathogens or symbionts to be used to help distinguish between them:

- Pathogens are generally clonal (Kennedy et al., 2008; Rich et al., 2001; Sarkar and Guttman, 2004; Tibayrenc and Ayala, 2002).
- Pathogens induce host responses. They usually cannot do their deeds without a trace.
- Infection with a pathogen can sometimes cause disease, which is a function of the interaction between the pathogen and the specific host, as determined by a matrix of host, microbial, and environment circumstances.
- The gene pool of the host includes the genes of its resident microorganisms, which may be why many members of the microbiota are selected for by the host: to prevent colonization by pathogens instead.

or should they be considered to be members of the indigenous microbiota that have evolved to live in perilous locations (Mazmanian et al., 2008), such as in respiratory tract-associated lymphatic tissue? From this viewpoint, the specific indigenous microbiota in such niches would regularly come into contact with elements of an immune system that hold them at bay most of the time (Pamer, 2007) but that occasionally fail to do so, resulting in disease. Immunization against these microorganisms not only protects against disease but also prevents host colonization, in an antigen-specific manner (reviewed in Segal and Pollard, 2004). Serotype replacement is an acknowledged deleterious effect of such immunization programmes, but may there be other deleterious effects that we have yet to uncover? It is crucial to improve our understanding of the relationships between humans and our microbiota, so that we can assess the risks, in addition to the potential benefits, of modifying the composition of the microbiota deliberately or inadvertently. The use of molecular Koch's postulates (Falkow, 2004) may assist in this risk assessment.

How Are the Microbiota Stabilized?

Is there any underlying biological regulation that stabilizes the diverse microbial populations that we carry, even in the face of host immunity, competing organisms and the daily continuous flux of newly introduced microorganisms? Cooperation between competing life forms is a challenging concept and, although much of the literature on microorganisms focuses on the evolution of virulence (Maynard Smith and Szathmary, 1995; Messenger et al., 1997) there is an increasing focus on the rules that govern the evolution of cooperation (Michod, 1997; Sachs and Bull, 2005).

In an earlier report, a hypothesis was presented that Nash equilibria enable pathogens such as *Mycobacterium tuberculosis* and *Salmonella enterica* subsp. *enterica* serovar Typhi to persist (Blaser and Kirshner, 2007). Such a view has also been applied to *Helicobacter pylori*, the dominant member of the gastric microbiota (Bik et al., 2006). In game theory, a Nash equilibrium represents a particular circumstance in which any player who deviates from the rules of the game is in an inferior position compared to those who have played by the rules (Nash 1950, 1951). Such conditions can exist in a limited number of situations in nature, and biological co-evolution is a prime example.

For any equilibrium to function, there must be boundary conditions or limits and strong penalties against transgressors that, in net, remove any advantage from "cheating" (Ma et al., 1988). This is characteristic of a Nash equilibrium, but the complex evolved biology of metazoans such as humans requires solutions that operate on multiple scales. A series of nested dynamic equilibria was envisioned, in which each stratum contributes to creating the boundary conditions for the others (Blaser and Kirschner, 2007). This is made possible, in part, by the fractal nature of biological communities, in which ecosystems nest within one another,

a structure that provides both stability and resilience in a world of opportunists, invaders and cheaters. In steady state, the equilibria form a continuum, analogous to a Mobius strip, with no beginning or end. This population structure permits the co-evolution of competing organisms (for example, host and microorganism) that would otherwise lead to an "arms race" (Obbard et al., 2006) and the destruction of one organism or the other. However, even in such a derived evolutionarily stable strategy (Smith, 1982), all is not fixed. The equilibrium has sufficient elasticity for invasions (by pathogens or "cheaters") to be contained under normal conditions of flux. However, even with such capacitance, there must be boundaries beyond which the systems lose equilibria and descend towards the extinction of one partner or the other.

In the human biosphere, the boundaries for flux are unknown. Even a catastrophe like the AIDS epidemic has so far been contained within the overall population, in part because of the inefficiencies of HIV transmission (Gray et al., 2001). However, scenarios involving more transmissible agents in a "smaller world" in which population connectivity selects for virulence and the ecosystem has been perturbed are worrisome (Galvani, 2003). This is directly germane to considerations of the stability of the microbiota. On the basis of humanity's long association with our indigenous microorganisms and their conservation (Dethlefsen et al., 2007; Ley et al., 2008), the equilibria that maintain the composition of our microbiota should be considered to be major pillars of our biological stability. Seen from this view, pathogens that arise are "cheaters" that can break the equilibria. In principle, cheaters' can cause the extinction of the microorganism, the host or even both, although in reality variants usually emerge and equilibria ultimately rule. For example, if the host-mediated penalties against "cheating" (such as host immunity to the microorganism or the microorganism's loss of a niche in the host) are sufficiently high, then less virulent variants that can evade biological surveillance can become successful.

The "Disappearing Microbiota" Hypothesis

In general, it seems that the human microbiota and their hosts evolved their equilibria together, in an orderly way (Ley et al., 2008), as diet, geography and occasional ecological disturbances had their effects on distinct, albeit diverse, human genetic backgrounds (Turnbaugh et al., 2009). Our complex protective commensal microbiota facilitate nutrient and vitamin acquisition, promote tissue development and integrity, and stimulate multiple aspects of immunity (Grodeon and Pesti, 1971; Mackowiak, 1982; Pamer, 2007; Rosebury, 1962; Smith et al., 2007). However, in the face of modern global ecological changes, has there been stability for the descendents of the microbiota that colonized humans during the ~2 million years that they lived in small groups as hunter–gatherers?

As human health and longevity have improved in developed countries, new diseases have arisen without obvious explanation. The "disappearing microbiota"

hypothesis (Blaser, 2006, 2008) has been advanced to explain the rise and fall of several common diseases in developed countries. Beginning in the nineteenth century and accelerating in the twentieth century, there have been dramatic changes in human ecology (Table A2-1), including cleaner water, smaller families, an increase in the number of caesarian sections, increased use of pre-term antibiotics, lower rates of breastfeeding and more than 60 years of widespread antibiotic use, particularly in young children. How have these changes affected the transmission and maintenance of the indigenous microbiota (Blaser, 2006)?

We postulate that the important factor in modern allergic and metabolic diseases might not be our decreased sampling of the microorganisms in food, air, water or soil, as has been postulated by the "hygiene hypothesis" (Strachan, 1989), but instead could reflect the loss of our ancestral microorganisms. As the representation of particular species diminishes in one generation, the potential for vertical transmission to the next generation (Nahar et al., 2009) can decrease in a stepwise manner (Figure A2-1). Diminished horizontal transmission resulting from changes in human ecology (Table A2-1) makes it more difficult to overcome losses in vertical transmission, and this then manifests as a birth cohort phenomenon. We believe that alterations in human macroecology have progressively affected the composition of our indigenous microbiota, which in turn has affected human physiology and, ultimately, disease risk. The increases that have occurred in recent years in the prevalence of conditions such as obesity and asthma, as well as oesophageal disorders that are a consequence of reflux, have been so rapid that an environmental cause must be present (Eder et al., 2006; Flegal et al., 2007; Pohl and Welch, 2005). Is one of these causes the loss of one or more constituents of the indigenous microbiota?

H. pylori, an ancient member of the human microbiota (Linz et al., 2007), generally dominates the gastric niche (Box A2-3). Accordingly, its presence can be used as a means to assess the status of our microbiota. Surprisingly, *H. pylori*

TABLE A2-1 Changes in Human Ecology That Might Affect Microbiota Composition

Change	Consequence
Clean water	Reduced faecal transmission
Increase in Caesarean sections	Reduced vaginal transmission
Increased use of pre-term antibiotics	Reduced vaginal transmission
Reduced breast-feeding	Reduced cutaneous transmission and a changed immunological environment
Smaller family size	Reduced early life transmission
Widespread antibiotic use	Selection for a changing composition
Increased bathing, showering, and use of antibacterial soaps	Selection for a changing composition
Increased use of mercury-amalgam dental fillings	Selection for a changing composition

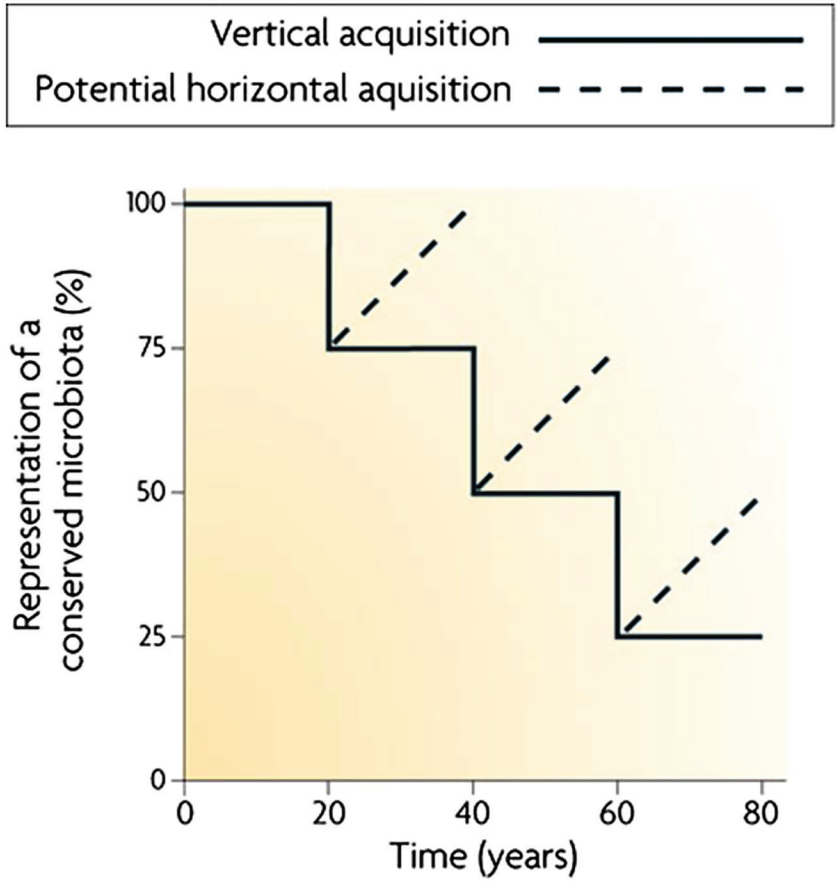


FIGURE A2-1 The effect of maternal status on the resident microbiota of the next generation. We propose that, since the earliest days of the evolution of mammals, there has been major maternal transmission of microbiota to their offspring (vertical transmission). However, loss of the conserved microbiota in one generation leads to its loss in the next. For humans, until recently, horizontal microbial transmission also occurred and could compensate for the loss of vertical transmission. Members of the microbiota were horizontally transmitted through faecally contaminated drinking and bathing water, and high physical contact as a result of social crowding and large families; in many modern societies, these routes have diminished. The progressive loss of vertically transmitted microorganisms without horizontal replacement represents a cumulative birth cohort phenomenon.

has been progressively disappearing (Banatvala et al., 1993; Harvey et al., 2002; Roosendaal et al., 1997) from individuals in developed countries (Figure A2-2) during the twentieth century, with secondary alterations in gastric secretory, hormonal and immune physiology (Blaser and Atherton, 2004; Chen and Blaser,

BOX A2-3
The Gastric Microbiome

The human body is colonized by a highly complex microbiota, but the stomach is an exceptional niche. When *Helicobacter pylori* can be detected by conventional means, including culture or biochemical assays, tissue histology and host serological responses, it is the numerically dominant organism, representing > 50% of all of the bacterial cells in the niche (Andersson et al., 2008; Bik et al., 2006). This phenomenon is unlike other human niches that house a large range of bacterial species but no single predominant organism (in the vagina, a single *Lactobacillus* species often predominates, but its identity differs in different women [Zhou et al., 2004]). The dominance of the gastric niche by *H. pylori* is consistent with its adaptations that enable gastric persistence (Blaser and Atherton, 2004). In persons in whom *H. pylori* cannot be detected by conventional means, the organism may still be detected by PCR-based techniques, usually as more minor populations (Bik et al., 2006). These observations suggest that there may be a "stealth" phenomenon in which *H. pylori* exists below the radar of histological, biochemical, and immunological fingerprints, but its characteristics are not yet understood.

In addition to *H. pylori*, numerous other bacterial species may be present, but whether they are resident in the stomach or transient from the upper gastrointestinal tract has not been determined (Bik et al., 2006). The phyla that are observed (chiefly Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes) seem to be similar in *H. pylori*-positive and *H. pylori*-negative hosts (Bik et al., 2006).

2008). These alterations have been associated with a progressively declining incidence of important illnesses with long latent periods, such as gastric cancer. We now have multiple tools to detect *H. pylori* (Suerbaum and Michetti, 2002). But if a lesser known ancestral indigenous microorganism disappeared in the colon, mouth, skin or vagina, could we identify that change and, crucially, could such a change (whether it was a loss or a replacement and subsequent 'overgrowth' by a different indigenous component) contribute to some of the diseases that are becoming more prevalent? The Human Microbiome Project (Turnbaugh et al., 2007) should begin to address such questions (Box A2-1).

The Consequences of Loss

Modelling of the specific host–microorganism relationships suggests that there are several potential physiological relationships that can occur (Figure A2-3). Several specific examples illustrate the concept that the loss of an indigenous microorganism will have consequences for the host.

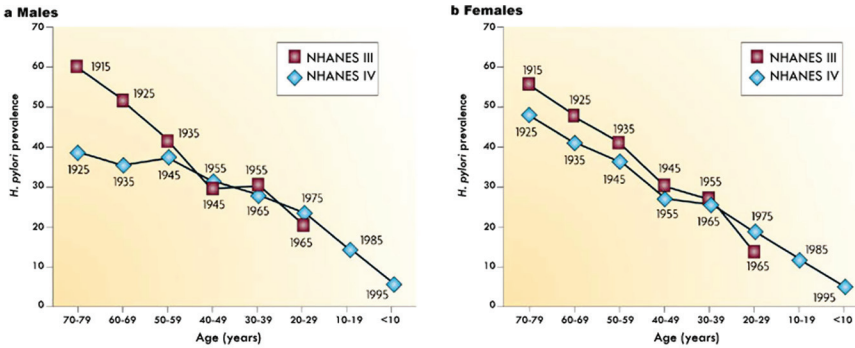


FIGURE A2-2 *Helicobacter pylori* prevalence in the United States by age and year of birth. *Helicobacter pylori* prevalence in males (**a**) and females (**b**) was determined in two large national studies (the National Health and Nutrition survey (NHANEs) III and NHANEs Iv), which were conducted in 1988–1991 and 1999–2000, respectively. These data, involving 15,000 subjects, are consistent with a now well-recognized birth cohort effect (Banatvala et al., 1993; Harvey et al., 2002; Roosendaal et al., 1997) in which *H. pylori* acquisition, and thus prevalence, has been declining in industrialized countries for >100 years. The numbers next to each point represent the midpoint year of birth for each group of survey subjects (with each group covering one decade of births).
NOTE: Image is modified, with permission, from Chen and Blaser (2008), University of Chicago Press.

Loss of H. pylori

H. pylori colonization and the response that it induces in the host affects the regulation of gastric hormones, including gastrin and somatostatin (Odum et al., 1994). As such, *H. pylori* status affects gastric pH and its regulation (Blaser and Atherton, 2004). Over the decades of *H. pylori* colonization, the mass of gastric acid-secreting glands in the host progressively decreases, owing to the long-term effects of inflammation (Kanno et al., 2009; Odum et al., 1994). Although the rates of glandular decrease reflect both host-specific and population-specific factors, overall there is a progressive decrease in acid production (Kulpers et al., 1995a) that is greater than the decrease observed in *H. pylori*-negative hosts (Kulpers et al., 1995a,b). The increasing gastric atrophy and hypochlorhydria that are associated with the presence of *H. pylori* (Argent et al., 2008; Kulpers et al., 1995a,b) contribute to the risk of gastric cancer (Kamangar et al., 2006; Peek and Blaser, 2002), but the sustained acidity in *H. pylori*-negative hosts increases the risk of gastroesophageal reflux disease (GERD) and its consequences, including oesophageal and gastric cardia adenocarcinomas (Islami and Kamangar, 2008; Kamangar et al., 2006).

As *H. pylori* is disappearing from human populations, reflecting both diminishing transmission and increasing antibiotic treatment (Table A2-1), both

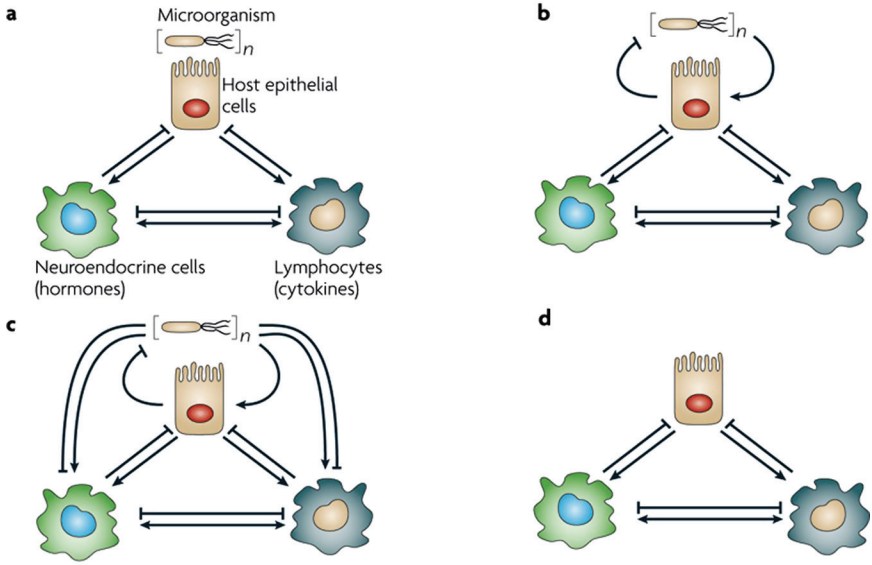


FIGURE A2-3 Interactions between host and microbiota. **(a)** For luminal microbiota that do not interact with host cells, the host-specific regulatory networks are essentially unaffected by transient microbiota or by luminal microbiota that are distant from the epithelium. This could represent a large class of organisms in the microbiota. **(b)** For microbiota that interact with epithelial cells, there is an equilibrium relationship involving signalling between the microbial populations and the host (Blaser and Kirschner, 2007). **(c)** For coevolved microbiota that interact with multiple cell types, the cross-signalling in the most fully coevolved states involves multiple host cell types, including epithelial, immunological, and neuroendocrine cells. The interaction of *Helicobacter pylori* with the gastric mucosa is representative of this model. The introduction of drugs, such as the commonly used proton pump inhibitors that reduce gastric acidity, affects equilibrium values and selects for a differing microbiota (Kanno et al., 2009; Vesper et al., 2009). **(d)** In the absence of a microbiota, the indigenous host regulatory mechanisms predominate and reach different homeostasis values than are reached when interacting microbiota are present. In the situation of a disappearing microbiome, (a) and (d) predominate.

"idiopathic" peptic ulcer disease and gastric cancer rates are diminishing (el-Serag and Sonnenberg, 1998; Howson et al., 1986), which is clearly salutary. However, oesophageal reflux, Barrett's oesophagus and adenocarcinoma are increasing, which is clearly deleterious (el-Serag and Sonnenberg, 1998; Pohl and Welch, 2005). Are these reciprocal phenomena? The *cag* pathogenicity island (Blaser and Atherton, 2004) (or perhaps a better term would be the *cag* ecological fitness island [Haker and Carniel, 2001]) status of the *H. pylori* population has a strong influence on the development of Barrett's oesophagus and adenocarcinoma,

as predicted by the strong interaction between c*ag*-positive strains and host cells (Bakert and Selbach, 2008; Kulpers et al., 1995a; Peek and Blaser, 2002; Nwokolo et al., 2003). The stomach also produces the hormones ghrelin and leptin, both of which have multiple roles in energy homeostasis (Blaser and Atherton, 2004). Patients in whom antibiotic treatment eliminates *H. pylori* have increased circulating ghrelin levels (Nwokolo et al., 2003). Investigations are ongoing, but it is already clear that in recent generations of children growing up in developed countries there has been little gastric *H. pylori*-mediated (Chen and Blaser, 2008) regulation of these adipokines at the developmental stage, when long-term adiposity is being programmed. It is possible that the disappearance of *H. pylori* might be contributing to the current epidemics of early-life obesity, type 2 diabetes and related metabolic syndromes.

Gastric T cell and B cell populations also differ in *H. pylori*-positive and *H. pylori*- negative hosts (Lundgren et al., 2005; Mattsson et al., 1998; Robinson et al., 2008). This might be expected, as the *H. pylori*-negative stomach has a diminished presence of the cellular elements of the immune system and reduced cytokine traffic, whereas the *H. pylori*-positive stomach has a rich population of immune cells (Figure A2-3). The enhanced T cell populations in *H. pylori*-positive hosts include greater proportions of particular T cell subsets, including cells expressing forkhead box protein P3 (FOXP3), which regulate immune functions (Lundgren et al., 2005; Robinson et al., 2008). The stomachs of *H. pylori*-negative hosts have much lower numbers of these cells, which have systemic, as well as local, activities. Several epidemiological studies (reviewed in Blaser et al., 2008) show that *H. pylori*-positive individuals (especially individuals carrying *cag*-positive strains) have lower risks of childhood asthma, allergic rhinitis and skin allergies than those without *H. pylori* (Chen and Blaser, 2007, 2008; Reibman et al., 2008). The rise in childhood asthma and related disorders has occurred while *H. pylori* has been disappearing; the loss of gastric T cell populations and their systemic effects could provide a mechanism for these allergic diseases (Codolo et al., 2008; Del Prete et al., 2008).

Subtherapeutic Antibiotic Treatment

In the early 1950s, not long after the discovery of antibiotics, scientists found that feeding low doses of antibiotics (termed subtherapeutic antibiotic treatment [STAT]) to farm animals increased their rate of growth and ability to convert food into body mass (otherwise known as their "feed efficiency"; reviewed in Jukes, 1972). The younger the animals are when STAT is started, the stronger the effect (Jukes, 1972). The fact that several antibiotics can produce this effect in poultry, cattle and swine (Butaye et al., 2003; Gaskins et al., 2002; Jukes, 1972) has led to the widespread usage of antibiotics as animal feed supplements in the United States and other developed countries (this practice has now been banned by the European Union because of the spread of antibiotic-resistant bacteria).

Why does giving STAT change energy homeostasis in this broad group of vertebrates? As growth promotion can be induced by many different antibacterial (but not antifungal) agents, this activity is not a side effect but a consequence of the antibacterial activities of these agents, which presumably affect the microbiota of the exposed animals (Gaskins et al., 2002). However, the affected microbial populations and metabolic pathways are unknown. Given that this manipulation of the microbiota has such effects on early life energy homeostasis and body mass development in farm animals, what might the effects be of the widespread exposure of children to antibiotics early in life? Instead of the continuous, low-dose antibiotics that are administered on the farm, we are giving our children short, high-dose pulses. How is this affecting their microbiota, energy homeostasis and "feed efficiency"? Even single courses of widely used antibiotics can change the stable structure of microbial communities, with the presence of selected organisms continuing for years after the antibiotic exposure has ceased (Sjölund et al., 2003, 2005). We speculate that the widespread treatment of young children with antibiotics has caused alterations in the compositions of their intestinal microbiota, and the lumenal signals to the host (Figure A2-3), that are directly contributing to the epidemic of obesity in developed countries.

The Pneumococcus and Staphylococcus aureus

S. pneumoniae (known as the pneumococcus) is an important human pathogen, causing pneumococcal pneumonia, infections of the upper respiratory tract and its appendages, and occasionally lethal diseases such as meningitis and endocarditis (Musher et al., 2000). Consequently, the development of a pneumococcal vaccine has been a priority for >100 years. However, pneumococci are carried by healthy persons in the nasopharynx, often for months, and are part of the consortia of microorganisms inhabiting this niche. Pneumococci are naturally transformable and vary extensively, with a major source of variation being the presence and type of polysaccharide capsule (MacLeod and Kraus, 1950). Most human disease is caused by encapsulated pneumococci, and particular serotypes predominate (Briles et al., 2005; Weinberger et al., 2008). Consequently, preventative strategies have focused on the most virulent capsular subtypes, and polyvalent vaccines have been developed (Ghaffar et al., 2004). These vaccines are effective and have reduced the incidence of serious pneumococcal infections in high-risk populations (Albrich et al., 2007). Immunization not only protects against disease but also prevents colonization by those pneumococci with the capsule types that are present in the vaccine (Ghaffar et al., 2004). One of the earliest concerns of researchers was that increasing the immunity to certain capsular types would lead to serotype replacement (Albrich et al., 2007; Ghaffar et al., 2004; Weinberger et al., 2008), which might result in new pneumococcal infections, but presumably at a lower rate than rates of infection by the more virulent pneumococci that are targeted by the vaccine. Although serotype replacement and the subsequent

pneumococcal disease would lower the net "effectiveness" of vaccination, there would still be residual utility.

All of these predicted consequences have come to pass (Albrich et al., 2007; Ghaffar et al., 2004; Madhi et al., 2007; Veenhoven et al., 2003). However, in addition to replacement with non-vaccine serotypes of *S. pneumoniae*, replacement with an unanticipated organism, *Staphylococcus aureus*, has occurred (Bogaert et al., 2004; Madhi et al., 2007; Regev-Yochay et al., 2004, 2008). A growing body of evidence indicates that the encapsulated pneumococci and *S. aureus* are ecological competitors (Bogaert et al., 2004; Regev-Yochay et al., 2004), and that the loss of the former is leading to the expansion of the latter (Regev-Yochay et al., 2004). This can take place through direct competition or through microbial manipulation of host immunity (Lysenko et al., 2008; Regev-Yochay et al., 2009; Selva et al., 2009). This is occurring just as we are witnessing an unprecedented epidemic of methicillin-resistant *S. aureus* (MRSA) infections in the community among persons who have not been recently hospitalized or received antibiotics. In the absence of obvious exposure or selection pressure, these community-acquired MRSA (CA-MRSA) strains are being widely transmitted from person to person and with occasional, but often serious, clinical phenotypes (Dufour et al., 2002; Herold et al., 1998). The ecology of *S. aureus* has clearly changed (Sachs and Bull, 2005). Whether or not this reflects the consequences of pneumococcal vaccination, antibiotic pressure selecting for particularly virulent clones, ecosystem degradation due to effects on the nasopharyngeal and cutaneous microbiota of widespread antibiotic use, or a combination of these factors remains to be determined.

Recolonizing a Vacated Niche

If the indigenous microbiota are disappearing, have there been replacements? Are there model systems in which to explore this question? As discussed above, *H. pylori*, the ancient and dominant microbial inhabitant of the human stomach, is disappearing with remarkable speed. In two or three generations, human societies have moved from near-ubiquitous (>80%) *H. pylori* prevalence rates to rates in single digits among contemporary native-born children in the United States (Figure A2-2) and western Europe (Chen and Blaser, 2008; Rothenbacher et al., 2002; Tindberg et al., 2001). This is an unprecedented change in human microecology.

What about the gastric niche itself: with *H. pylori* disappearing, will there be new tenants and, if so, how will they interact with the host? The stomach is bounded proximally by the oropharynx and oesophagus and distally by the intestine, all of which possess resident microbiota (Eckberg et al., 2005; Gill et al., 2006; Let et al., 2008; Pei et al., 2004). The gastrointestinal tract is also a portal to the outside world through ingested food and drink. Although *H. pylori* is the dominant gastric microorganism, there is DNA evidence for the presence

of other transient or residential microorganisms (Andersson et al., 2008; Bik et al., 2006) and indications that a more diverse microbial population is present in the *H. pylori*-vacated stomach (Andersson et al., 2008).

For occupancy of a vacated niche to occur, the transmission potential to other hosts is crucial (Brown et al., 2006). Several possible scenarios should be considered. The first possibility is that each individual will become colonized by their own host-specific microorganisms that can change over time but that are essentially unsuccessful at transmitting to new hosts. This would lead to billions of ongoing long-term experiments (one per human birth) in microbial transmission fitness. The second possibility is that the overall level of resident microorganisms will remain low, as other microorganisms cannot use the resources of the *H. pylori*-free stomach as efficiently as *H. pylori*. The third possibility is that, without *H. pylori* competition and inhibition, other microorganisms will better use the niche's resources. Such organisms could be a microbial consortium involving a few or many species, or a single species with a varied and ultimately robust pan-genome. Alternatively, they may be exogenous organisms that are acquired during ongoing environmental exposure. The transmission potential will determine whether the replacement organisms will be host-specific populations or emergent, highly selected organisms that are common between hosts. A potential consequence of the third scenario is the emergence of a highly transmissible microorganism that is well-adapted for the now sparsely populated niche. Because in this situation selection is based on the ability to transmit to new hosts, a range of virulence is possible, with the potential for positive selection for virulence (Brown et al., 2006). This could be dangerous, because the next main inhabitant of the gastric niche might not be as benign as *H. pylori,* which has coevolved with humans at a moderate level of virulence. A newly acquired organism that evolved to colonize and efficiently use the resources would probably be more virulent (Lenski and May, 1994) and could selectively sweep through the population.

These possibilities are not gastric specific but represent the general consequences that might accompany the extinction of species from the microbiota of any niche. The more dominant the species that are lost, the larger the void and the greater the potential risk of replacement with a highly virulent organism.

Future Therapeutics?

Will there be cases in which human health is improved by the replacement of some of the lost microbiota? Certainly, a trip through the dairy section of any supermarket in the Western world would lead one to believe so. Nonetheless, translating this marketing concept into scientifically based beneficial activities is limited by several outstanding questions. These include: which particular microorganisms should be replaced; what will their sources be; and what will the timing of their replacement be? We should begin to think about these options and their expected outcomes. Replacement will necessarily lead to the development of

new classes of probiotics; we speculate that one day we will see microbial additives that are specific for particular hosts, for specific durations of exposure and eradication. This is one vision for the future, but it will require a much deeper understanding of the metabolic, hormonal and immunological interactions with our microbiota (Figure A2-3).

One emerging concept in microbial pathogenesis is the notion of the "community as a pathogen," in which a conserved broad swathe of the microbial community, rather than any one specific member, contributes to disease. This concept might be relevant to a range of inflammatory processes of the skin and mucosa, including inflammatory bowel disease and chronic periodontitis. It suggests that studies of pathogenesis should consider the general properties of microbial communities, such as resilience, or conserved functional interactions, such as syntrophic interactions and the importance of gene transfer, rather than the role of single microorganisms, especially for the development of new approaches for maintaining or restoring health. Thus, under some circumstances, restoration of communities might be more appropriate than replacing single microorganisms.

Conclusions

It is predictable that social and medical progress that affects the composition of the microbiota will also have consequences for our physiology and health. However, the specific outcomes will only be learned empirically, as the human ecosystem is too complex for anything more than the most superficial predictions. Improving our prognostication is an important technical challenge. Is the selective disappearance of the microbiota contributing to oesophageal diseases, obesity and its consequences, asthma and related disorders, and the epidemic spread of high-grade pathogens? Further investigation will clarify these points, but the theoretical basis exists: ecological changes involving our ancient microbiota have the power to affect physiology and, ultimately, health. Individuals who are "normal" in modern societies might be representative of the population at large but not of our historical heritage (Marini et al., 2007). Studies of persons who are indigenous to those developing countries that have had little impact from modern health practices (including exposure to antibiotics) could be ideal to define our historic "norms."

Moving forward, we must learn to better distinguish between pathogens and amphibionts and to better assess in whom to eliminate, leave alone or restore the microorganism (or metabolic pathway) in question. Or is it even wise to contemplate any action until we understand more about our most intimate residents? Public health deals with trends in populations, but medical care inevitably involves individuals. A greater understanding of the characteristics of a host's genome and microbiota, and their interactions, will lead to individualized approaches to the prevention and treatment of specific diseases. We are at a scientific frontier.

Acknowledgements

M. Blaser gratefully acknowledges funding from the U.S. National Institutes of Health (NIH; grants RO1GM62370 and UH2AR057506) and from the Diane Belfer Program for Human Microbial Ecology. S. Falkow gratefully acknowledges funding from NIH (grants R01AI026195 and R01AI038459).

Databases

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj

Further Information

Human Microbiome Project: http://nihroadmap.nih.gov/hmp

International Human Microbiome Consortium: http://www.human-microbiome.org

Martin J. Blaser's homepage: http://www.med.nyu.edu/medicine/labs/blaserlab

References

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. & Dewhirst, F. E. Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**, 5721–5732 (2005).

Albrich, W. C., Baughman, W., Schmotzer, B. & Farley, M. M. Changing characteristics of invasive pneumococcal disease in Metropolitan Atlanta, Georgia, after introduction of a 7-valent pneumococcal conjugate vaccine. *Clin. Infect. Dis.* **44**, 1569–1576 (2007).

Andersson, A. F. *et al.* Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**, e2836 (2008).

Argent, R. H. *et al.* Toxigenic *Helicobacter pylori* infection precedes gastric hypochlorhydria in cancer relatives, and *H. pylori* virulence evolves in these families. *Clin. Cancer Res.* **14**, 2227–2235 (2008).

Backert, S. & Selbach, M. Role of type IV secretion in *Helicobacter pylori* pathogenesis. *Cell Microbiol.* **10**, 1573–1581 (2008).

Banatvala, N. *et al.* The cohort effect and *Helicobacter pylori*. *J. Infect. Dis.* **168**, 219–221 (1993).

Bik, E. M. *et al.* Molecular analysis of the bacterial microbiota in the human stomach. *Proc. Natl Acad. Sci. USA* **103**, 732–737 (2006).

Blaser, M. J. & Atherton, J. C. *Helicobacter pylori* persistence: biology and disease. *J. Clin. Invest.* **113**, 321–333 (2004).

Blaser, M. J. & Kirschner, D. The equilibria that allow bacterial persistence in human hosts. *Nature* **449**, 843–849 (2007).

Blaser, M. J. Disappearing microbiota: *Helicobacter pylori* protection against esophageal adenocarcinoma. *Cancer Prev. Res. (Phila. Pa)* **1**, 308–311 (2008).

Blaser, M. J. Who are we? Indigenous microbes and the ecology of human diseases. *EMBO Rep.* **7**, 956–960 (2006).

Blaser, M. J., Chen, Y. & Reibman, J. Does *Helicobacter pylori* protect against asthma and allergy? *Gut* **57**, 561–567 (2008).

Bogaert, D. *et al.* Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet* **363**, 1871–1872 (2004).

Briles, D. E., Novak, L., Hotomi, M., van Ginkel, F. W. & King, J. Nasal colonization with *Streptococcus pneumoniae* includes subpopulations of surface and invasive pneumococci. *Infect. Immun.* **73**, 6945–6951 (2005).

Brown, N. F., Wickham, M. E., Coombes, B. K. & Finlay, B. B. Crossing the line: selection and evolution of virulence traits. *PLoS Pathog.* **2**, e42 (2006).

Butaye, P., Devriese, L. A. & Haesebrouck, F. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on Gram-positive bacteria. *Clin. Microbiol. Rev.* **16**, 175–188 (2003).

Chen, Y. & Blaser, M. J. *Helicobacter pylori* colonization is inversely associated with childhood asthma. *J. Infect. Dis.* **198**, 553–560 (2008).

Chen, Y. & Blaser, M. J. Inverse associations of *Helicobacter pylori* with asthma and allergies. *Arch. Intern. Med.* **167**, 821–827 (2007).

Codolo, G. *et al.* The neutrophil-activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in ovalbumin-induced allergic asthma. *Cell Microbiol.* **10**, 2355–2363 (2008).

Crowe, C. C., Sanders, W. E. Jr & Longley, S. Bacterial interference. II. Role of the normal throat flora in prevention of colonization by group A *Streptococcus*. *J. Infect. Dis.* **128**, 527–532 (1973).

Del Prete, G. *et al.* Immunosuppression of TH2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* neutrophil-activating protein. *J. Allergy Clin. Immunol.* **122**, 908–913.e5 (2008).

Dethlefsen, L., McFall-Ngai, M. & Relman, D. A. An ecological and evolutionary perspective on humanmicrobe mutualism and disease. *Nature* **449**, 811–818 (2007).

Dufour, P. *et al.* Community-acquired methicillinresistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. *Clin. Infect. Dis.* **35**, 819–824 (2002).

Durack, D. T. Prevention of infective endocarditis. *N. Engl. J. Med.* **332**, 38–44 (1995).

Eckburg, P. B. *et al.* Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638 (2005).

Eder, W., Ege, M. J. & von Mutius, E. The asthma epidemic. *N. Engl. J. Med.* **355**, 2226–2235 (2006).

el-Serag, H. B. & Sonnenberg, A. Opposing time trends of peptic ulcer and reflux disease. *Gut* **43**, 327–333 (1998).

Falkow, S. Is persistent bacterial infection good for your health? *Cell* **124**, 699–702 (2006).

Falkow, S. Molecular Koch's postulates applied to bacterial pathogenicity — a personal recollection 15 years later. *Nature Rev. Microbiol.* **2**, 67–72 (2004).

Fierer, N., Hamady, M., Lauber, C. L. & Knight, R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl Acad. Sci. USA* **105**, 17994–17999 (2008).

Flegal, K. M., Graubard, B. I., Williamson, D. F. & Gail, M. H. Cause-specific excess deaths associated with underweight, overweight, and obesity. *JAMA* **298**, 2028–2037 (2007).

Galvani, A. P. Epidemiology meets evolutionary ecology. *Trends Ecol. Evol.* **18**, 132–139 (2003).

Gao, Z., Tseng, C. H., Pei, Z. & Blaser, M. J. Molecular analysis of human forearm superficial skin bacterial biota. *Proc. Natl Acad. Sci. USA* **104**, 2927–2932 (2007).

Gaskins, H. R., Collier, C. T. & Anderson, D. B. Antibiotics as growth promotants: mode of action. *Anim. Biotechnol.* **13**, 29–42 (2002).

Ghaffar, F. *et al.* Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. *Clin. Infect. Dis.* **39**, 930–938 (2004).

Gill, S. R. *et al.* Metagenomic analysis of the human distal gut microbiome. *Science* **312**, 1355–1359 (2006).

Gordon, H. A. & Pesti, L. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol. Rev.* **35**, 390–429 (1971).

Gray, R. H. *et al.* Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1- discordant couples in Rakai, Uganda. *Lancet* **357**, 1149–1153 (2001).

Hacker, J. & Carniel, E. Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. *EMBO Rep.* **2**, 376–381 (2001).

Harvey, R. F. *et al.* Relationship between the birth cohort pattern of *Helicobacter pylori* infection and the epidemiology of duodenal ulcer. *QJM* **95**, 519–525 (2002).

Herold, B. C. *et al.* Community-acquired methicillinresistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA* **279**, 593–598 (1998).

Hickman, C. S. in *Influence of Cooperative Bacteria on Animal Host Biology* (eds McFall-Ngai, M. J., Henderson, B. & Ruby, E. G.) 3–34 (Cambridge Univ. Press, Cambridge, UK, 2005).

Hoffmeister, M. & Martin, W. Interspecific evolution: microbial symbiosis, endosymbiosis and gene transfer. *Environ. Microbiol.* **5**, 641–649 (2003).

Howson, C. P., Hiyama, T. & Wynder, E. L. The decline in gastric cancer: epidemiology of an unplanned triumph. *Epidemiol. Rev.* **8**, 1–27 (1986).

Islami, F. & Kamangar, F. *Helicobacter pylori* and esophageal cancer risk: a meta-analysis. *Cancer Prev. Res. (Phila. Pa)* **1**, 329–338 (2008).

Iyer, L. M., Aravind, L., Coon, S. L., Klein, D. C. & Koonin, E. V. Evolution of cell-cell signaling in animals: did late horizontal gene transfer from bacteria have a role? *Trends Genet.* **20**, 292–299 (2004).

Jukes, T. Antibiotics in animal feeds and animal production. *Bioscience* **22**, 526–534 (1972).

Kamangar, F. *et al.* Opposing risks of gastric cardia and noncardia gastric adenocarcinomas associated with *Helicobacter pylori* seropositivity. *J. Natl Cancer Inst.* **98**, 1445–1452 (2006).

Kanno, T. *et al.* Gastric acid reduction leads to an alteration in lower intestinal microflora. *Biochem. Biophys. Res. Commun.* **381**, 666–670 (2009).

Kennedy, A. D. *et al.* Epidemic community-associated methicillin-resistant *Staphylococcus aureus:* recent clonal expansion and diversification. *Proc. Natl Acad. Sci. USA* **105**, 1327–1332 (2008).

Kuipers, E. J. *et al.* Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet* **345**, 1525–1528 (1995a).

Kuipers, E. J., Perez-Perez, G. I., Meuwissen, S. G. & Blaser, M. J. *Helicobacter pylori* and atrophic gastritis: importance of the *cagA* status. *J. Natl Cancer Inst.* **87**, 1777–1780 (1995b).

Lederberg, J. & McCray, A. T. 'Ome sweet 'omics. A genealogical treasury of words. *The Scientist* **15**, 8 (2001).

Lenski, R. E. & May, R. M. The evolution of virulence in parasites and pathogens: reconciliation between two competing hypotheses. *J. Theor. Biol.* **169**, 253–265 (1994).

Lepp, P. W. *et al.* Methanogenic Archaea and human periodontal disease. *Proc. Natl Acad. Sci. USA* **101**, 6176–6181 (2004).

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. & Gordon, J. I. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Rev. Microbiol.* **6**, 776–788 (2008).

Linz, B. *et al.* An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature* **445**, 915–918 (2007).

Lundgren, A. *et al.* Mucosal *FOXP3*-expressing CD4+CD25high regulatory T cells in *Helicobacter pylori*infected patients. *Infect. Immun.* **73**, 523–531 (2005).

Lysenko, E. S., Ratner, A. J., Nelson, A. L. & Weiser, J. N. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog.* **1**, e1 (2005).

Ma, C.-T., Moore, J. & Turnbull, S. Cheating and equilibrium. Stopping agents from 'cheating.' *J. Econ. Theory* **46**, 355–372 (1988).

Mackowiak, P. A. The normal microbial flora. *N. Engl. J. Med.* **307**, 83–93 (1982).

MacLeod, L. C. & Kraus, M. R. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed *in vitro*. *J. Exp. Med.* **92**, 1–9 (1950).

Madhi, S. A. *et al.* Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* and associated interactions with *Staphylococcus aureus* and *Haemophilus influenzae* colonization in HIV-infected and HIVuninfected children. *J. Infect. Dis.* **196**, 1662–1666 (2007).

Marini, E. *et al. Helicobacter pylori* and intestinal parasites are not detrimental to the nutritional status of Amerindians. *Am. J. Trop. Med. Hyg.* **76**, 534–540 (2007).

Mattsson, A., Lonroth, H., Quiding-Jarbrink, M. & Svennerholm, A. M. Induction of B cell responses in the stomach of *Helicobacter pylori*-infected subjects after oral cholera vaccination. *J. Clin. Invest.* **102**, 51–56 (1998).

Maynard Smith, J. & Szathmary, E. *The Major Transitions in Evolution* (Oxford Univ. Press, 1995).

Mazmanian, S. K., Round, J. L. & Kasper, D. L. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**, 620–625 (2008).

Messenger, S. L., Molineux, I. J. & Bull, J. J. Virulence evolution in a virus obeys a trade-off. *Proc. R. Soc. Lond. B* **266**, 1480–1482 (1997).

Michod, R. E. Evolution of the individual. *Am. Nat.* **150**, S5–S21 (1997).

Murphy, T. V., Pastor, P., Medley, F., Osterholm, M. T. & Granoff, D. M. Decreased *Haemophilus* colonization in children vaccinated with *Haemophilus influenzae* type b conjugate vaccine. *J. Pediatr.* **122**, 517–523 (1993).

Musher, D. M. *et al.* Bacteremic and nonbacteremic pneumococcal pneumonia. A prospective study. *Medicine (Baltimore)* **79**, 210–221 (2000).

Nahar, S. *et al.* Evidence of intra-familial transmission of *Helicobacter pylori* by PCR-based RAPD fingerprinting in Bangladesh. *Eur. J. Clin. Microbiol. Infect. Dis.* **28**, 767–773 (2009).

Nash, J. F. Equilibrium points in *n*-person games. *Proc. Natl Acad. Sci. USA* **36**, 48–49 (1950).

Nash, J. Non-cooperative games. *Ann. Math.* **54**, 286–295 (1951).

Nwokolo, C. U., Freshwater, D. A., O'Hare, P. & Randeva, H. S. Plasma ghrelin following cure of *Helicobacter pylori. Gut* **52,** 637-640 (2003).

Obbard, D. J., Jiggins, F. M., Halligan, D. L. & Little, T. J. Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Curr. Biol.* **16**, 580–585 (2006).

Odum, L., Petersen, H. D., Andersen, I. B., Hansen, B. F. & Rehfeld, J. F. Gastrin and somatostatin in *Helicobacter pylori* infected antral mucosa. *Gut* **35**, 615–618 (1994).

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. & Brown, P. O. Development of the human infant intestinal microbiota. *PLoS Biol.* **5**, e177 (2007).

Pamer, E. G. Immune responses to commensal and environmental microbes. *Nature Immunol.* **8**, 1173–1178 (2007).

Peek, R. M., Jr. & Blaser, M. J. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nature Rev. Cancer* **2**, 28–37 (2002).

Pei, Z. *et al.* Bacterial biota in the human distal esophagus. *Proc. Natl Acad. Sci. USA* **101**, 4250–4255 (2004).

Peterson, J. The NIH Human Microbiome Project. *Genome Res.* 9 October 2009 (doi:10.1101/gr.096651.109).

Pohl, H. & Welch, H. G. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J. Natl Cancer Inst.* **97**, 142–146 (2005).

Regev-Yochay, G. *et al.* Association between carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in children. *JAMA* **292**, 716–720 (2004).

Regev-Yochay, G. *et al.* Does pneumococcal conjugate vaccine influence *Staphylococcus aureus* carriage in children? *Clin. Infect. Dis.* **47**, 289–291 (2008).

Regev-Yochay, G. *et al.* The pneumococcal pilus predicts the absence of *Staphylococcus aureus* co-colonization in pneumococcal carriers. *Clin. Infect. Dis.* **48**, 760–763 (2009).

Reibman, J. *et al.* Asthma is inversely associated with *Helicobacter pylori* status in an urban population. *PLoS ONE* **3**, e4060 (2008).

Rich, S. M., Sawyer, S. A. & Barbour, A. G. Antigen polymorphism in *Borrelia hermsii*, a clonal pathogenic bacterium. *Proc. Natl Acad. Sci. USA* **98**, 15038–15043 (2001).

Roberts, G. J. Dentists are innocent! "Everyday" bacteremia is the real culprit: a review and assessment of the evidence that dental surgical procedures are a principal cause of bacterial endocarditis in children. *Pediatr. Cardiol.* **20**, 317–325 (1999).

Robinson, K. *et al. Helicobacter pylori*-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* **57**, 1375–1385 (2008).

Roos, K., Holm, S. E., Grahn-Hakansson, E. & Lagergren, L. Recolonization with selected αstreptococci for prophylaxis of recurrent streptococcal pharyngotonsillitis — a randomized placebo-controlled multicentre study. *Scand. J. Infect. Dis.* **28**, 459–462 (1996).

Roosendaal, R. *et al. Helicobacter pylori* and the birth cohort effect. Evidence of a continuous decrease of infection rates in childhood. *Am. J. Gastroenterol.* **92**, 1480–1482 (1997).

Rosebury, T. *Microorganisms Indigenous to Man* (McGraw Hill, New York, 1962).

Rothenbacher, D., Bode, G. & Brenner, H. History of breastfeeding and *Helicobacter pylori* infection in preschool children: results of a population-based study from Germany. *Int. J. Epidemiol.* **31**, 632–637 (2002).

Sachs, J. L. & Bull, J. J. Experimental evolution of conflict mediation between genomes. *Proc. Natl Acad. Sci. USA* **102**, 390–395 (2005).

Sarkar, S. F. & Guttman, D. S. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl. Environ. Microbiol.* **70**, 1992–2012 (2004).

Segal, S. & Pollard, A. J. Vaccines against bacterial meningitis. *Br. Med. Bull.* **72**, 65–81 (2004).

Selva, L. *et al.* Killing niche competitors by remotecontrol phage induction: the case of *Streptococcus pneumoniae–Staphylococcus aureus* interference. *Proc. Natl Acad. Sci. USA* **106**, 1234–1238 (2009).

Sjölund, M. *et al.* Long-term persistence of resistant *Enterococcus* species after antibiotics to eradicate *Helicobacter pylori. Ann. Intern. Med.* **139**, 483–487 (2003).

Sjölund, M. *et al.* Persistence of resistant *Staphylococcus epidermidis* after single course of clarithromycin. *Emerg. Infect. Dis.* **11**, 1389–1393 (2005).

Smith, J. M. in *Evolution and the Theory of Games* 10–27 (Cambridge Univ. Press, Cambridge, UK 1982).

Smith, K., McCoy, K. D. & Macpherson, A. J. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* **19**, 59–69 (2007).

Strachan, D. P. Hayfever, hygiene, and household size. *BMJ* **299**, 1259–1260 (1989).

Suerbaum, S. & Michetti, P. *Helicobacter pylori* infection. *N. Engl. J. Med.* **347**, 1175–1186 (2002).

Tibayrenc, M. & Ayala, F. J. The clonal theory of parasitic protozoa: 12 years on. *Trends Parasitol.* **19**, 405–410 (2002).

Tindberg, Y. *et al. Helicobacter pylori* infection in Swedish school children: lack of evidence of child-to-child transmission outside the family. *Gastroenterology* **121**, 310–316 (2001).

Turnbaugh, P. J. *et al.* A core gut microbiome in obese and lean twins. *Nature* **457**, 480–484 (2009).

Turnbaugh, P. J. *et al.* The human microbiome project. *Nature* **449**, 804–810 (2007).

Veenhoven, R. *et al.* Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* **361**, 2189–2195 (2003).

Vesper, B. J. *et al.* The effect of proton pump inhibitors on the human microbiota. *Curr Drug Metab* **10,** 84-89 (2009).

Viala, J. *et al.* Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. *Nature Immunol.* **5**, 1166–1174 (2004).

Weinberger, D. M. *et al.* Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. *J. Infect. Dis.* **197**, 1511–1518 (2008).

Whitman, W. B., Coleman, D. C. & Wiebe, W. J. Prokaryotes: the unseen majority. *Proc. Natl Acad. Sci. USA* **95**, 6578–6583 (1998).

Zhou, X. *et al.* Characterization of vaginal microbial communities in adult healthy women using cultivationindependent methods. *Microbiology* **150**, 2565–2573 (2004).

A3

PATHWAYS IN MICROBE-INDUCED OBESITY[6]

Laura M. Cox[7] *and Martin J.* ***Blaser***[8]

Diet, host gene composition, and alterations in the intestinal microbiota can contribute to obesity. In microbe-induced obesity, metabolic changes stem from primary perturbation of the microbiota, consequent to modern changes in human biology. Microbiota disruption during early development can result in syndromes of metabolic dysfunction. We focus on the pathways involved in these interactions, particularly related to energy extraction and the role of inflammation in the metabolic phenotypes. Model physiologic systems and perturbations including gastric bypass surgery, pregnancy, and hibernation provide insight into the respective roles of the critical participants.

Introduction

In the late 20th century, obesity has markedly increased in the U.S. (Flegal et al., 2012) as well as worldwide (Haslam and James, 2005). Much focus on the origins of obesity has centered on dietary excesses (diet-induced obesity [DIO]) or on host genes (gene-induced obesity [GIO]) (Hollopeter et al., 1998) (Figure A3-1). However, in addition to increased calories or alterations in host metabolism, the composition of the intestinal microbiota can contribute to the progression of obesity (Bäckhed et al., 2004; Turnbaugh et al., 2006, 2008); thus DIO and GIO have both microbe-independent and microbe-dependent mechanisms. Alternately, metabolic changes can stem from a primary perturbation of the microbiota (Cho et al., 2012), consequent to modern changes in human biology (Blaser and Falkow, 2009; Dominguez-Bello et al., 2011) in which the initiating factors lack microbe-independent effects (microbe-induced obesity [MIO]). Epidemiological studies in humans have shown that antibiotic treatment during the first 6 months of life (Trasande et al., 2013), or disrupted colonization from Caesarean section delivery (Blustein et al., 2013; Huh et al., 2012), can increase the risk of being overweight later in life. These two interventions have no direct contribution to host caloric intake or metabolism (Coates et al., 1963) but have large effects on the microbiome (Dethlefsen et al., 2008; Dominguez-Bello et al., 2010).

---

[6] Reprinted from *Cell Metabolism*, 17(6), L. M. Cox and M. J. Blaser, Pathways in microbe-induced obesity, Pp. 883-894, Copyright (2013), with permission from Elsevier.

[7] Department of Microbiology, New York University Sackler Institute of Graduate Biomedical Sciences, New York, NY 10016, USA.

[8] Department of Medicine, New York University School of Medicine, New York, NY 10016, USA-Medical Service, VA New York Harbor Healthcare System, New York, NY 10010, USA.

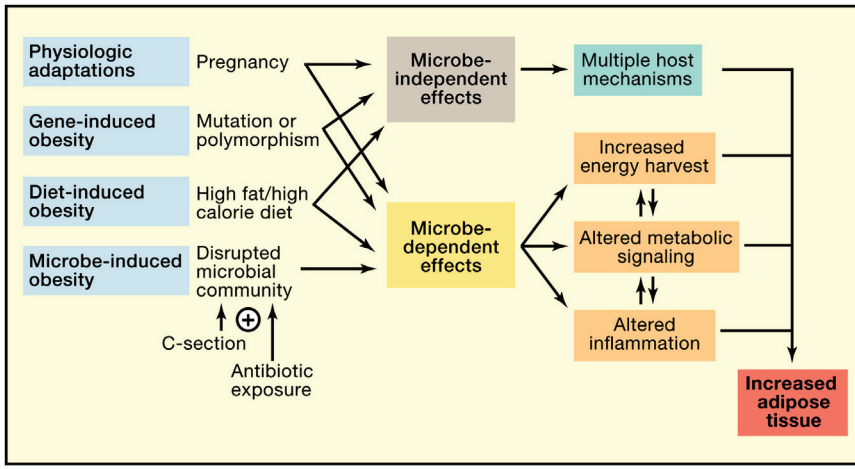


FIGURE A3-1 Pathways in microbe-induced obesity. Normal physiological adaptations (i.e., pregnancy), genetic mutations or polymorphisms (gene-induced obesity, GIO), or a diet with excessive fat or calories (diet-induced obesity, DIO) can promote weight gain and increase adiposity through microbe-independent and microbe-dependent mechanisms. Disrupting the founding microbial community through Caesarean section and/or early-life antibiotic exposure also can lead to increased weight gain and adiposity (microbe-induced obesity, MIO). The MIO effects all are microbe dependent, since the initiating factors do not contribute to obesity independent of microbes (e.g., antibiotics have no caloric value). For each pathway that leads to obesity, the altered microbiota can contribute to adiposity through increased energy harvest or by altering metabolic signals, inflammation, or immunity.

The microbes that colonize humans substantially outnumber human cells (Savage, 1977), and the collective unique microbial genes outnumber human genes by a factor of more than 100 (Arumugam et al., 2011; Human Microbiome Project Consortium, 2012; Qin et al., 2010). These coevolved microbes have a complex role in maintaining health (Human Microbiome Project Consortium, 2012) but can also contribute to disease (Cho and Blaser, 2012; Holmes et al., 2012). Model systems have been used to examine the effects of microbiome change in the context of different diets or on early life development. In this perspective, we examine mechanistic pathways in which the disrupted microbiome can contribute to obesity by altering energy extraction from food or altering inflammation and immunity (Figure A3-2). The microbiome is dynamic and resilient but subject to perturbations that can impact metabolism. We explore major recovery strategies in the microbiome in response to host metabolic perturbations (Figure A3-3) and discuss metabolic adaptations that aid particular classes of microbiota during recovery in the competitive environments of well-established ecosystems (Figure A3-4).

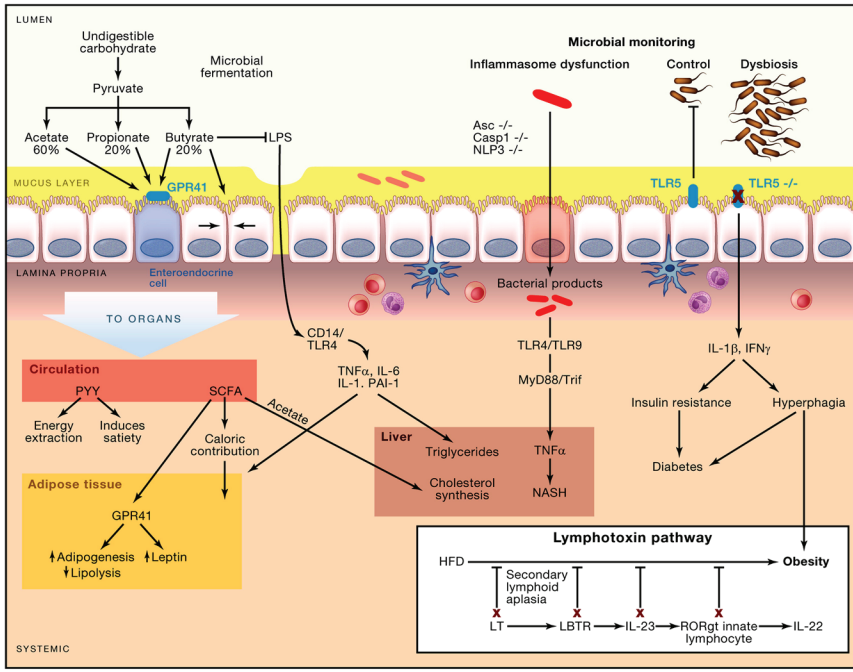


FIGURE A3-2 Pathways involving microbiome and immunity that contribute to obesity. Undigested carbohydrates are fermented by the intestinal microbiota to short-chain fatty acids (SCFA), primarily acetate (60%), propionate (20%–25%), and butyrate (15%–20%). SCFA can signal through G protein-coupled receptor 41 (GPR41) on enteroendocrine cells, inducing the secretion of peptide YY, which can contribute to obesity by slowing transit time and thus increasing energy extraction from food, or can protect against obesity by increasing satiety. SCFA are readily absorbed, contribute up to 10% of caloric intake, and can increase adipogenesis by binding GPR41 on adipocytes. In the liver, acetate is a substrate for cholesterol synthesis and lipogenesis. Locally, butyrate increases the epithelial tight junctions, blocking the translocation of LPS, a potent inflammatory mediator that increases weight gain, total body and liver adiposity, and insulin resistance. Similarly, the mucus layer can also block LPS translocation. Inflammasome dysfunction allows translocation of bacterial products, which induce hepatic TNF-α expression and drive nonalcoholic steatohepatitis (NASH) in a TLR4/TLR9-dependent manner in mice with other risk factors (e.g., methionine-choline-deficient diet). Loss of *Tlr5*, which senses bacterial flagellin, leads to a dysbiosis in the microbiome, increased inflammatory cytokines, hyperphagia, insulin resistance, and diabetes in the context of a high-fat diet. The NASH and metabolic syndrome phenotypes in inflammasome- and *Tlr5*-deficient mice are transferrable via the microbiome to germ-free wild-type (WT) recipients, demonstrating the roles of the altered microbiome in adiposity. (Inset) Deleting any component of the lymphotoxin pathway results in loss of control of the microbiome and blocks the ability of mice to gain weight on high-fat diet. Cohousing lymphotoxin β receptor-deficient and WT mice restores diet-induced obesity and partially restores a WT/HFD microbiota.

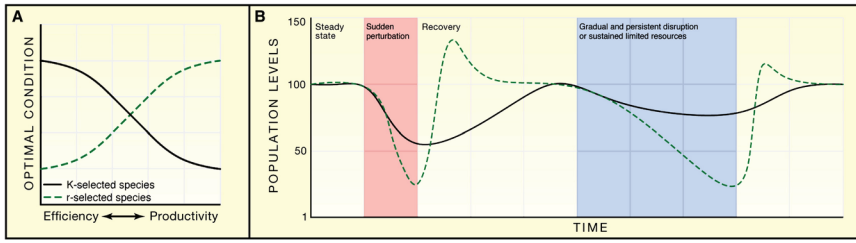


FIGURE A3-3 Response of r- and K-selected species to environmental stress. (A) r-species recover from environmental stress optimizing growth rate and productivity, while K-selected species resist environmental change by optimizing their efficiency when resources are scarce. (B) Following a large and sudden perturbation in which both r- and K-selected species suffer diminished in population levels, the r-selected species can rapidly bloom and dominate the environment due to utilization of unused resources, whereas the recovery from K-selected species is slower. During a longer, sustained environmental stress, K-selected species can resist large population losses because of their high-efficiency adaptation strategies, whereas r-selected species are more constrained to maintain populations, and therefore suffer losses.

Energy Extraction from Food

The diet provides nutrients to both the host and the microbial consortium, which have coevolved to derive and impart benefits to each other across a wide array of vertebrate lineages (Ley et al., 2008a,b). The microbiota improve the fitness of the host by increasing resistance to pathogens, providing essential vitamins, and increasing caloric extraction from food. The very presence of microbiota influences weight gain and fat storage, as germ-free mice have significantly lower weight and body fat percent, despite eating more calories than conventionalized mice (Bäckhed et al., 2004).

Current technologies have allowed more efficient production of food; consequently, the caloric intake has increased in both men and women in the last 30 years (Wright et al., 2004). Increased caloric intake for the host translates to increased energy availability for the microbiota, altering the environment they inhabit (Kau et al., 2011). In addition, readily available processed foods commonly substitute complex carbohydrates for simple refined sugars and contain food preservatives, which affect the composition and diversity of the microbiota (Bernbom et al., 2006; Kau et al., 2011; Payne et al., 2012). In obesity, both reduced microbial diversity and interconnectivity have been observed (Greenblum et al., 2012; Turnbaugh et al., 2009), consistent with our a priori notions of the importance of microbiota diversity to health.

An increase in caloric intake, whether from a high-fat diet (DIO) (Turnbaugh et al., 2008) or from overeating normal chow driven by the absence of the satiety hormone leptin in *ob/ob* mice (GIO) (Turnbaugh et al., 2006), selects

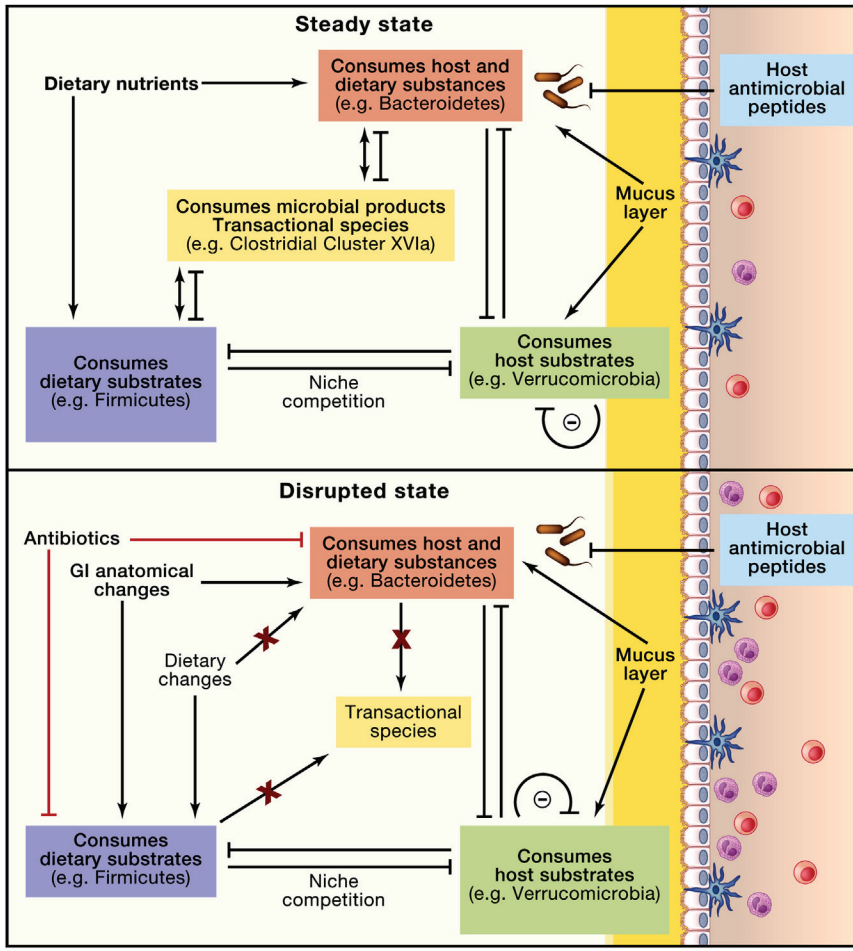


FIGURE A3-4 Microbial equilibrium and host effects in relation to energy substrates. Microbes in the intestine can derive energy from the diet, the host, or compounds secreted by other members of the consortium. Four classes of microbes may be considered, based on their predominant energy source: diet (purple), host (green), either diet or host (pink), or neither diet nor host (orange). Competition for niche colonization is affected by differential growth rates (r- and K-selected species), utilization of exclusive substrates, susceptibility to host defenses (e.g., antimicrobial peptides), production of inhibitory products, and community organization (e.g., autoinducers). Perturbation may occur, for example, via dietary change (e.g., changes in a micro- or macronutrient or long-term fasting), by antibiotics that differentially select the microbiota, or changes in gastrointestinal tract structure (e.g., gastric bypass). Importantly, in this model, competition between the major classes affects host mucus layer, epithelial permeability, and inflammation, which has downstream metabolic consequences.

for obesogenic microbiota. This was demonstrated by transferring the obesity phenotype to germ-free recipient mice via microbiome transplantation, providing evidence that, in addition to increased caloric consumption, the microbiota can contribute to obesity (Figure A3-1). Both DIO and *ob/ob* GIO caused phylum-level shifts in the intestinal microbiota, increasing Firmicutes and decreasing Bacteroidetes (Ley et al., 2005, 2006; Turnbaugh et al., 2008). That these broad phylum level changes have not been found consistently (Ley, 2010; Ravussin et al., 2012) may indicate that the important events involve lower taxonomic levels within these phyla.

Dietary composition can alter the microbiota, independent of the calories consumed. In a comparison of ad libitum-fed or caloric-restricted mice receiving either a DIO diet or normal chow, the dietary composition had a greater influence on shaping the microbiota than caloric intake or host obesity status (Ravussin et al., 2012). However, whether the metabolic phenotype was transferrable to germ-free recipients was not assessed, so it could not be determined whether the dietary composition or the increased calories select for an obesogenic microbiota. That excessive caloric intake in *ob/ob* mice receiving normal chow and in mice fed ad libitum on a DIO diet selects for an obesogenic microbiota argues that the increased caloric intake, not dietary composition, is the driving force.

In the large intestine, complex nutrients that have not been absorbed by the host are fermented to short-chain fatty acids (SCFAs), which contribute a small but significant number of calories to the host (Macfarlane and Macfarlane, 2012), estimated to account for up to 10% of caloric intake in humans (Bergman, 1990) (Figure A3-2). SCFA also play a role in maintaining intestinal health by acting as the major energy source for colonocytes and having anti-cancer activity (Wong et al., 2006). The total production of SCFAs, and thus calories contributed by microbiota, depends on both dietary and microbiota composition. The majority of SCFAs are produced from carbohydrates, but a smaller fraction can be produced from protein degradation, which yields SCFAs plus other metabolic end products, such as amines, phenols, thiols, and hydrogen sulfide (Macfarlane and Macfarlane, 2012). Leptin-deficient *ob/ob* mice harbor microbiota that produce more SCFA than wild-type (WT) mice and extract more calories from the diet (Turnbaugh et al., 2006). A similar increase in microbial energy harvest was observed in lean humans who switched from consuming 2,400 to 3,400 calories per day (Jumpertz et al., 2011). These findings show that in a GIO murine model and a DIO human simulation, the intestinal microbiota can adapt to an enriched diet and contribute additional calories to the host (Figure A3-1).

Metabolic disturbances can occur that are not caused by either abnormal host gene status or dietary changes but instead are driven by an aberrant microbiota (MIO). For example, the microbiota may become altered as a result of an abnormal founding population, as occurs with Caesarean section (Biasucci et al., 2010; Dominguez-Bello et al., 2010; Huurre et al., 2008; Pandey et al., 2012). Epidemiologic studies indicate an association of Caesarean section with

the development of childhood obesity (Blustein et al., 2013; Huh et al., 2012; Li et al., 2013). Similarly, the microbiota can be altered by antibiotic exposure (Antonopoulos et al., 2009; Dethlefsen et al., 2008; Ubeda and Pamer, 2012). In particular, antibiotic exposure in early life, just when host adipocyte populations are developing (Greenwood and Hirsch, 1974), has been associated with the development of adiposity in humans (Ajslev et al., 2011; Trasande et al., 2013). In a murine model of MIO, early life subtherapeutic antibiotic treatment (STAT) changed microbiome composition and increased fat mass (Cho et al., 2012). STAT increased the abundance of microbial genes involved in SCFA production and also increased acetate, butyrate, and propionate concentrations in the intestine. Thus, if SCFAs are markers of increased energy extraction, one driving force behind MIO has been identified.

SCFAs also provide important microbial signals to the host, in part via the G protein-coupled receptors GPR41 and GPR43 (FFAR2 and FFAR1, respectively). The GPR41 receptor on enteroendocrine cells senses SCFAs and induces secretion of peptide YY (Ichimura et al., 2009), which both slows intestinal transit time and increases satiety. *Gpr41*-deficient mice are leaner than their WT counterparts (Table A3-1), due to decreased PYY and increased intestinal transit rate (Samuel et al., 2008). However, germ-free *Gpr41*$^{-/-}$ mice exhibited no weight differences from germ-free WT mice, indicating that the effects are dependent on the microbiota. Absorbed SCFAs also provide metabolic signals to distant tissues. SCFAs bind GPR41 on adipocytes, increasing adipogenesis, inhibiting lipolysis, and inducing leptin secretion (Ichimura et al., 2009). The major product of microbial fermentation, acetate, is a substrate for hepatic cholesterol and triglyceride synthesis (Wong et al., 2006). Early life STAT increased intestinal acetate as well as the downstream (via the portal circulation) hepatic expression of genes involved in fatty acid metabolism and lipogenesis (Cho et al., 2012).

The extent of total energy extracted from the diet depends on the structure of the intestinal tract, the composition of the microbiota, and the composition of the diet. The dynamic metabolic relationships can be understood by considering continuous flow fermentation systems, in which the population of microbes is maintained by balancing nutrient infusion and waste removal. The evolution of a conserved and stable microbial consortium requires overcoming a considerable set of challenges. These concepts were first compiled by Hungate in his classic work *The Rumen and Its Microbes* (Hungate, 1966). Because of the constant downward movement of intestinal contents, a stable microbiota can only be achieved if the net microbial growth equals the flow rate of the system. Overnutrition can disrupt the microbial ecosystem and can have severe consequences for both host and microbiota. When a ruminant (i.e., cow or sheep) is rapidly switched from a grass-based diet to a grain-based diet, the sudden influx of starch in the digestive tract leads to an overgrowth of lactate-producing *Streptococcus bovis* (Hungate et al., 1952). Lactic acid accumulates in the rumen faster than can be utilized by other ruminant microbes (Nagaraja and Titgemeyer, 2007). The

TABLE A3-1 Metabolic Consequences of Specified Host and Dietary Interactions with the Microbiome

Host State	Phenotype with Microbiota	Phenotype in GF Mice or with Reduced Microbiota[a]	Major Microbial Changes	Transfer[b]	References
ob$^{-/-}$	↑ Weight and adiposity	Resistance to GIO[a]	↑ Firmicutes ↓ Bacteroidetes	Yes	Ley et al., 2005; Turnbaugh et al., 2006; Cani et al., 2008
High-calorie diet	↑ Weight and adiposity	Resistance to DIO	↑ Firmicutes ↓ Bacteroidetes	Yes	Turnbaugh et al., 2008
Gpr41$^{-/-}$	Lean	No difference from controls	Unknown	Unknown	Samuel et al., 2008
Pregnancy	↑ Weight and adiposity	Unknown	↑ Lactic acid bacteria ↓ Butyrate producers	Yes	Koren et al., 2012
Tlr5$^{-/-}$	Obese, insulin resistant, hyperphagic	No difference from controls[a]	Changes at the OTU level, no changes at the phylum level	Yes	Vijay-Kumar et al., 2010
Inflammasomes *Asc*$^{-/-}$, *Casp1*$^{-/-}$, *Nlp3*$^{-/-}$, *IL18*$^{-/-}$	↑ NASH	No difference from controls[a]	↑ Prevotellaceae and Porphyromonadaceae	Yes	Henao-Mejia et al., 2012
Lymphotoxin-deficient[c] *Ltbr*$^{-/-}$, *Lta*$^{-/-}$, *Ltb*$^{-/-}$	Resistant to DIO	Unknown	↑ SFB and Cytophaga ↓ Erysipelotrichi	Yes, but transient Obese WT → *ltbr*$^{-/-}$	Upadhyay et al., 2012
Roux-en-Y gastric bypass	Lean	Unknown	↑ Gammaproteobacteria ↑ *Akkermansia*	Yes	Liou et al., 2013

[a] Reduction in microbiota by high-dose broad-spectrum antibiotics.

[b] Transfer of the phenotype to conventional mice has been accomplished.

[c] Resistance to DIO also achieved by deleting IL23a and RORγt, elements downstream of the lymphotoxin pathway.

excess lactate decreases the pH of the rumen below 5, resulting in acute systemic acidosis, which can lead to death within 24 hr. This is an extreme example, since ruminants are substantially different from mice and humans in the fundamental digestive tract organization. Ruminants are foregut fermenters, while humans and mice are hindgut fermenters, which may have evolved as an adaptation enabling wider variations in diet. For mice and humans, most of the microbiota only receive nutrients after the host has extracted many of the simple molecules, and thus hindgut fermenters are not vulnerable to fatal indigestion from nutrient excess.

Major anatomical rearrangements of the gastrointestinal tract of mice and humans produce large effects on energy extraction and on the intestinal microbiota, in part due to changing factors related to nutrient flux and pH (Aron-Wisnewsky et al., 2012). In obese subjects undergoing Roux-en-Y gastric bypass (RYGB) surgery, metabolic improvements can be observed in the first week after the surgery, preceding significant weight loss (Ahn et al., 2010; Rubino et al., 2004). These weight-independent metabolic improvements may be mediated by rapid changes in microbiota composition, since major reconstruction of the gastrointestinal tract as part of gastric bypass significantly alters the microbiota shortly after the surgery (Graessler et al., 2012; Li et al., 2011). In a recent study by Liou et al., RYGB had a more substantial effect on the microbiome than sham surgery or caloric restriction, even though mice receiving caloric restriction also were significantly leaner than the sham-surgery mice (Liou et al., 2013), demonstrating that gut structure had greater impact on the microbiota than obesity status. RYBG was associated with increased populations of Proteobacteria (*Escherichia*) and Verrucomicrobia (*Akkermansia*), and a reduction in Firmicutes compared to sham surgery and weight restriction. Mice that underwent gastric bypass extracted significantly less energy from the diet than mice that underwent sham surgery or calorie-restricted mice, which may have been mediated by a combination of gut restructuring and changes in the intestinal microbiota. Germ-free mice colonized with microbiota from RYGB mice weighed significantly less than mice colonized with microbiota from sham surgery mice, showing that the RYGB-altered microbiota play an active role in weight loss and metabolic status. Both the RYGB cecal donors and their recipient mice had decreased total SCFA compared to sham donors and recipients, with major reductions in acetate levels and a relative increase in propionate levels, providing further evidence that SCFAs influence obesity.

Inflammation and Immunity

The interaction between diet, genes, and the intestinal microbiota in the context of obesity is complex (Parks et al., 2013), since each of these factors has the potential to modulate inflammation and immunity (Kau et al., 2011). Obesity is a low-grade inflammatory disorder, and the role of the microbiota via specific

immune pathways has been studied with the aid of experiments involving germ-free and knockout mice (Table A3-1).

LPS

High-fat diet (HFD) can increase absorption of lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, either by incorporation into chylomicrons or by increasing intestinal permeability (Cani et al., 2007, 2008). LPS is a potent inflammatory mediator that signals in a CD14/TLR4-dependent manner, and infusion of LPS alone can increase weight gain, adiposity, insulin resistance, and liver triglycerides similar to a HFD. High-dose antibiotic treatment or deletion of *CD14* reduces inflammatory cytokine expression and ameliorates weight gain on HFD, demonstrating the important interaction of microbiota and inflammatory signaling cascades. Prebiotic fibers fermented to SCFA by the intestinal microbiota can block LPS-mediated metabolic consequences by increasing colonic expression of tight junction proteins zona occludens 1 and claudin 3, improving gut barrier function and reducing systemic LPS (Neyrinck et al., 2012) (Figure A3-2).

Akkermansia muciniphila, a microbial specialist that can derive its carbon and energy sources from mucus lining the intestinal tract, increases with prebiotic treatment (Everard et al., 2011). In addition to the metabolic and inflammatory changes previously discussed, HFD reduces *A. muciniphila* levels and thins the mucus layer (Everard et al., 2013). Introduction of *A. muciniphila* by daily oral gavage to mice fed HFD restores the mucus layer, decreases circulating LPS levels, decreases fat mass gain, and increases glucose tolerance compared to mice gavaged with PBS. These changes in metabolism and inflammation were not produced by killed *A. muciniphila* or by the "probiotic" strain *Lactobacillus plantarum*, indicating that live *A. muciniphila* has specialist actions that improve the gut barrier and reduce the effects of DIO.

TLR5

Deletion of the innate immunity receptor *Tlr5*, which senses bacterial flagellin, induces inflammation, which if mild causes a metabolic syndrome including hyperphagia-dependent weight gain; insulin resistance; and increased adiposity, blood pressure, and cholesterolemia (Vijay-Kumar et al., 2010). These changes were linked with increased adipocyte proinflammatory cytokines IL-1β and INF-γ. An antibiotic regimen that reduced microbial density 100-fold abrogated the metabolic syndrome in $Tlr5^{-/-}$ mice, and conversely, germ-free mice that were colonized with $Tlr5^{-/-}$ microbiota showed many of the disease phenotypes. Together these results indicate the sufficiency of the altered microbiota to yield the pathologies that accompany *Tlr5* deficiency. However, some $Tlr5^{-/-}$ mice have an opposing phenotype of severe colitis leading to weight loss, not gain

(Vijay-Kumar et al., 2007). In a separate study, *Tlr5*$^{-/-}$ mice housed at two different animal facilities did not show an obesity phenotype or increased basal intestinal inflammation (Letran et al., 2011). The circulating microbes in separate facilities can lead to animals with differential microbiota colonization, altering host immune phenotypes (Ivanov et al., 2009). Since the genetic background was the same for the *Tlr5*$^{-/-}$ mice, the local microbiota in each animal facility most likely accounted for differences in the immune and metabolic phenotypes. Thus, in this model, the colonizing microbiota characteristics might determine inflammation extent and which pathway dominates.

Lymphotoxin Mediates Diet-Induced Obesity Through the Microbiota

Lymphotoxin, secreted by Th1 lymphocytes and implicated in obesity by GWAS, is involved in the control of mucosal intestinal microbiota (Upadhyay et al., 2012). Lymphotoxin β receptor-deficient (*Ltbr*$^{-/-}$) mice have reduced colonic IL-22 and RegIII antimicrobial peptide expression and elevated levels of segmented filamentous bacteria (SFB), consistent with diminished immune control at the mucosal surface (Upadhyay et al., 2012). Addition of HFD further shifts both immune responses and metabolism, including increasing colonic IL-23 expression, weight, and adiposity. When any of the components in the axis are eliminated, including HFD, lymphotoxin α or β subunits, lymphotoxin β receptor (*Ltbr*$^{-/-}$), IL-23, or RORγt (Th17) cells, mice become DIO resistant (Figure A3-2, inset). The dietary changes and the differences in *Ltbr* status account for 52% and 18% of the variation in the microbiome, respectively. Several expected microbiome shifts associated with introduction of a HFD, such as an increase in Firmicutes and a loss in diversity, were only observed in *Ltbr*$^{+/-}$ mice, but not in *Ltbr*$^{-/-}$ mice. GF WT mice conventionalized with microbiota from HFD-fed *Ltbr*$^{-/-}$ mice transiently resist DIO, but the phenotype gradually reverts, due to their (WT) immune development. When HFD-fed *Ltbr*$^{-/-}$ mice are cohoused with DIO-susceptible *Ltbr*$^{+/-}$ mice, the *Ltbr*$^{-/-}$ mice gain more weight, indicating the dominance of the transferred microbiota selected by HFD in the presence of a functional lymphotoxin pathway.

Inflammasomes and NASH

Multiprotein complexes that sense pathogen-associated molecular patterns (PAMPs), known as inflammasomes, aid in mucosal defenses by activating the inflammatory cytokine precursors pro-IL-1β and pro-IL-18. Inflammasomes have been implicated in the progression from nonalcoholic fatty liver disease to nonalcoholic steatohepatitis (NASH) (Henao-Mejia et al., 2012). Apoptosis-associated speck-like protein containing a CARD (ASC) is a key adaptor protein of the inflammasome complex. *Asc*-deficient mice show higher levels of TLR4 and TLR9 agonists in the portal circulation and higher hepatic TNF-α expression

than WT mice, indicating that microbial products translocate to the liver and induce inflammation. When given a methionine-choline-deficient diet known to induce NASH, mice lacking *Asc* or other components of the inflammasome complex (caspase-1, NLRP3) or downstream signaling (*IL-18*$^{-/-}$) develop NASH at a higher incidence than WT mice. The enhanced NASH phenotype in *Asc*$^{-/-}$ mice was ablated with deletion of either *Tlr4*, *Tlr9*, *MyD88:Trif*, or *TNF*-α, demonstrating the importance of innate immune pathways. However, the presence of the intestinal microbiota is also necessary because high-dose broad-spectrum antibiotics in *Asc*$^{-/-}$ mice reduce NASH incidence. When WT mice were cohoused with *Asc*$^{-/-}$ mice, WT mice developed NASH more frequently, which was associated with increased Prevotellaceae, showing the importance of the altered microbiome in disease risk.

All told, these models of DIO and GIO illustrate the importance of immune control of intestinal microbiota, whose relaxation or activation can result in pathologic metabolic consequences.

Metabolic Impact of Microbiome Perturbation

Systems Approaches

There are long-standing immunologic and metabolic relationships between the host and microbiota, and there can be consequences following natural and artificial perturbations. Microbial diversity and stability are important when considering the complex intracommunity interactions that lead to emergent properties of the system. Well-evolved ecosystems often have functional redundancy in which several species can perform the same task. This concept explains how the taxa that constitute the mammalian gut microbiome often are highly diverse and variable from person to person, yet share high-degree functional capacity (Human Microbiome Project Consortium, 2012; Kurokawa et al., 2007; Turnbaugh et al., 2009). However, ecosystems can be disrupted. In the case of forests, it might be fires, landslides, or droughts (Paine et al., 1998). For human microbiota, it might be famine, surfeit, antibiotic treatments, or even parturition. Each of these events has the potential to change the energy flow in tightly regulated systems, and consequences, including loss in diversity, vary according to the system's status and the extent of functional redundancy. As diversity declines, loss of function may not be immediately apparent until the last redundant member able to perform a function is gone; however, communities with lower diversity are less resilient when subject to further perturbations (Kau et al., 2011). For perturbations of host-associated microbiota, timing is important, because the effects and their consequences may differ according to stage of life.

Despite the obvious differences in geography, diet, and lifestyle, there are strong similarities in the age-specific features of the microbial composition in persons living in the United States, Malawi, and Venezuela (Yatsunenko et al.,

2012). Regardless of origin, babies begin life with low diversity, which progressively increases over the first years of life, and for all, the outline of the adult microbiome is essentially established by the age of three. Unsurprisingly, considering the long-standing differences in diet reflected in the three populations, the biochemical pathways represented in the metagenomes varied substantially. The study subjects differed substantially in the overall diversity in adulthood: the average adult in the U.S. had from 225 to 400 fewer operational taxonomic units (OTUs) (or approximately 15%–25% fewer) than did the Malawians or Venezuelan Amerindians. This observation is consistent with our earlier hypothesis of a "disappearing microbiota" due to the changes represented by modernity (Blaser and Falkow, 2009), and may be the harbinger of functional losses as well.

Pregnancy

Parturition is a major biological perturbation, yet is a critical event in the animal life cycle. The energetics are complex, since the needs of the mother and her offspring must be balanced, and in mammals the mother's role extends far beyond birth. Nature's challenge is to provide a sufficient endowment to the next generation while safeguarding the mother's own health for the essential tasks ahead. Multiple adaptations in the maternal immune system allow tolerance of the growing fetus (Erlebacher, 2013; Thellin and Heinen, 2003). The GI microbiota composition shifts remarkably over the course of pregnancy and continuing beyond (Koren et al., 2012). Its progressive nature implies that an optimum exists, timed to the events of parturition and consistent with a model of balanced outcome.

There are temporal shifts in both the diversity within a single microbial community, known as α-diversity, which can be measured by phylogenetic diversity, species richness, and Shannon's index, and in the diversity shared among different communities, known as β-diversity, which, for example, can be measured by UniFrac distances, changes in microbial abundance, and the Jaccard index (Lozupone and Knight, 2008). The microbiota in late pregnancy has reduced αdiversity but higher β-diversity compared to nonpregnant women or early pregnancy (Koren et al., 2012). In the third trimester, there is greater representation of lactic acid bacteria (*Lactobacillus*, *Streptococcus*, and *Enterococcus*), which are highly prevalent in the infant gut, whereas butyrate-producing bacteria (*Faecalibacterium*, *Blautia*, and *Ruminococcus*), which dominate the gut in adulthood, are enriched in early pregnancy. Since mammals have evolved to provide a portion of their microbes to their offspring, shifts occurring during pregnancy may reflect selection that increases the probability of offspring survival. The greater representation of lactic acid bacteria may be seen as an adaptation to prepare the mother for transfer of these organisms in the birth and perinatal period to her offspring to take maximum advantage of the main energy source for the child, lactose in

its mother's milk. The increased β-diversity reflects the variation in the different pathways by which nature accomplishes its goals of intergenerational transfer.

When germ-free mice were conventionalized with fecal microbiota from women in their first (T1) or third (T3) trimester of pregnancy, those receiving the T3 microbiota had increased intestinal cytokines, gained more weight, and were more glucose intolerant than those receiving the T1 microbiota. This result implies that the T3 microbiota induces an alternative metabolic state in its host, associated with greater energy storage. The findings in mice mimic those in pregnant women who also gain weight and develop insulin resistance, but vary in the degree of these phenotypes. The trimester-specific differences provide evidence for a microbiota-driven energy optimum to maximize maternal fitness and caloric transfer to the next generation. This concept is consistent with the notion of a coevolved microbiota that optimizes host survival through effects on its progeny.

Perturbation

Perturbation, an important constraint in biological systems, may be random, episodic, or programmed, like pregnancy or hibernation. Well-evolved ecosystems have long histories of perturbation, in part documented by and captured within their existing population structure (Paine et al., 1998). Ecosystem stability reflects both its resistance to change and its resilience following perturbation (Costello et al., 2012; Shade et al., 2012). Changes in diet in omnivorous hosts substantially perturb the gut microbiome (e.g., adding high fat [Turnbaugh et al., 2008], fiber [Cox et al., 2013], and protein [Faith et al., 2011]), and changes in the mode of birth delivery are associated with alterations in the patterns established for vertical microbiome transmission, comparing vaginal delivery and Caesarean sections (Dominguez-Bello et al., 2010). Direct exposure to antibacterial agents has the greatest potential for perturbation and occurs frequently in the U.S. Based on recent CDC data (Hicks et al., 2013), more than 250 million courses of antibiotics were prescribed in the US in 2010, which represents 880 courses of antibiotics per 1,000 population. Short antibiotic courses in adults cause profound but incompletely understood changes in community structure, with variable resilience (Dethlefsen et al., 2008). Perturbation during early life, when the gut community structure is emerging (Yatsunenko et al., 2012), may be most important, since it is occurring during a developmentally sensitive host window (Cho et al., 2012). The CDC data indicate that the average child in the U.S. is receiving about three antibiotic courses in first 2 years of life and nearly 11 courses by age 10 (Hicks et al., 2013). To understand the impact of these perturbations, ecological theory can guide us through the ground rules governing ecosystem responses.

Within ecosystems, there are r- or K-selected species, which have evolved alternate strategies to cope with environmental disruption (Reznick et al., 2002). r-selected species evolved to have a rapid growth rate to monopolize resources when competition is low following a large ecological disruption, whereas

K-selected species have evolved for efficiency to persist when the carrying capacity of the environment is low (Figure A3-3). In the intestine, large perturbations can result from antibiotic exposure, extreme changes in diet, surgical interventions, and acute gastrointestinal illnesses. Carrying capacity can be limited by availability of nutrients, electron acceptors (e.g., oxygen), or even physical niches. A weed is an example of an r-selected species with high proliferative potential. Weeds often are normal members of the ecosystem that live at the fringe in microhabitats, since competitors lock up the necessary resources for its expansion, but after a large perturbation, weeds can grow out of control. Whether native or introduced, the r-selected weeds are usually considered as invaders because of their unbalanced residency in the environment. Members of the phylum Proteobacteria or Verrucomicrobia usually represent a minor population within the intestinal microbiome but can dominate following antibiotic therapy (Dubourg et al., 2013; Ubeda and Pamer, 2012), gastric bypass (Graessler et al., 2012; Liou et al., 2013), or dietary shifts (Parks et al., 2013), and thus may be examples of r-selected species dominating an environment following a perturbation. The gradual shift from facultative anaerobic bacteria (Proteobacteria and Bacilli) to strict anaerobes (Bacteroidia, Clostridia, and Erysipelotrichi) in the developing infant gut (Pantoja-Feliciano et al., 2013) may be driven by a competitive advantage of the K-species strict anaerobes to maintain populations when oxygen is limited.

After an ecosystem is perturbed, exogenous organisms are more likely to successfully invade. Recovery from perturbation requires a full complement of species or of functional groups. In the gut, there appears to be enormous functional redundancy (Human Microbiome Project Consortium, 2012; Turnbaugh et al., 2009). However, there could be keystone species, defined as those that have disproportional influence on the behavior of the system as a whole, that when lost or gained have a profound influence on health. For example, on the plains, wolves have a very small population footprint, but their predatory presence affects much larger communities of grazers, and even larger populations of plants that the grazers eat. An important consequence of perturbations is loss of keystone species, in which small populations can oscillate down to zero and thus are lost.

Very successful organisms, whether they are r- or K-selected, often have bistable states. Bistability is an ancient adaptation to long-term niches that are subject to significant and frequent perturbations. Unpredictable food supplies, which constrain most animal populations, are an example of a typical perturbation. The alternative forms in bistable states allow optimization in changing milieus. When conditions change, rapid growers like *Clostridium* species can form spores to transmit to the next susceptible host. At the cost of not adding to the population of proliferating cells that stably colonize a given host, exchanging metabolic signals with the host and/or other microbiota, *Clostridium sp.* diverts its metabolism to the spore form that is most likely to survive transmission. There is fitness cost in diverting energy to formation of the seeds that spores represent, but there also is great fitness value, since host life span is finite. Such

dynamics affect the partitioning of energy in the gut lumen, with downstream effects on both microbiota and host. Unlike their animal hosts, bacteria have the ability to bloom on a very short timescale with logarithmic expansion of their share of the energy pie, ultimately affecting transmission at critical junctures. This characteristic is a factor, intrinsic to any well-evolved ecology, subject to competition by other organisms operating with parallel strategies, and to host constraints, which are obligatorily obeyed.

Energy Source and Perturbation: The Case of Hibernation

Throughout the biosphere, there is vigorous competition for energy. In well-evolved mature stable ecosystems, carbon metabolism is tightly regulated (Mackelprang et al., 2011), implying that checks exist against one species gaining a disproportionate share of the available nutrients. Hibernation is a massive physiological shift that is programmed that affects both central metabolism and the immune system (Bouma et al., 2010; Carey et al., 2003; Humphries et al., 2003). That energy storage and utilization are central to hibernation physiology provides an excellent model system to examine microbial characteristics associated with an annual perturbation cycle. Some colonizers of the mammalian colon have the ability to obtain their energy not from the food substrate passing through the intestine, but rather from digesting host-derived molecules (Berry et al., 2013). One important example is *Akkermansia muciniphila*, a member of the Verrucomicrobia phylum, which can use mucin as its sole carbon and nitrogen source (Derrien et al., 2004). That *Akkermansia* utilizes mucins for energy, which are virtually unlimited in supply in the vertebrate gut, represents an apparent paradox. With a readily available and apparently inexhaustible relatively exclusive food supply, why are Verrucomicrobia not always the dominant organism in the gut ecosystem? Similarly, what are the consequences to the host of an organism that has the ability to deplete its mucus, an important component of its barrier against potentially pathogenic microbiota and their toxic products?

Verrucomicrobia abundance is not constant in the intestinal microbiota; proportions increase with disruption and dysbiosis (e.g., antibiotic treatment) (Dubourg et al., 2013), or increase with an influx of fermentable fiber (Tachon et al., 2013). The change in microbiota in hibernating ground squirrels provides an excellent paradigm (Carey et al., 2013) with broad implications. Squirrels store energy in the summer and hibernate in the winter. In the late winter, essentially all of the energy available to the microbiota is host derived. Hibernation, rather than specific host, diet, or age, is the major determinant of the gut microbial community composition. During the seasons of feeding, Firmicutes, which are specialists in dietary carbohydrates (Flint et al., 2012), dominate, but after several months of fasting, their representation falls, replaced by Verrucomicrobia, as well as by Bacteroidetes, which can switch their expression of genes related to diet- or host-related energy sources (Martens et al., 2008; Salyers et al., 1977).

This programmed seasonal restructuring in relation to hibernation and its consequent dietary limitation is useful to model the energy relationships between the major gut microbiota taxa classes and the effects on host pathophysiology (Figure A3-4). In a steady state in most mammals (and in times of feeding for hibernators), the populations of (r-selected) organisms that utilize host-derived substrates are held in check by competing organisms that use the plentiful dietary food sources. Change in the mucus layer is minimized. Dietary utilizers also break down complex macronutrients into small monomers or ferment them to SCFA, which are required nutrients for other microbial consortia. Use of SCFA by these transactional species is a benefit to dietary utilizers because it is removing a waste product that could detrimentally lower pH. However, in perturbed states (and in times of fasting for hibernators), the organisms that use dietary sources are disrupted and fail to check the growth of the host substrate users or to provide crossfeeding metabolites. The resulting overgrowth of organisms that utilize host substrates depletes the mucus layer and diminishes its barrier function. Similarly, butyrate, produced by fermentation of dietary carbohydrates, and known to enhance tight junctions (Wong et al., 2006), is reduced during fasting. These changes are manifested as an increase in the cellularity of the lamina propria and a reduction in villus length (Kurtz and Carey, 2007). To the pathologist, this would be "colitis," except that in the hibernator it is the physiologic consequence of its lifestyle. The digestion of mucus and the loss of butyrate resulting from perturbations that affect the microbes requiring dietary energy sources are examples of secondary effects on the interface between the microbiota and the host. These can result in differential signaling to epithelial and immune cells, with both immune and metabolic consequences (Canani et al., 2011).

The dynamics of the interactions suggest that there are boundary conditions that govern the microbial population structure in each gut locality. However, in total, the conserved nature of the relationships of the major phyla to one another, and their fluctuation with perturbation, suggests the operation of a Nash equilibrium (Blaser and Kirschner, 2007), a game theory construct in which good behavior by players is rewarded and "cheaters" achieve inferior results (Nash, 1951). Within the boundary conditions of the gut, with competing organisms and alternative energy substrates that affect host viability, the development of a Nash equilibrium can explain the conserved dynamic changes observed between the microbe phyla and the host. The overall conservation of the mammalian colonic microbial community structure is an important observation that indicates shared solutions between hosts and their resident microbes that have persisted for >50 million years of evolution (Ley et al., 2008a). This timescale implies that important boundary conditions have selected against marked divergence from a common ancestry. In contrast, the presence of alternative macrolevel population structures in mammals (Arumugam et al., 2011) could be a condition inconsistent with a conserved Nash equilibrium.

Conclusions

Host phenotypes in obesity are dependent on the interactions between the diet, the resident microbiota, and host immunity. Evolutionary and ecological theories ultimately govern, but the principal actors include the differential microbial energetics and the host cells that monitor the microbial denizens. We are just beginning to explore a complex and ultimately crucial biological system, all the more important because it appears to be changing out of proportion to evolutionary time.

References

Ahn, S.M., Pomp, A., and Rubino, F. (2010). Metabolic surgery for type 2 diabetes. Ann. N Y Acad. Sci. 1212, E37–E45.

Ajslev, T.A., Andersen, C.S., Gamborg, M., Sørensen, T.I.A., and Jess, T. (2011). Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. Int. J. Obes. (Lond.) 35, 522–529.

Antonopoulos, D.A., Huse, S.M., Morrison, H.G., Schmidt, T.M., Sogin, M.L., and Young, V.B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect. Immun. 77, 2367–2375.

Aron-Wisnewsky, J., Doré , J., and Clement, K. (2012). The importance of the gut microbiota after bariatric surgery. Nat. Rev. Gastroenterol. Hepatol. 9, 590–598.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.-M., et al.; MetaHIT Consortium. (2011). Enterotypes of the human gut microbiome. Nature 473, 174–180.

Bäckhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F., and Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. USA 101, 15718–15723.

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70, 567–590.

Bernbom, N., Licht, T.R., Brogren, C.-H., Jelle, B., Johansen, A.H., Badiola, I., Vogensen, F.K., and Nørrung, B. (2006). Effects of Lactococcus lactis on composition of intestinal microbiota: role of nisin. Appl. Environ. Microbiol. 72, 239–244.

Berry, D., Stecher, B., Schintlmeister, A., Reichert, J., Brugiroux, S., Wild, B., Wanek, W., Richter, A., Rauch, I., Decker, T., et al. (2013). Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. Proc. Natl. Acad. Sci. USA 110, 4720–4725.

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., and Retetangos, C. (2010). Mode of delivery affects the bacterial community in the newborn gut. Early Hum. Dev. 86(Suppl 1), 13–15.

Blaser, M.J., and Falkow, S. (2009). What are the consequences of the disappearing human microbiota? Nat. Rev. Microbiol. 7, 887–894.

Blaser, M.J., and Kirschner, D. (2007). The equilibria that allow bacterial persistence in human hosts. Nature 449, 843–849.

Blustein, J., Attina, T., Liu, M., Ryan, A.M., Cox, L.M., Blaser, M.J., and Trasande, L. (2013). Association of caesarean delivery with child adiposity from age 6 weeks to 15 years. Int. J. Obes. Published online May 14, 2013. http://dx.doi.org/10.1038/ijo.2013.49.

Bouma, H.R., Carey, H.V., and Kroese, F.G.M. (2010). Hibernation: the immune system at rest? J. Leukoc. Biol. 88, 619–624.

Canani, R.B., Costanzo, M.D., Leone, L., Pedata, M., Meli, R., and Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J. Gastroenterol. 17, 1519–1528.

Cani, P.D., Amar, J., Iglesias, M.A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A.M., Fava, F., Tuohy, K.M., Chabo, C., et al. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56, 1761–1772.

Cani, P.D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A.M., Delzenne, N.M., and Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57, 1470–1481.

Carey, H.V., Andrews, M.T., and Martin, S.L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol. Rev. 83, 1153–1181.

Carey, H.V., Walters, W.A., and Knight, R. (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304, R33–R42.

Cho, I., and Blaser, M.J. (2012). The human microbiome: at the interface of health and disease. Nat. Rev. Genet. 13, 260–270.

Cho, I., Yamanishi, S., Cox, L., Methé , B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., et al. (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature 488, 621–626.

Coates, M.E., Fuller, R., Harrison, G.F., Lev, M., and Suffolk, S.F. (1963). A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br. J. Nutr. 17, 141–150.

Costello, E.K., Stagaman, K., Dethlefsen, L., Bohannan, B.J.M., and Relman, D.A. (2012). The application of ecological theory toward an understanding of the human microbiome. Science 336, 1255–1262.

Cox, L.M., Cho, I., Young, S.A., Anderson, W.H.K., Waters, B.J., Hung, S.-C., Gao, Z., Mahana, D., Bihan, M., Alekseyenko, A.V., et al. (2013). The nonfermentable dietary fiber hydroxypropyl methylcellulose modulates intestinal microbiota. FASEB J. 27, 692–702.

Derrien, M., Vaughan, E.E., Plugge, C.M., and de Vos, W.M. (2004). Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol. 54, 1469–1476.

Dethlefsen, L., Huse, S., Sogin, M.L., and Relman, D.A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 6, e280. http://dx.doi.org/10.1371/journal.pbio.0060280.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. USA 107, 11971–11975.

Dominguez-Bello, M.G., Blaser, M.J., Ley, R.E., and Knight, R. (2011). Development of the human gastrointestinal microbiota and insights from highthroughput sequencing. Gastroenterology 140, 1713–1719.

Dubourg, G., Lagier, J.-C., Armougom, F., Robert, C., Audoly, G., Papazian, L., and Raoult, D. (2013). High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment. Int. J. Antimicrob. Agents 41, 149–155.

Erlebacher, A. (2013). Immunology of the maternal-fetal interface. Annu. Rev. Immunol. 31, 387–411.

Everard, A., Lazarevic, V., Derrien, M., Girard, M., Muccioli, G.G., Neyrinck, A.M., Possemiers, S., Van Holle, A., Franc¸ ois, P., de Vos, W.M., et al. (2011). Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60, 2775–2786.

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G., Delzenne, N.M., et al. (2013). Crosstalk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc. Natl. Acad. Sci. USA. Published online May 13, 2013. http://dx.doi.org/10.1073/pnas.1219451110.

Faith, J.J., McNulty, N.P., Rey, F.E., and Gordon, J.I. (2011). Predicting a human gut microbiota's response to diet in gnotobiotic mice. Science 333, 101–104.

Flegal, K.M., Carroll, M.D., Kit, B.K., and Ogden, C.L. (2012). Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999–2010. JAMA 307, 491–497.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3, 289–306.

Graessler, J., Qin, Y., Zhong, H., Zhang, J., Licinio, J., Wong, M.-L., Xu, A., Chavakis, T., Bornstein, A.B., Ehrhart-Bornstein, M., et al. (2012). Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. Pharmacogenomics J. Published online October 2, 2012. http://dx.doi.org/10.1038/tpj.2012.43.

Greenblum, S., Turnbaugh, P.J., and Borenstein, E. (2012). Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proc. Natl. Acad. Sci. USA 109, 594–599.

Greenwood, M.R., and Hirsch, J. (1974). Postnatal development of adipocyte cellularity in the normal rat. J. Lipid Res. 15, 474–483.

Haslam, D.W., and James, W.P.T. (2005). Obesity. Lancet 366, 1197–1209. Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Strowig, T., Thaiss, C.A., Kau, A.L., Eisenbarth, S.C., Jurczak, M.J., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482, 179–185.

Hicks, L.A., Taylor, T.H., Jr., and Hunkler, R.J. (2013). U.S. outpatient antibiotic prescribing, 2010. N. Engl. J. Med. 368, 1461–1462.

Hollopeter, G., Erickson, J.C., and Palmiter, R.D. (1998). Role of neuropeptide Y in diet-, chemical- and genetic-induced obesity of mice. Int. J. Obes. Relat. Metab. Disord. 22, 506–512.

Holmes, E., Li, J.V., Marchesi, J.R., and Nicholson, J.K. (2012). Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. Cell Metab. 16, 559–564.

Huh, S.Y., Rifas-Shiman, S.L., Zera, C.A., Edwards, J.W.R., Oken, E., Weiss, S.T., and Gillman, M.W. (2012). Delivery by caesarean section and risk of obesity in preschool age children: a prospective cohort study. Arch. Dis. Child. 97, 610–616.

Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. Nature 486, 207–214.

Humphries, M.M., Thomas, D.W., and Kramer, D.L. (2003). The role of energy availability in Mammalian hibernation: a cost-benefit approach. Physiol. Biochem. Zool. 76, 165–179.

Hungate, R.E. (1966). The Rumen and Its Microbes (New York: Academic Press).

Hungate, R.E., Dougherty, R.W., Bryant, M.P., and Cello, R.M. (1952). Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet. 42, 423–449.

Huurre, A., Kalliomäki, M., Rautava, S., Rinne, M., Salminen, S., and Isolauri, E. (2008). Mode of delivery—effects on gut microbiota and humoral immunity. Neonatology 93, 236–240.

Ichimura, A., Hirasawa, A., Hara, T., and Tsujimoto, G. (2009). Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis. Prostaglandins Other Lipid Mediat. 89, 82–88.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139, 485–498.

Jumpertz, R., Le, D.S., Turnbaugh, P.J., Trinidad, C., Bogardus, C., Gordon, J.I., and Krakoff, J. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. Am. J. Clin. Nutr. 94, 58–65.

Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L., and Gordon, J.I. (2011). Human nutrition, the gut microbiome and the immune system. Nature 474, 327–336.

Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Bäckhed, H.K., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., et al. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 150, 470–480.

Kurokawa, K., Itoh, T., Kuwahara, T., Oshima, K., Toh, H., Toyoda, A., Takami, H., Morita, H., Sharma, V.K., Srivastava, T.P., et al. (2007). Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. 14, 169–181.

Kurtz, C.C., and Carey, H.V. (2007). Seasonal changes in the intestinal immune system of hibernating ground squirrels. Dev. Comp. Immunol. 31, 415–428.

Letran, S.E., Lee, S.J., Atif, S.M., Flores-Langarica, A., Uematsu, S., Akira, S., Cunningham, A.F., and McSorley, S.J. (2011). TLR5-deficient mice lack basal inflammatory and metabolic defects but exhibit impaired CD4 T cell responses to a flagellated pathogen. J. Immunol. 186, 5406–5412.

Ley, R.E. (2010). Obesity and the human microbiome. Curr. Opin. Gastroenterol. 26, 5–11.

Ley, R.E., Bäckhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D., and Gordon, J.I. (2005). Obesity alters gut microbial ecology. Proc. Natl. Acad. Sci. USA 102, 11070–11075.

Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I. (2006). Microbial ecology: human gut microbes associated with obesity. Nature 444, 1022–1023.

Ley, R.E., Hamady, M., Lozupone, C.A., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., and Gordon, J.I. (2008a). Evolution of mammals and their gut microbes. Science 320, 1647–1651.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776–788.

Li, J.V., Ashrafian, H., Bueter, M., Kinross, J., Sands, C., le Roux, C.W., Bloom, S.R., Darzi, A., Athanasiou, T., Marchesi, J.R., et al. (2011). Metabolic surgery profoundly influences gut microbial-host metabolic cross-talk. Gut 60, 1214–1223.

Li, H., Ye, R., Pei, L., Ren, A., Zheng, X., and Liu, J. (2013). Caesarean delivery, caesarean delivery on maternal request and childhood overweight: a Chinese birth cohort study of 181 380 children. Pediatr. Obes. Published online March 19, 2013. http://dx.doi.org/10.1111/j.2047-6310.2013.00151.x.

Liou, A.P., Paziuk, M., Luevano, J.M., Machineni, S., Turnbaugh, P.J., and Kaplan, L.M. (2013). Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci. Trans. Med. 5. http://dx.doi.org/10.1126/scitranslmed.3005687, 178ra141.

Lozupone, C.A., and Knight, R. (2008). Species divergence and the measurement of microbial diversity. FEMS Microbiol. Rev. 32, 557–578.

Macfarlane, G.T., and Macfarlane, S. (2012). Bacteria, colonic fermentation, and gastrointestinal health. J. AOAC Int. 95, 50–60.

Mackelprang, R., Waldrop, M.P., DeAngelis, K.M., David, M.M., Chavarria, K.L., Blazewicz, S.J., Rubin, E.M., and Jansson, J.K. (2011). Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw. Nature 480, 368–371.

Martens, E.C., Chiang, H.C., and Gordon, J.I. (2008). Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe 4, 447–457.

Nagaraja, T.G., and Titgemeyer, E.C. (2007). Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. J. Dairy Sci. 90(Suppl 1),E17–E38.

Nash, J. (1951). Non-cooperative games. Ann. Math. 54, 286–295.

Neyrinck, A.M., Van Hée, V.F., Piront, N., De Backer, F., Toussaint, O., Cani, P.D., and Delzenne, N.M. (2012). Wheat-derived arabinoxylan oligosaccharides with prebiotic effect increase satietogenic gut peptides and reduce metabolic endotoxemia in diet-induced obese mice. Nutr Diabetes 2, e28. http://dx.doi.org/10.1038/nutd.2011.24.

Paine, R.T., Tegner, M.J., and Johnson, E.A. (1998). Compounded perturbations yield ecological surprises. Ecosystems 1, 535–545.

Pandey, P.K., Verma, P., Kumar, H., Bavdekar, A., Patole, M.S., and Shouche, Y.S. (2012). Comparative analysis of fecal microflora of healthy full-term Indian infants born with different methods of delivery (vaginal vs cesarean): Acinetobacter sp. prevalence in vaginally born infants. J. Biosci. 37, 989–998.

Pantoja-Feliciano, I.G., Clemente, J.C., Costello, E.K., Perez, M.E., Blaser, M.J., Knight, R., and Dominguez-Bello, M.G. (2013). Biphasic assembly of the murine intestinal microbiota during early development. ISME J. 7, 1–4.

Parks, B.W., Nam, E., Org, E., Kostem, E., Norheim, F., Hui, S.T., Pan, C., Civelek, M., Rau, C.D., Bennett, B.J., et al. (2013). Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. Cell Metab. 17, 141–152.

Payne, A.N., Chassard, C., and Lacroix, C. (2012). Gut microbial adaptation to dietary consumption of fructose, artificial sweeteners and sugar alcohols: implications for host-microbe interactions contributing to obesity. Obes. Rev. 13, 799–809.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., et al.; MetaHIT Consortium. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.

Ravussin, Y., Koren, O., Spor, A., LeDuc, C., Gutman, R., Stombaugh, J., Knight, R., Ley, R.E., and Leibel, R.L. (2012). Responses of gut microbiota to diet composition and weight loss in lean and obese mice. Obesity (Silver Spring) 20, 738–747.

Reznick, D., Bryant, M.J., and Bashey, F. (2002). R- and K-selection revisited: the role of population regulation in life-history evolution. Ecology 83, 1509– 1520.

Rubino, F., Gagner, M., Gentileschi, P., Kini, S., Fukuyama, S., Feng, J., and Diamond, E. (2004). The early effect of the Roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism. Ann. Surg. 240, 236–242.

Salyers, A.A., Vercellotti, J.R., West, S.E., and Wilkins, T.D. (1977). Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. Appl. Environ. Microbiol. 33, 319–322.

Samuel, B.S., Shaito, A., Motoike, T., Rey, F.E., Backhed, F., Manchester, J.K., Hammer, R.E., Williams, S.C., Crowley, J., Yanagisawa, M., and Gordon, J.I. (2008). Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc. Natl. Acad. Sci. USA 105, 16767–16772.

Savage, D.C. (1977). Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. 31, 107–133.

Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B.H., et al. (2012). Fundamentals of microbial community resistance and resilience. Front Microbiol. 3, 417.

Tachon, S., Zhou, J., Keenan, M., Martin, R., and Marco, M.L. (2013). The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. FEMS Microbiol. Ecol. 83, 299–309.

Thellin, O., and Heinen, E. (2003). Pregnancy and the immune system: between tolerance and rejection. Toxicology 185, 179–184.

Trasande, L., Blustein, J., Liu, M., Corwin, E., Cox, L.M., and Blaser, M.J. (2013). Infant antibiotic exposures and early-life body mass. Int J Obes (Lond) 37, 16–23.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.

Turnbaugh, P.J., Bäckhed, F., Fulton, L., and Gordon, J.I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3, 213–223.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., et al. (2009). A core gut microbiome in obese and lean twins. Nature 457, 480–484.

Ubeda, C., and Pamer, E.G. (2012). Antibiotics, microbiota, and immune defense. Trends Immunol. 33, 459–466.

Upadhyay, V., Poroyko, V., Kim, T.J., Devkota, S., Fu, S., Liu, D., Tumanov, A.V., Koroleva, E.P., Deng, L., Nagler, C., et al. (2012). Lymphotoxin regulates commensal responses to enable diet-induced obesity. Nat. Immunol. 13, 947–953.

Vijay-Kumar, M., Sanders, C.J., Taylor, R.T., Kumar, A., Aitken, J.D., Sitaraman, S.V., Neish, A.S., Uematsu, S., Akira, S., Williams, I.R., and Gewirtz, A.T. (2007). Deletion of TLR5 results in spontaneous colitis in mice. J. Clin. Invest. 117, 3909–3921.

Vijay-Kumar, M., Aitken, J.D., Carvalho, F.A., Cullender, T.C., Mwangi, S., Srinivasan, S., Sitaraman, S.V., Knight, R., Ley, R.E., and Gewirtz, A.T. (2010). Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 328, 228–231.

Wong, J.M.W., de Souza, R., Kendall, C.W.C., Emam, A., and Jenkins, D.J.A. (2006). Colonic health: fermentation and short chain fatty acids. J. Clin. Gastroenterol. 40, 235–243.

Wright, J., Kennedy-Stephenson, J., Wang, C., McDowell, M., and Johnson, C. (2004). Trends in intake of energy and macronutrients—United States, 1971–2000. JAMA 291, 1193–1194.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P., et al. (2012). Human gut microbiome viewed across age and geography. Nature 486, 222–227.

A4

MICROBIAL EXPOSURE DURING EARLY LIFE HAS PERSISTENT EFFECTS ON NATURAL KILLER T CELL FUNCTION[9]

Torsten Olszak,[10,*] *Dingding An*,[11,*] *Sebastian Zeissig*,[12] *Miguel Penilla Vera*,[13] *Julia Richter*,[14] *Andre Franke*,[15] *Jonathan N. Glickman*,[16] *Reiner Siebert*,[14] *Rebecca M. Barron*,[13] *Dennis L. Kasper*,[11,†‡] *and Richard S.* ***Blumberg***[10,†‡]

Abstract

Exposure to microbes during early childhood is associated with protection from immune-mediated diseases such as inflammatory bowel disease (IBD) and asthma. Here, we show that in germ-free (GF) mice, invariant natural killer T (iNKT) cells accumulate in the colonic lamina propria and

[9] From "Microbial exposure during early life has persistent effects on natural killer T cell function," *Science* 336(6080):489-493, 2012. Reprinted with permission from the AAAS.

[10] Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

[11] Channing Laboratory, Brigham and Women's Hospital and Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA.

[12] Department of Internal Medicine I, University Medical Center Schleswig-Holstein, Kiel 24105, Germany.

[13] Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

[14] Institute of Human Genetics, Christian-Albrechts University Kiel and University Medical Center Schleswig-Holstein, Kiel 24105, Germany.

[15] Institute of Clinical Molecular Biology, Christian-Albrechts University, Kiel 24105, Germany.

[16] GI Pathology, Caris Diagnostics, Caris Life Sciences, Newton, MA 02464, USA.

* These authors contributed equally to this work.

† These authors contributed equally to this work.

‡ To whom correspondence should be addressed. E-mail: rblumberg@partners.org (R.S.B.);dennis_kasper@hms.harvard.edu (D.L.K.).

lung, resulting in increased morbidity in models of IBD and allergic asthma as compared with that of specific pathogen-free mice. This was associated with increased intestinal and pulmonary expression of the chemokine ligand CXCL16, which was associated with increased mucosal iNKT cells. Colonization of neonatal—but not adult—GF mice with a conventional microbiota protected the animals from mucosal iNKT accumulation and related pathology. These results indicate that age-sensitive contact with commensal microbes is critical for establishing mucosal iNKT cell tolerance to later environmental exposures.

The mammalian host immune system is broadly stimulated with the first exposure to microorganisms during neonatal life (Kelly et al., 2007). The inner surfaces of the gastrointestinal tract and lungs are particularly affected because they are predominant sites of microbial contact (Renz et al., 2012). Epidemiologic observations further suggest that the immune effects of early-life microbial exposure are durable and persist into later life because they can be associated with prevention of diseases such as inflammatory bowel disease (IBD) and asthma (Ege et al., 2011; López-Serrano et al., 2011; von Mutius, 2007).

Invariant natural killer T (iNKT) cells probably play an important role in the pathogenesis of ulcerative colitis (UC)—a major form of IBD—and in asthma (Akbari et al., 2003; Fuss et al., 2004). Such cells recognize endogenous and exogenous lipid antigens presented by the nonpolymorphic major histocompatibility complex (MHC) class I–like protein CD1d and secrete abundant amounts of proinflammatory cytokines such as interleukin-4 (IL-4) and IL-13 upon activation (Cohen et al., 2009; Kronenberg, 2005). We therefore investigated age-dependent regulation of iNKT cells by use of microbes in mouse models of IBD and asthma.

We first examined the appearance of iNKT cells in tissues of 8-week-old germ-free (GF) and specific pathogen-free (SPF) Swiss-Webster (SW) mice. Relative and absolute numbers of iNKT cells were increased in GF mice in colonic lamina propria (LP) (Figure A4-1, A to C). These differences in colonic iNKT cell numbers between GF and SPF mice were detectable after weaning and stable for life, suggesting early and persistent effects of the microbiota (Figure A4-1D). iNKT cells were not increased in the ileal LP (ileum) of GF mice, and the liver, spleen, and thymus contained even fewer iNKT cells under GF relative to SPF conditions (fig. S1), which is consistent with a recent report (Wei et al., 2010). GF C57BL/6 mice (B6) exhibited similar increases of iNKT cells in the colonic LP as well as the liver, in contrast to GF SW mice (fig. S2). Although increased in number, the iNKT cell expression of several activation and memory markers was unaltered in GF SW, relative to SPF, mice (fig. S3).

To examine the relevance of these findings, we investigated the susceptibility of GF and SPF mice to oxazolone-induced colitis, a model of intestinal inflammation that possesses features of UC and is dependent on IL-13 production by

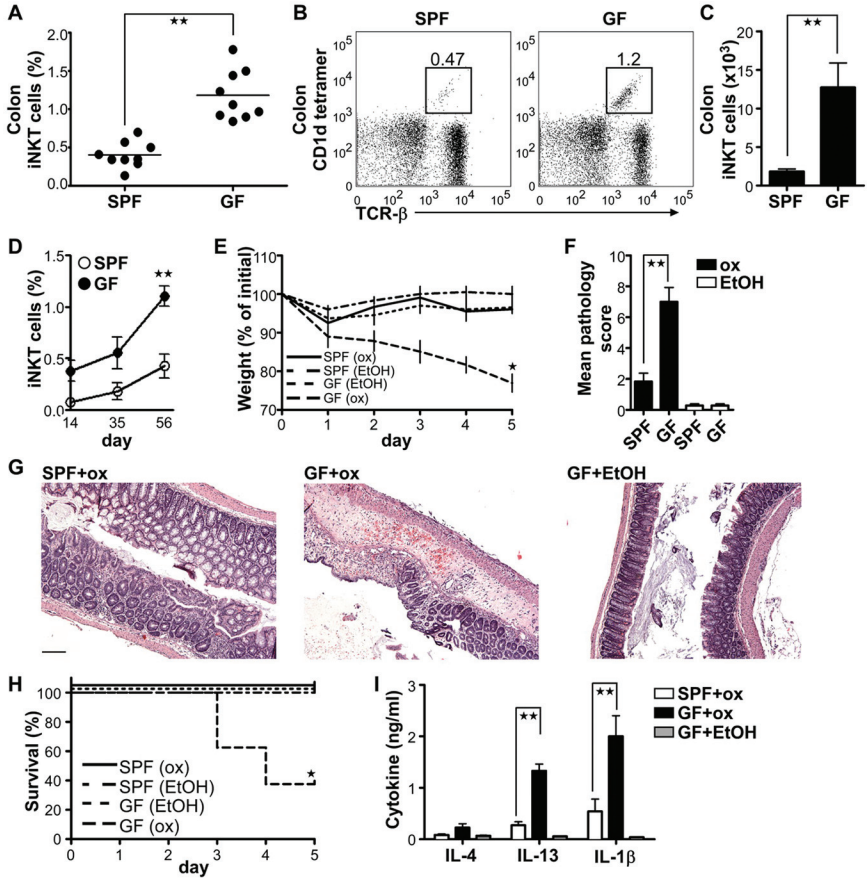


FIGURE A4-1 Intestinal bacteria-dependent accumulation of colonic iNKT cells in GF mice leads to high mortality in oxazolone-induced colitis. (A to C) The percentage of CD1d tetramer–positive cells (iNKT cells) within the live lymphocytes from the lamina propria (LP) of gender-matched GF and SPF SW mice (7 to 8 weeks old) was analyzed by means of flow cytometry (A). Representative dot-plots are shown in (B), and the absolute number of iNKT cells is shown in (C). Each circle in (A) represents an individual mouse. (D) Percentages of colonic iNKT cells of live LP lymphocytes in gender-matched SPF and GF mice were analyzed at different ages by means of flow cytometry ($n = 4$ mice per group). (E to H) Eight-week-old GF and SPF mice were monitored and scored after rectal challenge with 1% oxazolone (ox) or 50% ethanol for survival and body weight loss for 5 days. On day 5, the colons were collected and dissected for histological analysis ($n = 5$ mice per group). (G) Scale bar, 50 μm. (I) The concentration of IL-4, IL-13, and IL-1β in the supernatant of 24 hours–colon organ explant cultures determined by means of enzyme-linked immunosorbent assay (ELISA) on day five ($n = 5$ mice per group). All data were obtained from three independent experiments with similar results. In all panels, error bars represent the SD. $^*P < 0.05$, $^{**}P < 0.01$, unpaired t test and $^*P < 0.05$, log-rank test in (H).

CD1d-restricted iNKT cells (Heller et al., 2002; Schiechl et al., 2011). Although GF or germ-reduced mice exhibit exacerbated inflammation in an innate mouse model of colitis (Maslowski et al., 2009; Rakoff-Nahoum et al., 2004), colitis is typically prevented under GF conditions in models dependent on an adaptive immune response (*Strober* et al., 2002). Surprisingly, GF mice were more sensitive to oxazolone-induced colitis, as revealed by severe weight loss, pathology, and a high mortality rate in contrast to SPF mice (Figure A4-1, E to H). This was not due to overexpression of cell-surface CD1d expression on intestinal epithelial (fig. S4A) and hematopoietic cells (fig. S4B). Consistent with previous studies (*Heller* et al., 2002), the colitis in GF mice was characterized by a marked increase in production of IL-13 and IL-1β in comparison with SPF mice (Figure A4-1I).

To confirm the CD1d-restriction of this colitis in GF mice, we investigated the effects of CD1d blockade. Adult (8- to 9-week-old) GF and SPF mice were treated with a blocking monoclonal antibody (Ab) specific for CD1d (19G11) or an isotype control Ab (Roark et al., 1998). Treatment of GF mice with 19G11, but not the isotype control, protected against colitis-induced mortality and associated pathology (Figure A4-2, A to C, and fig. S4, C and D). Furthermore, CD1d blockade of GF or SPF mice did not lead to significant functional changes in dendritic cells (Yue et al., 2005) or B cells (Mizoguchi and Bhan, 2006) as demonstrated by stable IL-12 or IL-10 production (fig. S4, E and F), respectively.

We next examined whether reestablishing microbiota in adult GF mice would normalize iNKT cell levels in the colon. Quite surprisingly, exposure of adult GF mice to SPF conditions (GF/a) did not restore iNKT cells in the colon to the levels observed in SPF mice or reverse the mortality and severe pathology after oxazolone administration (Figure A4-2, D to F). We therefore considered whether the ability to normalize iNKT cell levels and function in the colon was dependent on the age at which microbial contact occurred. Indeed, when we colonized pregnant GF female mice just before delivery and therefore exposed neonatal GF mice to SPF conditions on their first day of life (GF/n), we observed a complete normalization of iNKT cell levels that persisted even two months after colonization (Figure A4-2D). Consistent with this, GF/n exhibited reduced susceptibility to oxazolone-induced colitis (relative to that of GF mice) two months after colonization, with a degree of severity identical to that observed in SPF animals, as shown by an analysis of mortality, weight loss, pathology, and cytokine production (Figure A4-2, E and F, and fig. S5).

To verify that the early life events associated with the absence of normal microbial colonization on iNKT cell homeostasis were CD1d-dependent, we treated GF mice with 19G11 Ab for their first 6 weeks of life. 19G11, but not control Ab, treatment blocked accumulation of iNKT cells in all tissues examined and susceptibility to oxazolone-induced colitis in later life (fig. S6). Similar to GF mice, SPF mice (B6) born under germ-reduced conditions exhibited an increase in colonic iNKT cells and excessive oxazolone-induced colitis at 4 weeks of life

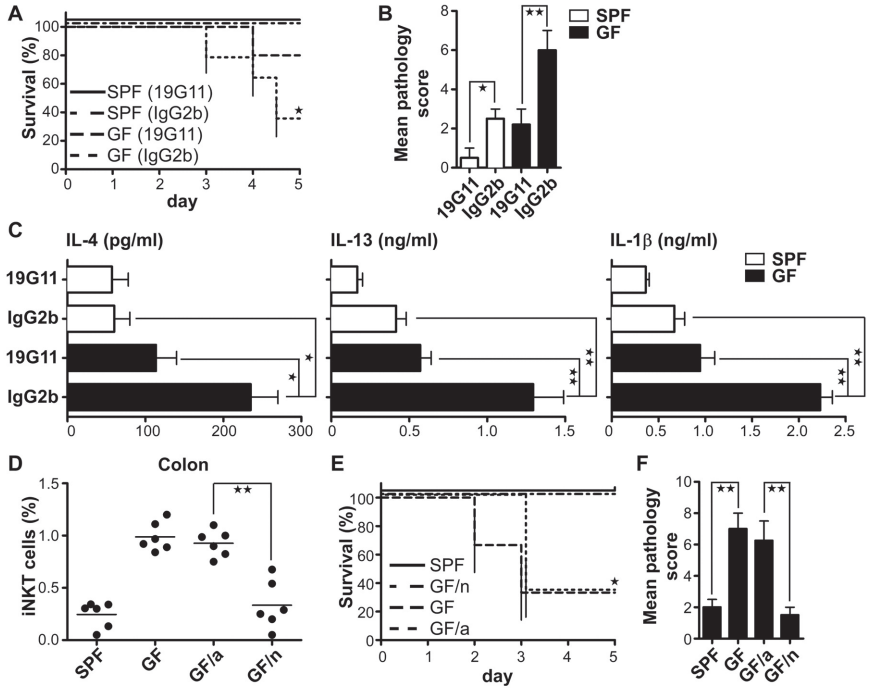


FIGURE A4-2 Microbial colonization during early life prevents the CD1d/iNKT cell-dependent high mortality in oxazolone colitis in GF mice. (A to C) Eight-week-old GF and SPF mice were treated once with 1 mg of 19G11 or isotype control IgG2b antibody before presensitization and rectal oxazolone challenge. (A) Survival after oxazalone challenge is shown. (B) On day 5, the colons were collected for histological analysis, and (C) the cytokine concentration in the supernatant of 24 hours-colon organ explant cultures was measured with ELISA ($n \geq 4$ mice per group). (D) Colonic LP lymphocytes were analyzed for iNKT cells by flow cytometry. (GF/a) mice were GF mice that were exposed to SPF environmental conditions at the age of 5 weeks and maintained for 4 more weeks. (GF/n) mice were pups exposed to SPF conditions on their first day of life and maintained under SPF environmental conditions for 8 to 9 weeks. Each circle represents a mouse. (E to F) Colonized mice were treated as above with oxazolone ($n = 5$ mice per group). All data were obtained from more than two independent experiments with similar results. Error bars indicate SD. $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired t test and $^{*}P < 0.05$, log-rank test in (A) and (E).

(fig. S7). As predicted (Heller et al., 2002), *Cd1d*$^{-/-}$ and *Jα18*$^{-/-}$ mice, both of which lack iNKT cells, were not susceptible to oxazolone-induced colitis either under SPF or germ-reduced conditions (fig. S7).

Animal models of asthma demonstrate an important role for iNKT cells (Meyer et al., 2008; Wingender et al., 2011), although the contribution to human

disease remains controversial (Iwamura and Nakayama, 2010). We found that the lungs of GF SW and B6 mice contained significantly higher relative and absolute numbers of iNKT cells in comparison with that of the lungs of the respective SPF mice (Figure A4-3, A to C, and fig. S8). Because commensal microbes may affect the induction of experimental asthma (Herbst et al., 2011), we tested an ovalbumin (OVA)–driven allergic-asthma mouse model in GF mice. We observed that similar to the colon, GF mice developed an allergic airway response to OVA significantly greater than that observed in SPF mice, as demonstrated by increases in airway resistance, total bronchial alveolar lavage fluid (BALF) cell numbers, BALF eosinophilia, serum immunoglobulin E (IgE) levels, proinflammatory cytokine production in the BALF, and lung tissue eosinophil infiltration (Figure A4- 3, D to F, and fig. S9). The asthma in GF mice was CD1d- and antigen-dependent because elimination of the asthmatic response was only observed with 19G11, but not control, Ab treatment (Figure A4-3, D to F, and fig. S9) of OVA-sensitized mice but not mice exposed to phosphate-buffered saline (PBS), which did not induce allergic asthma (fig. S10).

We therefore next investigated whether early-life exposure to a conventional microbiota also protects animals from CD1d-restricted inflammation in the lungs. As observed in the intestinal mucosa, neonatal (GF/n)—but not adult-life (GF/a)—exposure of GF mice to a conventional microbiota abrogated the increased accumulation of iNKT cells in the lungs and protected adult mice in the allergic asthma model from the pathology (Figure A4-3, G to I, and fig. S11).

The chemokine receptor CXCR6 on iNKT cells (Germanov et al., 2008) and its ligand CXCL16, which is expressed at high levels by human epithelial cells (*Day* et al., 2009) and increased in inflammation (Diegelmann et al., 2010), plays an important role in iNKT cell homeostasis. Therefore, we examined the serum of GF and GF/a mice for the presence of CXCL16 and observed significantly higher levels than that observed in SPF and GF/n mice (Figure A4-4A). This was due to significant increases of *Cxcl16* mRNA expression levels in the colon and lung—but not ileum, liver, and thymus—of GF and GF/a relative to SPF and GF/n mice (Figure A4-4B and fig. S12A), and mainly immunolocalized to the epithelium (fig. S12B). Although *Cxcl16* mRNA levels in the colon, ileum, and lung were similar immediately after birth in the GF and SPF animals at the beginning of colonization, they increased significantly and specifically only in the colon and lungs of the GF mice during later life (Figure A4-4C). These results suggest that microbial exposure provides signals that determine *Cxcl16* gene expression in specific tissues. GF and SPF mice, however, exhibited similar levels of *Cxcr6* mRNA expression in tissues and CXCR6 expression on the cell surface of iNKT cells (fig. S12, C and D).

CXCL16 was responsible for the accumulation of iNKT cells because treatment of newborn SPF and GF mice with a CXCL16-neutralizing Ab caused a decrease in iNKT cells in the colon and lung (Figure A4-4D) but had no effect on iNKT cell levels in the ileum or thymus (fig. S13A). However, it was associated

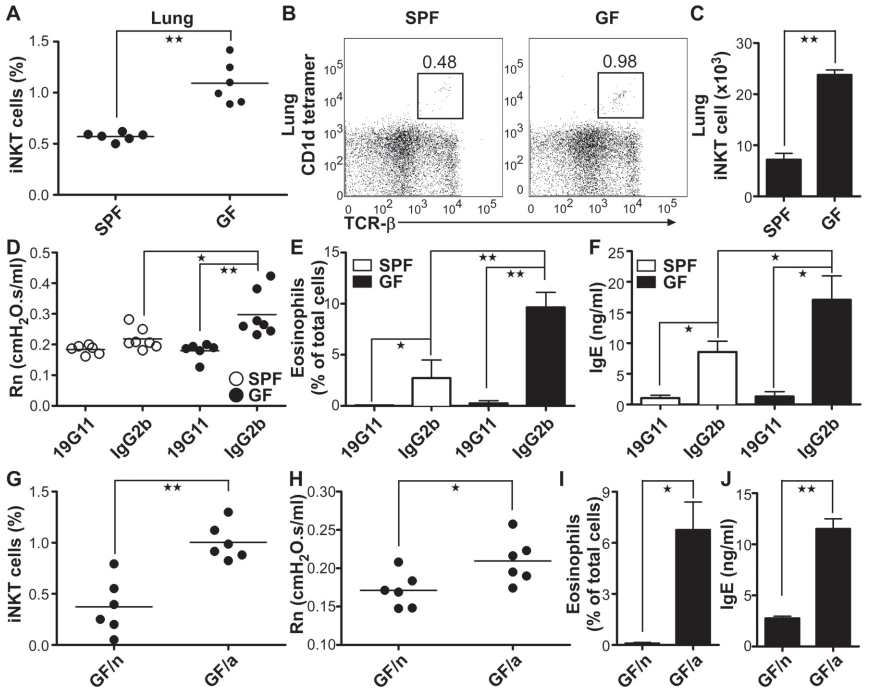


FIGURE A4-3 The increased CD1d/iNKT cell-mediated allergic response sensitivity of GF mice is dependent on age of colonization. (**A** to **C**) Lymphocytes from lungs of 8-week-old mice in each group were analyzed for iNKT cells by means of flow cytometry (A). Representative dot-plot is shown in (B), and the absolute number of iNKT cells is shown in (C) (n = 6 mice per group). (**D** to **F**) Age-matched mice from each group were treated once before OVA presensitization with 1 mg of 19G11 or IgG2b isotype control antibody and with 0.5 mg of 19G11 or IgG2b antibody before the first and the third serial OVA exposure. Twenty-four hours after the last challenge with 5% aerosolized OVA, the mice were analyzed for (D) airway resistance (Rn), (E) percentage of eosinophils of total BALF cells, and (F) IgE (n = 4 mice per group). (**G**) Lung lymphocytes were analyzed for iNKT cells by means of flow cytometry of age- and gender-matched GF/n and GF/a mice. (**H** to **J**) Mice were presensitized with OVA and analyzed after the last serial OVA challenge as described above ($n \geq 4$ mice per group). Each circle in (A), (D), (G), and (H) represents an individual mouse. All data were obtained from more than two independent experiments with similar results. Error bars indicate the SD. $*P \leq 0.05$, $**P < 0.01$, unpaired t test.

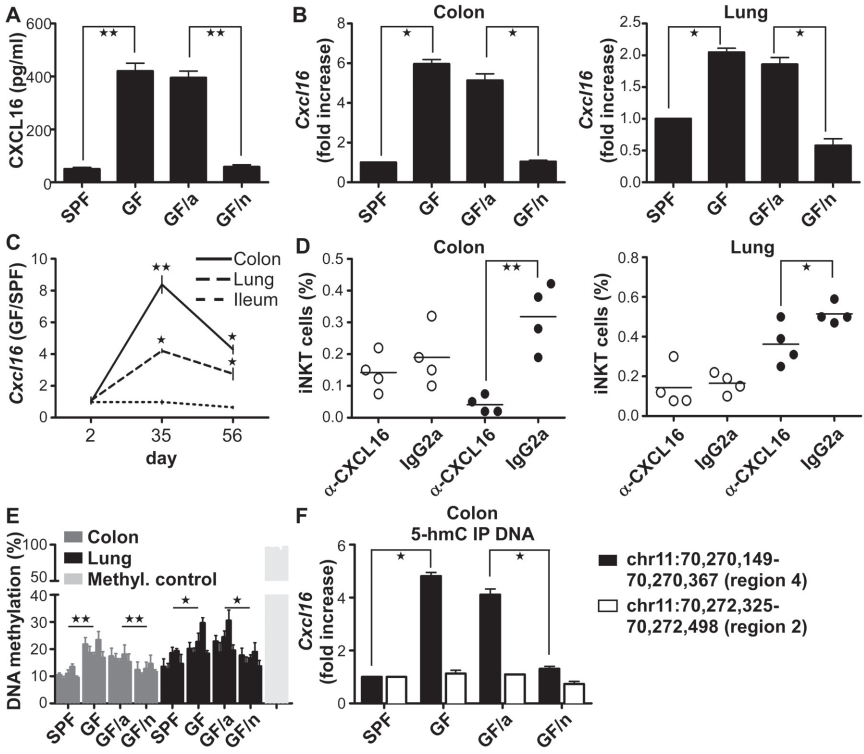


FIGURE A4-4 Microbiota affects tissue specific iNKT cell accumulation by genetic modifications of *Cxcl16*. (**A**) The CXCL16 concentration in the serum of each group was measured with ELISA in SPF, GF, GF/a, and GF/n as described above (n = 4 mice per group). (**B**) Colon and lung tissue samples were harvested from age-matched mice from each group and analyzed for *Cxl16*expression (n = 4 mice per group). (**C**) Colon, ileum, and lung tissue samples were analyzed from age-matched SPF and GF mice at different time points for *Cxcl16* expression (n = 5 mice per group). (**D**) SPF (open circles) and GF (closed circles) newborn mice were treated three times a week intraperitoneal with 25 µg of a neutralizing CXCL16 antibody (αCXCL16) or its isotype control IgG2a antibody and analyzed at the age of 2 weeks for iNKT cell percentages by means of flow cytometry. Each circle represents a mouse. (**E**) Analysis of DNA methylation of five CpG sites of *Cxcl16* by bisulfite pyrosequencing. For each group, the mean of DNA methylation of 5 CpG sites is shown as a percentage according to the methylated control (Methyl. control) ($n \geq 3$ mice per group). (**F**) *Cxcl16* qPCR analysis of colonic DNA after performing a 5-hydroxymethylated DNA immunoprecipitation (5-hmC IP DNA) ($n \geq 3$ mice per group). All data were obtained from at least two independent experiments with similar results. Error bars indicate the SD. $^*P \leq 0.05$, $^{**}P < 0.01$, unpaired t test.

with an increased accumulation of iNKT cells in the liver (fig. S13A), suggesting CXCL16 blockade caused a redirection of iNKT cells to this organ. Consequently, Ab-to-CXCL16– but not isotype-treated GF mice were protected from oxazolone colitis–induced mortality and pathology (fig. S13, B to D).

We next examined the epigenetic content of the *Cxcl16* gene of GF and SPF mice. A region 5′ of the *Cxcl16*gene of SW mice that contains five potential CpG sites was determined by means of bisulfite pyrosequencing to be hypermethylated in the colon and lungs (Figure A4-4E)—but not in other tissues such as the spleen and liver (fig. S14)—under GF conditions as compared with SPF conditions. Colonization of neonatal —but not adult—GF mice with a conventional microbiota decreased the hypermethylation of the *Cxcl16* gene to SPF levels (Figure A4-4E). Gene activation due to hypermethylation may occur as a consequence of specific types of environmental exposure (Rishi et al., 2010). To confirm this, we treated SPF neonates on their first day of life by oral gavage with high doses of folinic acid as previously described (Pu) so as to force *Cxcl16* methylation. Compared with PBS-treated control mice, folinic acid administration resulted in elevated methylation of the *Cxcl16* gene in the colon, ileum, and lung; at least a three- to fourfold increase of *Cxcl16* mRNA expression in the same tissues; increased CXCL16 serum levels; and accumulation of iNKT cells in these tissues as observed in GF mice (fig. S15). Alternatively, we investigated whether elimination of *Cxcl16* methylation in GF mice would reverse the elevation of CXCL16 expression by treating GF newborn littermates with the DNA methyltransferase inhibitor 5-Azacytidine (5-Aza). When examined at 2 weeks of life, 5-Aza treatment inhibited methylation and mRNA expression of *Cxcl16* in the colon, ileum, and lung (fig. S16, A and B), diminished the levels of CXCL16 in the serum, and reduced iNKT cells in these tissues (fig. S16, C and D). 5-Aza treatment had no effect on other cell populations such as $CD45^{+}CD11c^{+}$ double positive cells (fig. S17).

Epigenetic marks provided by 5-hydroxymethylcytosine (5-hmC) incorporation into DNA have been suggested to be different from those provided by 5-methylcytosine (5-mC) and a distinct signature for elevated levels of transcription (Song et al., 2011). We therefore performed DNA immunoprecipitation with a monoclonal 5-hmC Ab followed by quantitative polymerase chain reaction (PCR) analysis for *Cxcl16* in seven different regions of *Cxcl16* in DNA samples obtained from the colons of SPF, GF, GF/a, and GF/n mice. We observed that *Cxcl16* was highly increased in three out of the seven investigated regions (with region 4 as a representative) within the 5-hmC–modified DNA of GF and GF/a mice but not in the DNA acquired from SPF and GF/n mice (Figure A4-4F). No differences were observed in the other four regions (with region 2 as a representative) and in the DNA samples obtained from the ileum demonstrating the colonic specificity for 5-hmC modification of *Cxcl16* (fig. S18). A map summarizing this information is provided in fig. S19.

Our studies indicate that CXCL16 is an age- and organ-dependent microbially regulated factor that modulates the quantities and function of iNKT cells in the colon and lungs and, consequently, susceptibility to tissue inflammation. The exact mechanism by which the microbiota regulates CXCL16 expression and thus iNKT cell accumulation in these organs is unknown, although it is independent of the Toll-like receptor adapter protein MyD88 (fig. S20). These observations are in accordance with previous epidemiological studies collectively known as the "hygiene hypothesis," which proposes that early-life exposure to specific microbe-enriched environments decreases susceptibility to diseases such as IBD and asthma (Ege et al., 2011; Neish, 2009; Strachan, 1989; von Mutius, 2007), whereas its absence, as in antibiotic treatment during childhood, may have the opposite effect (Goksör et al., 2011; Shaw et al., 2010). Our results suggest that CD1d-restricted iNKT cell pathways and their relationship to microbes play a central role in these aforementioned processes. Early-life microbial exposure elicits long-lasting effects on iNKT cells, and in their absence, later-life exposure to factors that stimulate these cells may induce an autoinflammatory response. These findings are predicted to extrapolate to humans, given the extensive similarities between the mouse and human CD1d and iNKT cell systems (Sullivan and Kronenberg, 2007).

Acknowledgements

We thank E. Troy and S. Edwards for handling of the GF mice, J. Cusick for technical assistance, E. Nieuwenhuis and K. Schneeberger for assistance with epigenetics studies, A. Bellacosa and K. Baker for helpful discussions and manuscript preparation, and the KinderKrebsInitiative Buchholz/Holm-Seppensen for providing pyrosequencing infrastructure. The work was supported by NIH grants DK44319, DK51362, DK53056, and DK88199 (R.S.B.) and AI090102 (D.L.K.); Crohn's Colitis Foundation of America Senior Research Award (D.L.K.) and Crohn's and Colitis Foundation of America Postdoctoral Fellowship Award (D.A. and S.Z.); the Harvard Digestive Diseases Center (DK034854); the Medizinausschuss Schleswig-Holstein, German Ministry of Education Research through the National Genome Research Network (A.F.); The Medical Faculty, Kiel (R.S.) and the Deutsche Forschungsgemeinschaft (DFG) (OL 324/1-1, SZ 814/1-1, 814/4-1); as well as DFG Excellence Cluster "Inflammation at Interfaces" (A.F. and S.Z.). The data reported in this manuscript are tabulated in the main paper and in the supplementary materials.

References

D. Kelly, T. King, R. Aminov, Importance of microbial colonization of the gut in early life to the development of immunity. Mutat. Res. 622, 58 (2007).

H. Renz et al., The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat. Rev. Immunol. 12, 9 (2012).

M. J. Ege, et al., GABRIELA Transregio 22 Study Group, Exposure to environmental microorganisms and childhood asthma. N. Engl. J. Med. 364, 701 (2011).
P. López-Serrano et al., Environmental risk factors in inflammatory bowel diseases. Investigating the hygiene hypothesis: A Spanish case-control study. Scand. J. Gastroenterol. 45, 1464 (2010).
E. von Mutius, Allergies, infections and the hygiene hypothesis—The epidemiological evidence. Immunobiology 212, 433 (2007).
O. Akbariet al., Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat. Med. 9, 582 (2003).
I. J. Fusset al., Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. J. Clin. Invest. 113, 1490 (2004).
N. R. Cohen, S. Garg, M. B. Brenner, Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. Adv. Immunol. 102, 1 (2009).
M. Kronenberg, Toward an understanding of NKT cell biology: Progress and paradoxes. Annu. Rev. Immunol. 26, 877 (2005).
B. Wei et al., Commensal microbiota and CD8+ T cells shape the formation of invariant NKT cells. J. Immunol. 184, 1218 (2010).
F. Heller, I. J. Fuss, E. E. Nieuwenhuis, R. S. Blumberg, W. Strober, Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity 17, 629 (2002).
G. Schiechl et al., Tumor development in murine ulcerative colitis depends on MyD88 signaling of colonic F4/80+CD11b(high)Gr1(low) macrophages. J. Clin. Invest. 121, 1692 (2011).
K. M. Maslowskiet al., Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461, 1282 (2009).
S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, R. Medzhitov, Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118, 229 (2004).
W. Strober, I. J. Fuss, R. S. Blumberg, The immunology of mucosal models of inflammation. Annu. Rev. Immunol. 20, 495 (2002).
J. H. Roark et al., CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. J. Immunol. 160, 3121 (1998).
S. C. Yue, A. Shaulov, R. Wang, S. P. Balk, M. A. Exley, CD1d ligation on human monocytes directly signals rapid NF-κB activation and production of bioactive IL-12. Proc. Natl. Acad. Sci. U.S.A. 102, 11811 (2005).
A. Mizoguchi, A. K. Bhan, A case for regulatory B cells. J. Immunol. 176, 705 (2006).
E. H. Meyer, R. H. DeKruyff, D. T. Umetsu, T cells and NKT cells in the pathogenesis of asthma. Annu. Rev. Med. 59, 281 (2008).
G. Wingender et al., Invariant NKT cells are required for airway inflammation induced by environmental antigens. J. Exp. Med. 208, 1151 (2011).
C. Iwamura, T. Nakayama, Role of NKT cells in allergic asthma. Curr. Opin. Immunol. 22, 807 (2010).
T. Herbst et al., Dysregulation of allergic airway inflammation in the absence of microbial colonization. Am. J. Respir. Crit. Care Med. 184, 198 (2011).
E. Germanov et al., Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. J. Immunol. 181, 81 (2008).
C. Day, R. Patel, C. Guillen, A. J. Wardlaw, The chemokine CXCL16 is highly and constitutively expressed by human bronchial epithelial cells. Exp. Lung Res. 35, 272 (2009).
J. Diegelmann et al., Expression and regulation of the chemokine CXCL16 in Crohn's disease and models of intestinal inflammation. Inflamm. Bowel Dis. 16, 1871 (2010).
V. Rishi et al., CpG methylation of half-CRE sequences creates C/EBPαbinding sites that activate some tissue-specific genes. Proc. Natl. Acad. Sci. U.S.A. 107, 20311 (2010).
M. Pufulete et al., Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. Gut 54, 648 (2005).

C. X. Song et al., Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat. Biotechnol. 29, 68 (2011).
A. S. Neish, Microbes in gastrointestinal health and disease. Gastroenterology 136, 65 (2009).
D. P. Strachan, Hay fever, hygiene, and household size. BMJ 299, 1259 (1989).
S. Y. Shaw et al., Am. J. Gastro. 105, 2687 (2010).
E. Goksör et al., Preschool wheeze—Impact of early fish introduction and neonatal antibiotics. Acta Paediatr. 100, 1561 (2011).
B. A. Sullivan, M. Kronenberg, TCR-mediated recognition of glycolipid CD1 complexes. Curr. Top. Microbiol. Immunol. 314, 165 (2007).
K. A. Sauer, P. Scholtes, R. Karwot, S. Finotto, Isolation of CD4+ T cells from murine lungs: A method to analyze ongoing immune responses in the lung. Nat. Protoc. 1, 2870 (2006).
J. L. Matsuda et al., Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J. Exp. Med. 192, 741 (2000).
R. M. Baronet al., Nitric oxide synthase-2 down-regulates surfactant protein-B expression and enhances endotoxin-induced lung injury in mice. FASEB J. 18, 1276 (2004).
B. Lamprecht et al., Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. Nat. Med. 16, 571 , 1p, 579 (2010).

A5

THE APPLICATION OF ECOLOGICAL THEORY TOWARD AN UNDERSTANDING OF THE HUMAN MICROBIOME[17]

Elizabeth K. Costello,[18] *Keaton Stagaman*,[19] *Les Dethlefsen*,[18,20] *Brendan J. M.* ***Bohannan***,[19] *David A. Relman*[18,20,21,*]

Abstract

The human-microbial ecosystem plays a variety of important roles in human health and disease. Each person can be viewed as an island-like "patch" of habitat occupied by microbial assemblages formed by the fundamental processes of community ecology: dispersal, local diversification, environmental selection, and ecological drift. Community assembly theory, and metacommunity theory in particular, provides a framework for understanding the ecological dynamics of the human microbiome, such as compositional

[17] From "The application of ecological theory toward an understanding of the human microbiome," *Science* 336(6086):1255-1262, 2012. Reprinted with Permission from the AAAS.

[18] Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA.
[19] Institute of Ecology and Evolution, University of Oregon, Eugene, OR 97403, USA.
[20] Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA.
[21] Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA.

* To whom correspondence should be addressed. E-mail: relman@stanford.edu

variability within and between hosts. We explore three core scenarios of human microbiome assembly: development in infants, representing assembly in previously unoccupied habitats; recovery from antibiotics, representing assembly after disturbance; and invasion by pathogens, representing assembly in the context of invasive species. Judicious application of ecological theory may lead to improved strategies for restoring and maintaining the microbiota and the crucial health-associated ecosystem services that it provides.

Each person is an assemblage of not only human cells but also many symbiotic species. The abundant and diverse microbial members of the assemblage play critical roles in the maintenance of human health by liberating nutrients and/or energy from otherwise inaccessible dietary substrates, promoting differentiation of host tissues, stimulating the immune system, and protecting the host from invasion by pathogens. A number of clinical disorders are associated with alterations in host-associated microbial communities (the "microbiota"), including obesity, malnutrition, and a variety of inflammatory diseases of the skin, mouth, and intestinal tract. Thus, the human body can be viewed as an ecosystem, and human health can be construed as a product of ecosystem services delivered in part by the microbiota.

There is growing interest in the use of theoretical methods to study microbial community ecology in general and host-associated microbiota in particular (Mihaljevic, 2012; Prosser et al., 2007). Recent discoveries of unexpected variation in the composition of the microbiome of healthy individuals (Palmer et al., 2007; Ravel et al., 2011; Wu et al., 2011) highlight the importance of identifying the processes that could possibly give rise to such variation. Ecological theory seeks to explain and predict observable phenomena, such as temporal and spatial patterns of diversity. Here, we explore how community assembly theory can be used to understand the human-associated microbiota and its role in health and disease.

Ecological Processes Within Humans

The essential building blocks of community assembly theory encompass the processes that create and shape diversity in local assemblages: dispersal, diversification, environmental selection, and ecological drift (Vellend, 2010). In addition, coevolution provides another lens through which to view the human-microbial ecosystem (Dethlefsen et al., 2007), although in this review we focus on shorter-term dynamics at the level of individual hosts.

Dispersal, or the movement of organisms across space, is a fundamental process by which diversity accumulates in local microbial communities. The concept put forth in the late 19th and early 20th centuries that "everything is everywhere, but the environment selects" had a powerful impact on thinking about community

assembly (O'Malley, 2007), but a more recent appreciation of microbial dispersal limitation suggests that this conceptualization was overly simplistic. Thinking in terms of dispersal leads to a view of the human body as an "island," a patch of habitat that is continually sampling the pool of available colonists. The list of available colonists may be influenced by microbial traits—those affecting dispersal efficiency, transmission routes, and "ex-host" survivability—and by patterns of host contact and carriage, among other factors. Control of infectious disease transmission depends on accurate models of host-to-host microbial dispersal (Koopman, 2004), and these could guide investigations into the dissemination of the human microbiome. Selection favors efficient dispersal in pathogens, but perhaps less so among beneficial bacteria, because the host is harmed by the first and not by the second; for beneficial microbes, transmission routes such as direct or close contact may be more important. The density and spatial arrangement of host habitat patches has been highly dynamic throughout human history, as a result of human migration, urbanization, changes in family living structure, air travel, and other factors.

A second process operating in microbial communities is local diversification. Unlike in most plant and animal communities, this process can take place over short ecological time scales for microbes. Large microbial population sizes, high growth rates, and strong selective regimes, all of which can be found in the human body, result in rapid microbial adaptation via mutation or recombination. Recombination via horizontal gene transfer may be especially common among members of the human microbiota, especially those sharing the same ecological niche (e.g., body site) (Smillie et al., 2011). Microbial diversification may also be driven by interactions with phage in the human body. The processes of dispersal and diversification may interact (Urban et al., 2008); for example, immigration may suppress adaptive radiations (Fukami et al., 2007).

Relative abundances in local communities are shaped over time by a third process: environmental selection. When considering environmental selection, or niche-based interactions, the human body can be viewed in two ways. First, it can be viewed as a "habitat filter," a collection of resources and conditions that allow the growth of some microbes but not others, resulting in the selection of microbial traits that permit survival and growth in the host. In this view, the host shapes the microbiota, but not the other way around. Body temperature is an example of such filtering, because microbes alter body temperature (causing fever) only when they transgress host anatomic boundaries. Second, the human body and its symbionts can be viewed as a community of interacting cells. This view differs from the habitat filter view in that it assumes strong feedbacks between hosts and microbes as well as among microbes. This view assumes that the host shapes the microbiota, and vice versa. Interactions between the host immune system and the microbiota might be best represented by this view (see Hooper et al., 2012). The overall patterns that arise from dispersal and environmental selection can vary as a function of the spatial scale over which these processes occur (Kerr et al., 2002).

In addition to selection-driven changes, species abundances may fluctuate as the result of a fourth process, known as ecological drift or demographic stochasticity. As a result of this process, low-abundance species (e.g., recent immigrants, strains nearly eliminated by antibiotics, or strains occupying niches with low carrying capacity) are more likely to proceed toward local extinction and become lost from the system, unless they have (or can gain) a competitive advantage, access a different niche, or become replenished by dispersal from outside the community. Thus, dispersal can effectively "rescue" species from the brink of local extinction.

Finally, the human habitat can be understood as a host-symbiont "holobiont"—an ecological system under selection to minimize conflict between individual members. This view emphasizes a dominant role of coevolution in the assembly and dynamics of the human ecosystem and reminds us that long- and short-term selective pressures on the human microbiota are not necessarily aligned. Any mutualistic trait that imposes a cost on the microbes that express it—such as producing dedicated molecules to interfere with pathogens or modulate host immune activity—represents a trade-off between the immediate selection against that cost and the long-term selection in favor of mutualism (Dethlefsen et al., 2007).

In summary, different views of humans as microbial habitats make different assumptions about the processes most important to the assembly and dynamics of the human microbiome. Community assembly can be conceptualized as being niche-based, dispersal-limited, historically contingent, or random, depending on the relative contributions of habitat conditions, colonist availability, arrival order (and timing), or chance-driven events, respectively, in shaping observed patterns (Figure A5-1). Metacommunity theory integrates the four processes described above and provides a useful framework for considering community assembly in the human body (Vellend, 2010).

Metacommunity Theory and the Human Microbiome

Various theoretical frameworks are used to study community assembly; one key framework is neutral theory (*Hubbell*, 2001), in which it is assumed that dispersal, diversification, and ecological drift are purely chance-driven processes. It is a neutral model because it invokes neither environmental selection nor inherent differences in species' ability to disperse or diversify. Although neutral theory on its own is quite valuable in testing this null hypothesis, a broader framework for the assembly of the human microbiome might accommodate alternative theories and combine the strengths of transmission dynamic models (e.g., inclusion of host contact and carriage dynamics) with those of community ecology (e.g., focus on communities rather than individual pathogens). One such approach is metacommunity theory (Leibold et al., 2004), which could be especially useful for modeling host-associated communities.

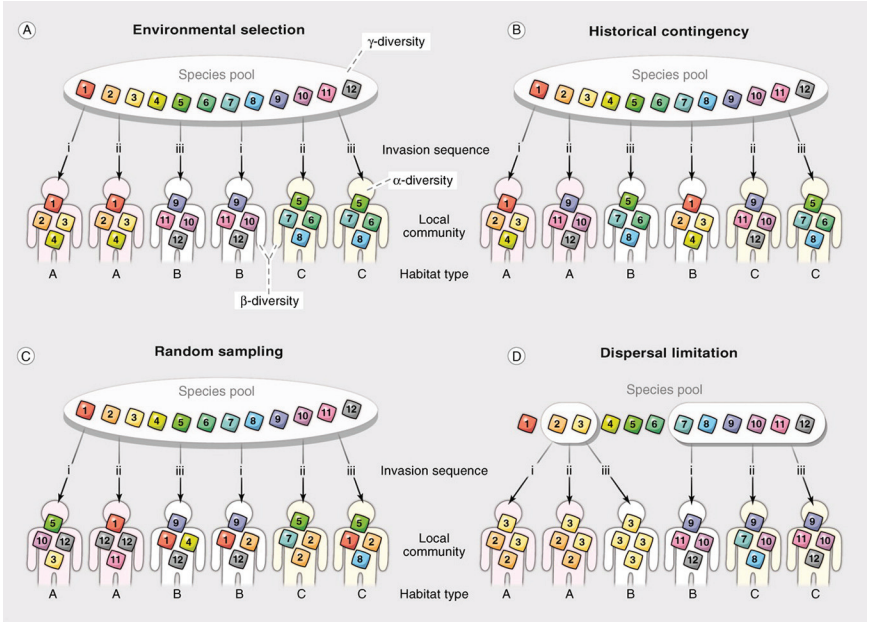


FIGURE A5-1 Alternative community assembly scenarios could give rise to the compositional variations observed in the human microbiota. Each panel shows the assembly of local communities in different habitat types from a pool of available species. (A to C) Each local community has access to all available colonists, but the order of invasion varies. In (A), local species composition is determined primarily by environmental selection: Regardless of invasion order, habitats with initially similar conditions select for similar assemblages. In (B), the opposite is true: Regardless of initial habitat conditions, historical contingencies (i.e., differences in the timing and order of species invasions) determine assemblage composition. In (C), neither habitat nor history matter: Local communities assemble via random draws from the species pool. (D) Dispersal barriers result in local communities that assemble from different species pools. For each of the pools, local communities may assemble as in (A), (B), or (C). The meaning of three different diversity measures is shown in (A): γ-diversity refers to the "regional" species pool (i.e., the total diversity of the local communities connected via dispersal); β-diversity refers to the differences between local communities (species turnover); and α-diversity refers to the diversity within a local community. Although multiple scenarios are likely to apply to any real-world setting, one may dominate. For example, differences between body habitats may be best explained by environmental selection, differences between siblings for the same habitat may be best explained by historical contingency, differences between monozygotic twins prior to weaning highlight the role of stochasticity, and differences between neonates born by cesarean section versus vaginal delivery are likely to be explained by dispersal limitation. [Adapted from (Chase, 2003; Fukami, 2010)].

Metacommunity theory views the world as a collection of patches—spatially distinct areas of suitable habitat surrounded by a matrix of unsuitable habitat. These patches each contain a community of organisms, and these spatially distinct communities are connected together to form a metacommunity by the dispersal of organisms from patch to patch. Human populations can be viewed likewise, with host-to-host dispersal linking microbial communities. Metacommunity theory is especially helpful for understanding the relative importance of dispersal and environmental selection in shaping host-associated communities (*Mihaljevic, 2012*), an issue that has received relatively little attention in studies of the human microbiome.

The predictions of metacommunity theory depend on the frequency and extent of dispersal, differences in the traits of individual organisms, and the degree to which patches vary in their environmental conditions (Ellis et al., 2006; Leibold et al., 2004). Dispersal can be infrequent and localized, or widespread and frequent, as discussed above. In some metacommunity models, patches are assumed to be environmentally identical, with movement among patches determining variation in community membership. Such models might be especially appropriate for populations of closely related hosts. Other models assume that patches vary strongly in their available niches, and that variation in community membership results at least in part from environmental selection (e.g., underpinned by host genetics or diet).

Metacommunity theory enables one to predict the conditions under which community dynamics within a patch are driven by immigration from outside versus local adaptation (Urban et al., 2008). Low dispersal rates favor adaptation within a patch, whereas high dispersal favors immigration. This concept could be useful, for example, in understanding responses to antibiotic use. If acquisition of antibiotic resistance were primarily a result of immigration, then interventions focused on quarantine and hygiene would be more effective than those focused on altering antibiotic duration or dose (see below).

Metacommunity theory has been used to elucidate the drivers of non–host-associated microbial community membership and dynamics (Logue et al., 2011; Ofiteru et al., 2010; Van der Gucht et al., 2007), but it has rarely been used to study host-associated communities (Hovatter et al., 2011; Sloan et al., 2006)—for example, to explore the stringency of host selection and its dependence on the microbial group or the age of the host. Ultimately this information will result in a better understanding of how microbes are "filtered" by the host and, conversely, how microbes evade this filtering. This information is crucial if clinicians are to directly manipulate host-associated communities—for example, by designing probiotics that can evade host filtering and establish within a host.

The effective application of metacommunity theory (and assembly theory in general) to the human microbiome requires a preliminary understanding of how the microbiome varies across hosts and over time. We next review our current understanding of this variation, focusing on the dynamics of communities in

newly created habitats (e.g., neonatal colonization), after disturbance (e.g., after antibiotic treatment), and after invasion (e.g., by a pathogen). We chose these scenarios because they represent and reveal the fundamental types of assembly relevant to human health.

Postnatal Acquisition and Development of the Human Microbiome

Babies are born essentially sterile and acquire their microbiome from their surroundings. The postnatal assembly of the human microbiota plays an important role in infant health, providing resistance to pathogen invasion, immune stimulation, and other important developmental cues early in life (Mackie et al., 1999). Acute and chronic disorders, such as necrotizing enterocolitis, antibiotic-associated diarrhea, malnutrition, inflammatory bowel disease, and asthma have been linked to inadequate, inappropriate, or disrupted postnatal microbiome acquisition and development (Murgas and Neu, 2011). Mechanisms controlling the appearance of bacteria in healthy infants have been studied for well over a century (Escherich, 1988), with microbiome development having been likened to ecological succession (Mackie et al., 1999; Savage, 1977). The view of succession as a mode of community assembly has largely emphasized niche-based processes, but the importance of stochastic and/or historical events has also long been recognized.

In the absence of microbial invasion of the amniotic cavity, which is thought to be a rare, pathologic condition, rupture of membranes signals the moment when microbes, most likely of maternal vaginal origin, first gain access to the neonate. Vaginally delivered infants clearly receive a strong input of vaginal and possibly other urogenital or fecal microbiota as they pass through and exit the birth canal (Dominguez-Bello et al., 2010; Mändar and Mikelsaar, 1996). Vaginal microbiome composition in nonpregnant, reproductive-age women is highly dynamic and is characterized by at least five compositional classes delineated by different, dominant *Lactobacillus* species or a lack of *Lactobacillus* dominance. There is frequent class switching over time, including switching to and from compositions indicative of bacterial vaginosis, even in the absence of symptoms (Brotman et al., 2010; Gajer et al., 2012; Ravel et al., 2011). Whether these dynamics occur similarly in pregnant and postpartum women has important implications for the initial colonization of vaginally delivered infants; if they do, infant-to-infant variation in the composition of initial colonists may be imposed in some cases by maternal vaginal microbiome class at the time of delivery. Likewise, maternal gut microbiome types (Arumugam et al., 2011; Wu et al., 2011) may also determine the pool of colonists available to vaginally delivered infants at birth. Thus, variation among neonate microbiomes may reflect variation in maternal microbiomes, but this has not been widely tested for maternal habitats other than the vagina. At the time of delivery, microbiomes do not differ consistently among infant body sites (Dominguez-Bello et al., 2010), which implies

that sampling is driving initial community assembly, with minimal filtering by the infant host.

Delivery mode also determines microbial exposure at the time of birth. For example, infants delivered by cesarean section do not receive contributions from the vaginal microbiota, and instead are exposed initially to what appears to be ambient skin microbiota (Dominguez-Bello et al., 2010). Incidental exposures to maternal (or other) gut or vaginal microbiota may occur later in cesarean section infants, at low density or low frequency, but may be inadequate for outcompeting already established strains. For example, cesarean section infants display reduced abundances and/or incidences of colonization by the genera *Bacteroides* and *Bifidobacterium* early in development relative to infants born by vaginal delivery (Bennet and Nord, 1987; Penders et al., 2006). The effects of delivery mode can persist for months and may have consequences for infant health; cesarean section infants have a higher risk for some immune-mediated diseases (Kuitunen et al., 2009; van Nimwegen et al., 2011). The ambient environment may also play a role in colonization at delivery; infants delivered at home versus the hospital were colonized differently at 1 month of age (Penders et al., 2006). Thus, dispersal limitation imposed by certain medical interventions may contribute to interindividual variation early in life.

Over the first few months—roughly up until the first solid foods are introduced—a well-constrained range of stereotypical bacteria appear in the feces (distal gut), diversity generally increases, and aerobes are thought to be supplanted by facultative and then strict anaerobes (Mackie et al., 1999). Exclusive breast-feeding selects for increased abundance of particular *Bifidobacterium* species whose genome sequences reflect specialized use of human milk oligosaccharides and similar host-derived substrates (Sela et al., 2008), or for other bacteria such as *Bacteroides* that could compete for the same ecological niche (Marcobal et al., 2010). Strikingly, during this early phase, microbiota composition is highly dynamic within and between infants (Favier et al., 2002; Koenig et al., 2011; Mackie et al., 1999; Palmer et al., 2007; Trosvik et al., 2010), with temporal variation characterized by periods of relative stability (for varying lengths of time) punctuated by abrupt shifts in composition and structure. In some cases, these shifts can be linked with life events that likely impose environmental selection, such as fever, formula feeding, or antibiotic therapy (Koenig et al., 2011; Palmer et al., 2007; Savino et al., 2011). Extraordinarily parallel transitions observed in a pair of dizygotic twins suggest that stochastic exposures (shared exposures in their case) can also play an important role during this phase, driving within- and between-infant variation (Palmer et al., 2007). This finding underscores the need to better understand how infants sample their environment over time (e.g., whether outside-of-host environmental reservoirs, or host exchange scenarios—either direct or indirect, such as via fomites—prevail) and with regard to the frequency and extent of dispersal (as discussed above). Abrupt shifts might reflect opportunistic invasions by better-adapted species or changes in filtering by

the host. An infant's unique developmental path through this early unstable phase may have longer-term health implications. For example, recent work has shown that colonization during the neonatal period has a particularly important effect on mucosal immune development (Olszak et al., 2012).

The introduction of solid foods and weaning are associated with the onset of a transition toward an adult-like gut microbiome. Differences attributable to early exposures such as delivery mode fade as microbiota compositions become more canalized. Life events such as illness, diet modification, and antibiotic therapy can still impose disturbances, although specific compositions appear to recover. Taxa characteristic of the adult eventually establish, but the process of microbial community assembly appears to extend past the first year of life and into childhood (Palmer et al., 2007; Koenig et al., 2011). If there is an imprint of microbial flow from parents to children, it is difficult to detect at early ages and/or emerges gradually later in life. In one study, fecal patterns of bacterial taxonomic diversity in 1-year-olds were not found to be more similar to those of their parents than to those of unrelated adults (Palmer et al., 2007); but in another study, patterns of microbial diversity in adult twins were slightly more similar to those of their mother (Turnbaugh et al., 2009). These findings suggest that we acquire microbes from sources other than, or in addition to, our family members. Further, there may be strong selection for an individualized microbiota. Describing the adult state as "stable" may not suffice when stability is defined as the permanent coexistence of locally occurring species, because even adult gut composition appears to change over time (Caporaso et al., 2011). In summary, microbiome assembly in newly created habitats likely involves a gradual shift from conditions under the strong influence of dispersal limitation (as well as stochastic and/or historical factors) toward conditions increasingly influenced by environmental selection (e.g., by diet), with weaning as a strong catalyst, and with development toward adult-like composition continuing into childhood.

Community Assembly After Disturbance: Antibiotics as a Paradigm

The assembly of human-associated microbial communities does not, in general, proceed smoothly to a stable climax state, which then resists further changes in composition. Disturbances often remove or kill some fraction of the community, providing an opportunity for remaining community members or new colonists to increase in abundance. For example, personal oral hygiene removes bacterial biofilm from teeth, and antibiotics affect not only the targeted pathogen but also members of the normal microbiota (Figure A5-2A). The former case represents a deliberate attempt to interrupt the development of microbial communities that might be associated with periodontitis; the latter case is an inadvertent consequence of medical intervention. In addition to directly inducing a shift in the community state, disturbance may also involve a change in the community's habitat—for example, as the result of a change in host diet (Figure A5-2B). In

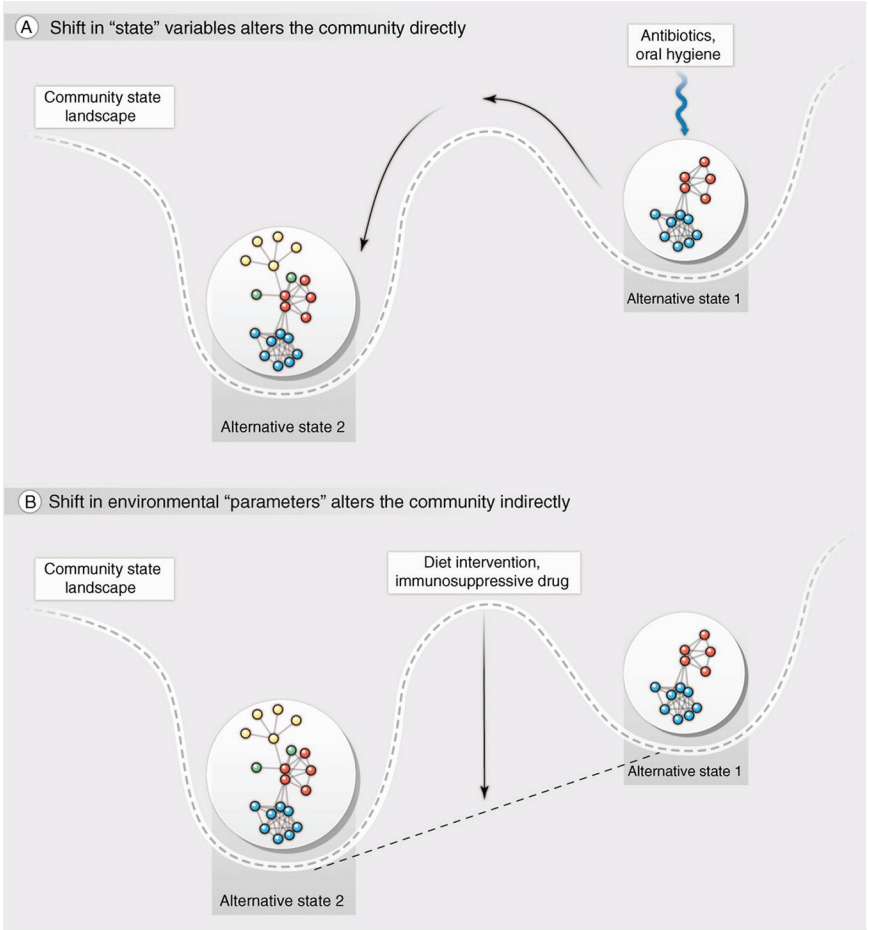


FIGURE A5-2 Disturbance can be illustrated using a stability landscape schematic. The ball represents the community; the changing horizontal position of the ball represents the changing community state. The depth of a basin indicates the likelihood of a community remaining in that basin despite frequent "buffeting" by minor disturbance (Walker et al., 2004) and hence the relative stability of the community state. Disturbance can alter the community directly (A) by changing its composition or activity, or indirectly (B) by changing the environmental parameters. In either case, the community can shift to an alternative state. In reality, continuous feedback between the community and its environment means that they change together. See (Lemon et al., 2012) for applications to therapy.

many cases, a crucial unknown is resilience—that is, the degree to which the post-disturbance community returns to its former state (Walker et al., 2004). Although most work on resilience has considered resilience in terms of taxonomic composition, assessment of function and ecosystem services may be even more important.

The effect of antibiotics on the gut microbiota serves as a paradigm for disturbances in human-associated communities. Antibiotics are now one of the most common and important forms of disturbance of the human microbiota; on any given day, approximately 1 to 3% of people in the developed world are exposed to pharmacologic doses of antibiotics (Goossens et al., 2005). Over the past several decades there has been increasing concern about the spread of antibiotic resistance among pathogens, as well as growing concern that antibiotic use may disrupt the host-microbe interactions that contribute to human health.

Antibiotic therapy is meant to achieve a sufficient concentration of the drug for a sufficient duration in a particular body site so that the targeted pathogen is eliminated. Even if this aim were always attained, the antibiotic will be found at a range of concentrations at many locations in the body, depending on the mode of administration and its pharmacodynamic properties. Where members of the indigenous microbiota are exposed to antibiotics that affect their growth without killing them, there is selection for resistance. Human gut and oral communities are recognized as reservoirs for the evolution and horizontal transfer of antibiotic resistance determinants, including to pathogens (Roberts and Mullany, 2010; Salyers et al., 2004; Smillie et al., 2011). However, antibiotic resistance among the microbiota is one of several mechanisms that may act to enhance the resilience of indigenous communities, hence preserving their beneficial ecosystem services. Others may include population-level resistance via stress-response signaling (Lee et al., 2010) and the existence of dormant persister cells (Vega et al., 2012) or refugium-like locations (e.g., mucus layer).

Only a handful of studies have used cultivation-independent surveys to examine the consequences of therapeutic doses of antibiotics on the human gut microbiota (e.g., Dethlefsen et al., 2008; Dethlefsen and Relman, 2011; Jernberg et al., 2007; Jakobsson et al., 2010; Vega et al., 2012; Young and Schmidt, 2004). These studies—although they examined different antibiotics by means of various sampling strategies, treatment durations, and analytical approaches—all have found that antibiotics alter the composition of the gut microbiota, and that the abundance of most taxa begins to return to prior levels within several weeks. However, the studies are also consistent in showing that various taxa recover to different extents and that some do not recover over the duration of the study. The antibiotic effect is greater than the routine temporal variability of community composition (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011; Jernberg et al., 2007). Some studies have revealed that the composition of strains within a taxon is sometimes altered, even if the relative abundance of the taxon as a whole has returned to pre-antibiotic levels. In both of the studies that involved measurements of the prevalence of antibiotic-resistant strains, elevated levels of

resistance persisted to the end of the study (Jernberg et al., 2007; Jakobsson et al., 2010).

Overall, research suggests that the human gut microbiota of generally healthy adults is largely, but not entirely, resilient to short courses of antibiotic therapy, whereas clinical evidence indicates that extended or repeated courses are more likely to result in serious complications such as the invasion and bloom of *Clostridium difficile* (Owens et al., 2008). Perhaps over short courses of antibiotics, a sufficient, although possibly quite small, number of residual cells from most of the large, preexisting populations survives to recolonize the gut. An increasing number of these residual cells may be lost with longer or repeated courses of antibiotics. Thus, reassembly of the microbial community after extended antibiotic treatment may require colonization from outside the host—a process that would likely be more variable and require a longer period of time than reassembly via the filtering of existing populations in the host. In addition, the microbiome may be highly vulnerable to invasion by (and/or blooms of) pathogens during recovery after disturbance, because resources are in high abundance and resident populations are diminished. The longer recovery time required after extended antibiotic treatment could lead to a higher probability of invasion by pathogenic strains. One can envision a more enlightened strategy for the clinical use of antibiotics that includes pretreatment estimates of a patient's microbial community resilience, based on the use of a standardized disturbance and monitoring of key community products, mapping of the community stability landscape, and assessment of the likelihood for community displacement and adoption of a disadvantageous, altered state. Assessments of elevated risk, or of loss of resilience, might then prompt efforts at restoration (see Lemon et al., 2012).

Little is known regarding the response of the microbiome to frequent antibiotic use. When disturbances take place with a magnitude or frequency beyond what a community has had an opportunity to adapt to, ecological surprises may occur (Paine et al., 1998). Such frequent disturbances may allow the persistence of microbial taxa that are inferior competitors within a given host but are nonetheless maintained across hosts because they have traits that result in widespread and frequent dispersal (i.e., "fugitive" taxa). Such a scenario is analogous to the patch dynamics paradigm of metacommunity theory (Leibold et al., 2004).

Assembly of the Human Microbiome in the Context of Invaders (Pathogens)

It is naïve to consider only host and pathogen when predicting the likelihood of microbial disease. Host-associated microbial communities play an important role in preventing disease, and although outside the scope of this discussion, they may also promote pathology, as in the case of inflammatory bowel disease (Elinav et al., 2011). It may be useful to view some pathogens as invasive species, and to view the consequences of invasion as a special case of community assembly. As

with invasive species in more traditionally studied settings, the ability of a species to invade a particular community depends largely on niche opportunities—that is, the filters imposed by the abiotic environment and the resistance of the community to colonization by an exotic species (Shea and Chesson, 2002). A successful invasion involves dispersal of an invader to a new community, initial colonization, and proliferation; these steps are influenced by the same processes as in community assembly more generally.

The environment created by the host determines the number of potential niche opportunities. The nature of this environment is influenced by a number of conditions, including "abiotic" factors (such as oxygen levels, pH, and temperature) as well as the abundance and types of available resources (such as the composition of the host's diet) (Turnbaugh et al., 2006) and carbon sources provided directly by the host (such as mucosal poly- and oligosaccharides) (Sonnenburg et al., 2004). In addition, the host immune system acts as an important environmental filter to limit the spatial extent of the microbiota's available niches. The main functions of the mucosal immune system are to create an inhospitable buffer zone between the microbiota and the host epithelium, and to minimize the incidence of systemic inflammation that would normally be induced in the face of so many bacterial products (Duerkop et al., 2009; Hooper et al., 2012; Macpherson et al., 2012). The immune system performs these functions through three general mechanisms: (i) physical barriers such as the inner mucus layer of the colon and stomach, which is generally impenetrable to bacterial cells (Johansson et al., 2008); (ii) antimicrobial peptides and mucosal antibodies in the mucus layer that further hinder bacterial colonization of the epithelium (Duerkop et al., 2009); and (iii) innate and adaptive immune responses within the regional lymphatic tissues (Macpherson et al., 2012). These three mechanisms, in most healthy hosts, select for bacterial species that do well at or near mucosal surfaces or strong barriers such as the skin. However, host filtering is not the only factor influencing the ability of pathogens to invade the host-microbiota community.

One of the most important roles of the microbiota in mediating host-pathogen interactions is protection of the host from pathogen invasion, or "colonization resistance" (Duerkop et al., 2009; Sekirov and Finlay, 2009). Protection is achieved through induction of the innate and adaptive branches of the immune system, creating an environment that is unfavorable to pathogens (as illustrated by the observation that axenic mice [Macpherson et al., 2004] and zebrafish [Kanther and Rawls, 2010] have diminished immune responses and impaired barriers to infection), and through direct competition (or community filtering, e.g., by lowering vaginal pH). In the latter case, pathogens are kept at bay by competition with the microbiota for space and resources. This protective effect is demonstrated by the increased susceptibility to infection of hosts that have had their microbiota altered by antibiotics, a phenomenon well documented by Miller and Bohnhoff in the early 1960s with *Salmonella* invasion of mice pretreated with antibiotics (Bohnhoff and Miller, 1962). The ability of certain anaerobes to limit the invasion

and growth of *Clostridium perfringens* in a diet-dependent manner is an example of competition for resources (Yurdusev et al., 1989). *Bifidobacterium breve* produces an exopolysaccharide that protects it from the immune response; this allows it to compete for space and colonize the mouse gut at high loads both in the lumen and at the epithelial surface without inducing inflammation (Fanning et al., 2012). Even if invaders do gain a foothold, the indigenous microbiota can block lethality: In mice, some *B. longum* strains can protect against enterohemorrhagic *E. coli*–mediated death by inhibiting translocation of Shiga toxin from lumen to blood (Fukuda et al., 2011).

By viewing pathogens as invasive species, we see that the contexts in which they are able to cause disease are the same as those required for any other species that invades and proliferates in a community. Niche opportunities can result from exploiting novel or excess resources (from the host's food), outcompeting a commensal species for the same resource, or, perhaps most important, exploiting niches left open after a disturbance. The importance of exploiting disturbance is well illustrated by the increasing number of cases of disease caused by *C. difficile* (Kelly and LaMont, 2008), which is a "weedy" exotic (or native) species that can rapidly fill niches once they are vacant but, in most cases, is eventually removed or kept at low abundance in the absence of disturbance. *Salmonella enterica* serovar Typhimurium is an example of an exotic invasive that exploits disturbance, but in this case, it also causes the disturbance it exploits. *S*. Typhimurium expresses many virulence factors that induce inflammation in the mammalian intestine. A mutant *S*. Typhimurium strain lacking these virulence factors is unable to invade the gut community and cause disease; however, if inflammation is provided by some other mechanism, avirulent strains are able to invade the host communities (Stecher et al., 2007). Inflammation likely reduces the abundances of other bacteria that would compete with pro-inflammatory pathogens for space. As one possible mechanism, inflammation causes the intestine to produce tetrathionate, which *S*. Typhimurium uses as an electron acceptor for respiring ethanolamine, a carbon source that cannot be exploited by other bacteria, thus avoiding competition for nutrients (Thiennimitr et al., 2011). By causing acute inflammation, the pathogen is able to alter the native microbiota and effectively colonize and proliferate.

In contrast to the above examples, a reduction in disturbance frequency can also promote invasion by pathogens. Patients with cystic fibrosis produce thickened mucus, which inhibits the ability of the cilia to remove foreign material from normally sterile lung airways. This lack of constant removal (i.e., impaired innate immune host-filtering mechanism), among other factors, allows for the establishment of bacterial communities that would normally not be able to persist at that site (Klepac-Ceraj et al., 2010).

In summary, predicting the success and outcome of infection by pathogens can be aided by framing the issue as an ecological problem of community assembly. Invasion ecology highlights the importance of niche opportunities as

determinants of success of invasion, and the manipulation of which might help in pathogen control and disease prevention. Experimental models using gnotobiotic organisms such as mice and zebrafish will be helpful in understanding the role of community diversity, as well as the role of particular community members in conferring colonization resistance through indirect inhibition and resource competition. In addition, the frequency and magnitude of disturbance plays a crucial role in facilitating colonization by exotic invasives as well as the expansion of native species. Finding ways, through prebiotics, probiotics, or pharmabiotics, to alter pathogen or other bacterial species abundances (or to inhibit their detrimental effects on the host) in a specific manner without causing additional disturbance to the community will be very important for preventing and treating disease caused by invasive species.

Translating Ecological Understanding into Clinical Practice

An improved understanding, informed by ecological theory, of how microbiomes assemble could alter clinical practice by changing the perspective clinicians bring to the treatment of infectious disease and chronic inflammatory disorders. The traditional perspective has been to think of the human body as a battleground on which physicians attack pathogens with increasing force, occasionally having to resort to a scorched-earth approach to rid a body of disease. Although this perspective has been very successful for several diseases, it has come at a great cost. Even for those diseases for which it has worked, the collateral damage can be severe. As we have discussed, antibiotics often kill more than the target organisms (Dethlefsen and Relman, 2011) and can increase the chance of invasion by unwanted organisms (such as *C. difficile* [Kelly and LaMont, 2008]).

The body-as-battleground approach ignores the community context of infectious disease and chronic inflammation, and does not take into account our increasing knowledge regarding the assembly of the human microbiome. We suggest that it is time for clinicians to abandon the war metaphor (Lederberg, 2000). Given the ecological parallels between assembly of the human microbiome and assembly of other ecological communities, we suggest that human medicine has more in common with park management than it does with battlefield strategy. To effectively manage a plant or animal community requires a multipronged approach of habitat restoration, promotion of native species, and targeted removal of invasives. We describe below some examples of how such a human-as-habitat approach might alter clinical practice.

An Ecological Approach to Managing Invasions

An understanding of the dominant mechanisms of community assembly could directly alter how clinicians treat disease. Consider, for example, the rise of drug-resistant pathogens during the course of drug treatment. We can consider

this a "special case" of community assembly, much as we did invasion by a pathogen (see above). We can ask: What is the relative importance of dispersal, diversification, environmental selection, and ecological drift in the successful invasion by this drug-resistant strain? If the source of these strains is primarily through random sampling of the external environment, then the most effective preventive strategy may be quarantine and enhanced hygiene. In contrast, if such strains arise primarily through diversification of resident taxa, then multidrug treatment may be more effective (to make successful evolution more difficult). If the drug-resistant strains are already present at the outset of treatment and increase in abundance via environmental selection, then drug cycling may be the most effective treatment to reduce the overall competitive advantage of the resistant strains. If drug-resistant strains establish primarily through ecological drift, then disturbance may be crucial to their establishment (freeing up ecological "space" for their invasion). In this case, reducing disturbance of the resident microbiota may be most effective. In this way, a detailed understanding of the relative importance of different community assembly processes can be used to tailor the treatment of disease.

Health as a Product of Ecosystem Services

Humans benefit from a variety of processes supplied by natural ecosystems. Collectively these benefits are known as ecosystem services (Daily et al., 1997). There is growing evidence that human health is a collective property of the human body and its associated microbiome, and thus could be considered a net effect of ecosystem services. We envision clinical medicine focused on managing the human body and its associated microbiome to preserve these ecosystem services. How might this be accomplished? In general ecology, the management of an ecosystem service requires four basic steps (Allan and Stankey, 2009): (i) identification of ecosystem service providers (ESPs; taxa that provide specific ecosystem services) and characterization of their functional roles; (ii) determination of how community context influences the function of these providers; (iii) assessment of key environmental factors influencing the provision of services; and (iv) measurement of the spatial and temporal scales at which these providers and their functions operate. This general framework would work equally well for human health-associated ecosystem services. If studies of the human microbiome were structured around these four priorities, the development of an ecological approach to medicine could be accelerated. Progress has been made in identifying ESPs ("biomarkers"; see Lemon et al., 2012); for example, declines in *Faecalibacterium prausnitzii* are associated with inflammatory bowel disease, and this organism may be an ESP for health in the human gut (Sokol et al., 2008).

Adaptive Management of the Human Body

Transitioning clinical practice from the body-as-battleground to the human-as-habitat perspective will require rethinking how one manages the human body. In the management of plant and animal communities, a system-level approach known as "adaptive management" has become popular. This approach is a structured, iterative process of decision making, one that uses system monitoring to continually update management decisions (Allan and Stankey, 2009). It has been successfully used to manage biodiversity in a variety of habitats, including communities in highly disturbed environments affected by overfishing and by climate change (Allan and Stankey, 2009). For the human body, we envision that this approach would involve monitoring of the microbiome during health, to establish a healthy baseline, with more intensive monitoring during disease and treatment. This will require the development of new diagnostic tools that are both accurate and sufficiently rapid to inform decisions regarding therapeutics [see Lemon et al., 2012). Such diagnostics are not yet feasible, but given recent advances in our ability to survey the human microbiome, this possibility is not far in the future, especially if we are able to identify particular components of the human microbiome that contribute disproportionately to the maintenance of human health. An adaptive management approach to clinical medicine provides a wonderful example of personalized medicine, with treatments tailored to individuals on the basis of diagnostic changes in an individual's microbiome, and continually adjusted through regular monitoring. Such an information-intensive approach, guided by ecological theory, has the potential to revolutionize the treatment of disease.

Acknowledgments

We thank members of the Relman and Bohannan laboratories, and in particular E. Bik, N. Qvit-Raz, G. Schmid, and K. Shelef (Stanford), and K. Guillemin, J. Green, Z. Stephens, and A. Burns (Oregon) for valuable ideas and critique. E.K.C. is a Walter V. and Idun Berry Postdoctoral Fellow. B.J.M.B. is supported by NIH grant R01GM095385. D.A.R. is supported by a Distinguished Clinical Scientist Award from the Doris Duke Charitable Trust, NIH Pioneer Award DP1OD000964, and the Thomas C. and Joan M. Merigan Endowment at Stanford University. There are no conflicts of interest.

References

J. R. Mihaljevic, Linking metacommunity theory and symbiont evolutionary ecology. Trends Ecol. Evol. 10.1016/j.tree.2012.01.011 (2012). 10.1038/nrmicro1643

J. I. Prosser et al., The role of ecological theory in microbial ecology. Nat. Rev. Microbiol. 5, 384 (2007).

C. Palmer, E. M. Bik, D. B. DiGiulio, D. A. Relman, P. O. Brown, Development of the human infant intestinal microbiota. PLoS Biol. 5, e177 (2007).

J. Ravel et al., Vaginal microbiome of reproductive-age women. Proc. Natl. Acad. Sci. U.S.A. 108 (suppl. 1), 4680 (2011).
G. D. Wu et al., Linking long-term dietary patterns with gut microbial enterotypes. Science 334, 105 (2011).
M. Vellend, Conceptual synthesis in community ecology. Q. Rev. Biol. 85, 183 (2010).
L. Dethlefsen, M. McFall-Ngai, D. A. Relman, An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811 (2007).
M. A. O'Malley, The nineteenth century roots of 'everything is everywhere'. Nat. Rev. Microbiol. 5, 647 (2007).
J. Koopman, Modeling infection transmission. Annu. Rev. Public Health 25, 303 (2004).
C. S. Smillie et al., Ecology drives a global network of gene exchange connecting the human microbiome. Nature 480, 241 (2011).
M. C. Urban et al., The evolutionary ecology of metacommunities. Trends Ecol. Evol. 23, 311 (2008).
T. Fukami, H. J. Beaumont, X. X. Zhang, P. B. Rainey, Immigration history controls diversification in experimental adaptive radiation. Nature 446, 436 (2007).
L. V. Hooper, D. R. Littman, A. J. Macpherson, Interactions between the microbiota and the immune system. Science 336, 1268 (2012). 10.1038/nature00823
B. Kerr, M. A. Riley, M. W. Feldman, B. J. Bohannan, Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418, 171 (2002).
S. P. Hubbell, *The Unified Neutral Theory of Biodiversity and Biogeography* (Princeton Univ. Press, Princeton, NJ, 2001).
M. A. Leibold et al., The metacommunity concept: A framework for multi-scale community ecology. Ecol. Lett. 7, 601 (2004). 10.1111/j.1461
A. M. Ellis, L. P. Lounibos, M. Holyoak, Evaluating the long-term metacommunity dynamics of tree hole mosquitoes. Ecology 87, 2582 (2006). 10.1890/0012-9658(2006)87[2582
J. B. Logue, N. Mouquet, H. Peter, H. Hillebrand, Metacommunity Working Group, Empirical approaches to metacommunities: A review and comparison with theory. Trends Ecol. Evol. 26, 482 (2011).
I. D. Ofiteru et al., Combined niche and neutral effects in a microbial wastewater treatment community. Proc. Natl. Acad. Sci. U.S.A. 107, 15345 (2010).
K. Van der Gucht et al., The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. Proc. Natl. Acad. Sci. U.S.A. 104, 20404 (2007).
S. R. Hovatter, C. Dejelo, A. L. Case, C. B. Blackwood, Metacommunity organization of soil microorganisms depends on habitat defined by presence of *Lobelia siphilitica* plants. Ecology 92, 57 (2011). 10.1890/10
W. T. Sloan et al., Quantifying the roles of immigration and chance in shaping prokaryote community structure. Environ. Microbiol. 8, 732 (2006). 10.1111/j.1462
R. I. Mackie, A. Sghir, H. R. Gaskins, Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. 69, 1035S (1999). 10232646
R. Murgas Torrazza, J. Neu, The developing intestinal microbiome and its relationship to health and disease in the neonate. J. Perinatol. 31 (suppl. 1), S29 (2011).
Th. Escherich, The intestinal bacteria of the neonate and breast-fed infant. Rev. Infect. Dis. 10, 1220 (1988).
D. C. Savage, Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. 31, 107 (1977).
M. G. Dominguez-Bello et al., Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U.S.A. 107, 11971 (2010).
R. Mändar, M. Mikelsaar, Transmission of mother's microflora to the newborn at birth. Biol. Neonate 69, 30 (1996).
R. M. Brotman, J. Ravel, R. A. Cone, J. M. Zenilman, Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. Sex. Transm. Infect. 86, 297 (2010).

P. Gajer et al., Temporal dynamics of the human vaginal microbiota. Sci. Transl. Med. 4, 132ra52 (2012). 10.1136/sti.2009.040592

M. Arumugam et al., MetaHIT Consortium, Enterotypes of the human gut microbiome. Nature 473, 174 (2011).

R. Bennet, C. E. Nord, Development of the faecal anaerobic microflora after caesarean section and treatment with antibiotics in newborn infants. Infection 15, 332 (1987).

J. Penders et al., Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511 (2006). 10.1542/peds.2005

M. Kuitunen et al., Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. J. Allergy Clin. Immunol. 123, 335 (2009).

F. A. van Nimwegen et al., Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. J. Allergy Clin. Immunol. 128, 948 , e1 (2011).

D. A. Sela et al., The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. Proc. Natl. Acad. Sci. U.S.A. 105, 18964 (2008).

A. Marcobal et al., Consumption of human milk oligosaccharides by gut-related microbes. J. Agric. Food Chem. 58, 5334 (2010).

C. F. Favier, E. E. Vaughan, W. M. De Vos, A. D. Akkermans, Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. 68, 219 (2002). 10.1128/AEM.68.1.219

J. E. Koenig et al., Succession of microbial consortia in the developing infant gut microbiome. Proc. Natl. Acad. Sci. U.S.A. 108 (suppl. 1), 4578 (2011).

P. Trosvik, N. C. Stenseth, K. Rudi, Convergent temporal dynamics of the human infant gut microbiota. ISME J. 4, 151 (2010).

F. Savino et al., Faecal microbiota in breast-fed infants after antibiotic therapy. Acta Paediatr. 100, 75 (2011). 10.1111/j.1651

T. Olszak et al., Microbial exposure during early life has persistent effects on natural killer T cell function. Science 336, 489 (2012).

P. J. Turnbaugh et al., A core gut microbiome in obese and lean twins. Nature 457, 480 (2009).

J. G. Caporaso et al., Moving pictures of the human microbiome. Genome Biol. 12, R50 (2011). 10.1186/gb-2011-12-5

B. Walker, C. S. Holling, S. R. Carpenter, A. Kinzig, Resilience, adaptability and transformability in social-ecological systems. Ecol. Soc. 9, 5 (2004). 10.1186/gb-2011-12-5

H. Goossens, M. Ferech, R. Vander Stichele, M. Elseviers, ESAC Project Group, Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. Lancet 365, 579 (2005). 15708101

A. P. Roberts, P. Mullany, Oral biofilms: A reservoir of transferable, bacterial, antimicrobial resistance. Expert Rev. Anti Infect. Ther. 8, 1441 (2010).

A. A. Salyers, A. Gupta, Y. Wang, Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 12, 412 (2004).

H. H. Lee, M. N. Molla, C. R. Cantor, J. J. Collins, Bacterial charity work leads to population-wide resistance. Nature 467, 82 (2010).

N. M. Vega, K. R. Allison, A. S. Khalil, J. J. Collins, Signaling-mediated bacterial persister formation. Nat. Chem. Biol. 8, 431 (2012).

L. Dethlefsen, S. Huse, M. L. Sogin, D. A. Relman, The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 6, e280 (2008).

L. Dethlefsen, D. A. Relman, Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. U.S.A. 108 (suppl. 1), 4554 (2011).

H. E. Jakobssonet al., Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS ONE 5, e9836 (2010).

C. Jernberg, S. Löfmark, C. Edlund, J. K. Jansson, Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 1, 56 (2007).

V. B. Young, T. M. Schmidt, Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. J. Clin. Microbiol. 42, 1203 (2004). 10.1128/JCM.42.3.1203

R. C. Owens Jr., C. J. Donskey, R. P. Gaynes, V. G. Loo, C. A. Muto, Antimicrobial-associated risk factors for Clostridium difficile infection. Clin. Infect. Dis. 46 (suppl. 1), S19 (2008).

K. P. Lemon, G. C. Armitage, M. A. Fischbach, Microbiome-targeted therapies: An ecological perspective. Sci. Transl. Med. 4, 137rv5 (2012). 10.1126/science.1208344

R. T. Paine, M. J. Tegner, E. A. Johnson, Compounded perturbations yield ecological surprises. Ecosystems 1, 535 (1998).

E. Elinavet al., NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 145, 745 (2011). 10.1016/S0169-5347(02)02495

K. Shea, P. Chesson, Community ecology theory as a framework for biological invasions. Trends Ecol. Evol. 17, 170 (2002). 10.1016/S0169-5347(02)02495

P. J. Turnbaugh et al., An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027 (2006).

J. L. Sonnenburg, L. T. Angenent, J. I. Gordon, Getting a grip on things: How do communities of bacterial symbionts become established in our intestine? Nat. Immunol. 5, 569 (2004).

B. A. Duerkop, S. Vaishnava, L. V. Hooper, Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31, 368 (2009).

A. J. Macpherson, M. B. Geuking, E. Slack, S. Hapfelmeier, K. D. McCoy, The habitat, double life, citizenship, and forgetfulness of IgA. Immunol. Rev. 245, 132 (2012). 10.1111/j.1600

M. E. Johansson et al., The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. U.S.A. 105, 15064 (2008).

I. Sekirov, B. B. Finlay, The role of the intestinal microbiota in enteric infection. J. Physiol. 587, 4159 (2009).

A. J. Macpherson, N. L. Harris, Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. 4, 478 (2004).

M. Kanther, J. F. Rawls, Host-microbe interactions in the developing zebrafish. Curr. Opin. Immunol. 22, 10 (2010).

M. Bohnhoff, C. P. Miller, Enhanced susceptibility to *Salmonella* infection in streptomycin-treated mice. J. Infect. Dis. 111, 117 (1962).

N. Yurdusev, M. Ladire, R. Ducluzeau, P. Raibaud, Antagonism exerted by an association of a Bacteroides thetaiotaomicron strain and a *Fusobacterium necrogenes* strain against *Clostridium perfringens* in gnotobiotic mice and in fecal suspensions incubated in vitro. Infect. Immun. 57, 724 (1989). 2537255

S. Fanning et al., Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc. Natl. Acad. Sci. U.S.A. 109, 2108 (2012).

S. Fukuda et al., Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543 (2011).

C. P. Kelly, J. T. LaMont, *Clostridium difficile*—more difficult than ever. N. Engl. J. Med. 359, 1932 (2008).

B. Stecher et al., *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 5, e244 (2007).

P. Thiennimitr et al., Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. Proc. Natl. Acad. Sci. U.S.A. 108, 17480 (2011).

V. Klepac-Ceraj et al., Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. Environ. Microbiol. 12, 1293 (2010). 10.1111/j.1462

J. Lederberg, Infectious history. Science 288, 287 (2000).

G. C. Daily et al., Ecosystem services: Benefits supplied to human societies by natural ecosystems. Issues Ecol. 2, 1 (1997).

C. Allan, G. H. Stankey, Eds., *Adaptive Environmental Management: A Practitioner's Guide* (Springer, Heidelberg, 2009).

H. Sokol et al., *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc. Natl. Acad. Sci. U.S.A. 105, 16731 (2008).

J. M. Chase, Community assembly: When should history matter? Oecologia 136, 489 (2003). 10.1038/nature00823

T. Fukami, in *Community Ecology: Processes, Models, and Applications*, H. A. Verhoef, P. J. Morin, Eds. (Oxford Univ. Press, Oxford, 2010), pp. 45–54.

A6

SEASONAL RESTRUCTURING OF THE GROUND SQUIRREL GUT MICROBIOTA OVER THE ANNUAL HIBERNATION CYCLE[22]

*Hannah V. **Carey***,[23] *William A. Walters*,[24] *and Rob Knight*[25,26]

Abstract

Many hibernating mammals suspend food intake during winter, relying solely on stored lipids to fuel metabolism. Winter fasting in these species eliminates a major source of degradable substrates to support growth of gut microbes, which may affect microbial community structure and host-microbial interactions. We explored the effect of the annual hibernation cycle on gut microbiotas using deep sequencing of 16S rRNA genes from ground squirrel cecal contents. Squirrel microbiotas were dominated by members of the phyla Bacteroidetes, Firmicutes, and Verrucomicrobia. UniFrac analysis showed that microbiotas clustered strongly by season, and maternal influences, diet history, host age, and host body temperature had minimal effects. Phylogenetic diversity and numbers of operational taxonomic units were lowest in late winter and highest in the spring after a 2-week period of

[22] Reprinted with permission from the American Physiological Society. Carey HV, Walters WA, Knight R. Seasonal restructuring of theground squirrel gut microbiota over the annual hibernation cycle. *Am J Physiol Regul Integr Comp Physiol* 304: R33–R42, 2013. First published November 14, 2012; doi:10.1152/ajpregu.00387.2012.

[23] Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin.

[24] Department of Molecular, Cell, and Developmental Biology, University of Colorado, Boulder, Colorado.

[25] Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado.

[26] Howard Hughes Medical Institute, Boulder, Colorado.

refeeding. Hibernation increased relative abundance of Bacteroidetes and Verrucomicrobia, phyla that contain species capable of surviving on host-derived substrates such as mucins, and reduced relative abundance of Firmicutes, many of which prefer dietary polysaccharides. Hibernation reduced cecal short-chain fatty acid and ammonia concentrations, and increased and decreased concentrations of acetate and butyrate, respectively. These results indicate that the ground squirrel microbiota is restructured each year in a manner that reflects differences in microbial preferences for dietary vs. host-derived substrates, and thus the competitive abilities of different taxa to survive in the altered environment in the hibernator gut.

The coevolution of mammals with their gut microbes has produced complex relationships that provide benefits for both partners (Ley et al., 2008a, b). Microbes shape the biology of their hosts in multiple ways: they enhance resistance to pathogen colonization, influence gastrointestinal structure and function, drive the development of the immune system, and increase energy harvest from the diet (Backhed et al., 2004; Crawfored et al., 2009; Hooper et al., 2001; Velagapudi et al., 2010). In turn, the host provides a nutrient-rich environment that supports the development of diverse microbial communities that inhabit multiple niches (Van den Abbeele et al., 2011; Velagapudi et al., 2010). The structure of the microbiota is shaped by several factors, including host genetics, maternal effects, interactions with the immune system, interactions among members of the microbial consortium, and the availability of metabolizable substrates. Although host diet provides the major source of substrates to support microbial growth, microbes can also use host-derived substrates, including mucus glycans, nutrients in sloughed epithelial cells, and pancreatic and biliary secretions (Backhed and Ley, 2005; Hooper et al., 2002; Leser et al., 2000; Martens et al., 2008; Sonnenburg et al., 2005). Gut microbes vary in their abilities to degrade different substrate types, and some species can rapidly adapt their metabolic machinery to use alternative substrates, such as dietary vs. host-derived glycans (Koropatkin et al., 2012; Sonnenburg et al., 2005). It is now well established that changes in diet type and amount alter microbiota composition in mammals (Ley et al., 2008; Muegge et al., 2011), although most studies have used species that do not experience severe dietary extremes as part of their normal lifestyles.

Circannual cycles of feeding and fasting are a key feature of the biology of many hibernating mammals (Carey et al., 2003; Florant and Healy, 2012; Lyman et al., 1982). In these species, hyperphagia drives the accumulation of large fat stores in summer and early fall, followed by voluntary fasting, which can last up to 8 mo (Carey et al., 2003; Florant and Healy, 2012; Lyman et al., 1982). This natural cycle of food intake provides the opportunity to examine the response of the gut microbiota to changes in substrate availability free from experimental manipulations, such as imposed fasts, which can increase host stress. In addition

to dietary change, other aspects of the hibernation phenotype could potentially alter microbial communities. Most of the hibernation season is spent in a metabolically depressed state known as torpor, when body temperature (T_b) falls to <10°C and metabolism is <4% of active values (Carey et al., 2003). Although T_b during torpor is below the temperature optimum for most gut microbes, bouts of torpor are interrupted periodically by interbout arousals to normothermia that last for 12–24 h (Carey et al., 2003), providing sufficient time at high temperature for microbial degradation of any host-derived substrates that may be present in the gut lumen. Hibernation is also accompanied by a remodeling of the intestinal immune system (Kurtz and Carey, 2007), which is an aspect of host biology that is both influenced by, and can itself influence, the microbiota. Here, we demonstrate an annual reorganization of the cecal microbiota in 13-lined ground squirrels that reflects the dominant role of host diet in shaping microbial community structure.

Methods

Animals

All procedures were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. Six pregnant female 13-lined ground squirrels (*Ictidomys tridecemlineatus*) were collected in early May, 2010, around Madison, Wisconsin, and housed individually in a 22°C room with a 12:12-h light-dark cycle. In the wild, these squirrels eat a plant-based diet (leaves, flowers, seeds) supplemented with animal material when available (e.g., insects, bird eggs). After capture, water and rat chow (Harlan Teklad no. 7001) were provided ad libitum, and diets were supplemented once per week with fruit (apples, strawberries) and sunflower seeds. After parturition, pups remained with mothers for 5 wk, after which they were moved to individual cages with ad libitum chow + fruit for an additional 2 wk. The pups' food was then restricted to 12 g of chow/day to prevent excessive weight gain, supplemented with 1 g sunflower seeds once per week. Mothers were euthanized in July for sample collection. Pups were randomly assigned to one of six seasonal groups (Figure A6-1B), such that each season contained at least one pup from each mother. One group of pups was euthanized in August (summer). The remaining pups were transferred to a 4°C room with constant darkness, except for brief ~5 min) periods of dim light once per day to check activity states using the sawdust method (Pengelley and Fisher, 1961). Food and water were removed after squirrels began using torpor. Hibernating squirrels were euthanized either in early winter ~30 days of hibernation) or late winter ~130 days of hibernation) in one of two activity states: torpor (T_b, ~6°C) or interbout arousal (T_b, ~36°C). Spring squirrels were returned to the warm room after ~130 days of hibernation and were euthanized 14 days later. Details on squirrel body masses, Tbs, and hibernation patterns are presented in Table A6-1.

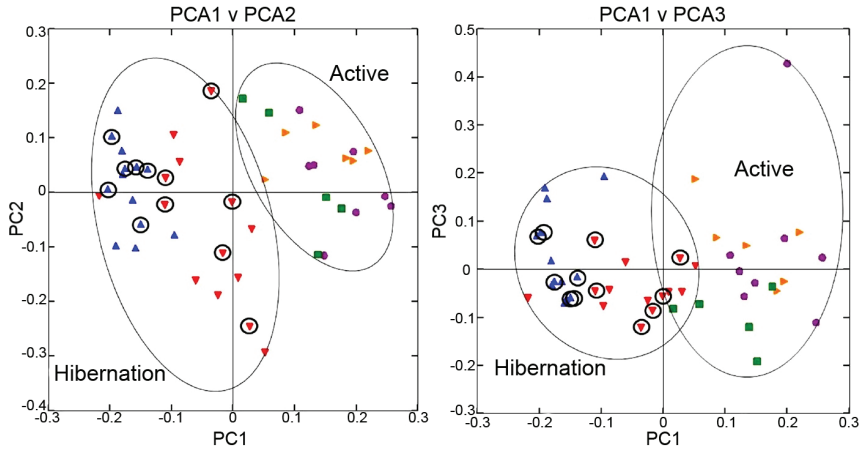


FIGURE A6-1 Principal coordinate analysis plots of unweighted UniFrac metrics for squirrel miocrobiotas. Each dot represents individual microbiota samples obtained from Mothers (yellow triangles) and their pups in Summer (purple circles), Early Winter (red triangles), Late Winter (blue triangles), and Spring (green squares). The first three axes are affected by seasonal variation. *Left*: PCA1 vs. PCA2; *Right*: PCA1 vs. PCA3. For hibernators, samples from torpid squirrels are circled. Numbers of squirrels in each seasonal group are shown in Table A6-1.

Sample Collection

Squirrels were euthanized by isoflurane followed by decapitation, except for torpid hibernators, which did not receive isoflurane. T_b was measured immediately by insertion of a thermal probe into the body cavity, and intact ceca were rapidly removed. Cecal contents were harvested and weighed, and one portion frozen immediately in liquid nitrogen followed by storage at −80°C until DNA extraction. The remainder was sonicated and centrifuged (10,000 rpm, 10 min), and one aliquot was stored at −80°C for later analysis of ammonia concentration. The remaining supernatant was acidified with 36N sulfuric acid (2%/volume) and stored −80°C for later analysis of short-chain fatty acids (SCFA). The empty cecal tissue was weighed to determine cecal wet mass.

SCFA and Ammonia Analyses

After thawing, an aliquot of the acidified cecal contents was analyzed by gas chromatography for total SCFA concentrations and molar proportions of propionate, acetate, and butyrate. A separate aliquot was used to measure ammonia concentrations using a kit (no. AA0100 Sigma-Aldrich).

TABLE A6-1 Ground Squirrel Body Mass, Cecal Tissue Mass, Body Temperature, and Torpor Characteristics

Group	n	Age, days	Body mass, g	Cecal mass, g	Tb, °C	Total Days Hibernating	Total Days in Torpor	Days in Last Torpor Bout
Mothers	6	>420*	202±10[a]	1.15±0.07[a]	34.5±0.5[a,b]			
Summer	8	98±1[a]	156±10[b]	1.03±0.04[a]	35.0±0.8[b]			
Early Winter-Aroused	8	157±1[b]	158±6[b]	0.78±0.11[b]	35.8±0.4[a,b]	34.3±1.3[a]	29.1±1.1[a]	6.1±0.4
Early Winter-Torpid	6	155±1[b]	157±8[b]	0.62±0.05[b]	6.5±0.2[c]	33.5±0.6[a]	28.8±0.6[a]	6.2±0.4
Late Winter-Aroused	7	253±3[c]	132±7[c]	0.68±0.08[b]	36.2±0.5[a,b]	132.0±2.9[b]	115.0±1.5[b]	6.1±0.7
Late Winter-Torpid	6	252±1[c]	138±4[b,c]	0.71±0.04[b]	6.5±0.3[c]	130.0±1.7[b]	116.7±1.5[b]	7.2±0.9
Spring	5	307±1[d]	160±10[b]	0.80±0.02[b]	36.5±0.5[a]			

Values are expressed as means ± SE. For aroused hibernators, days in last torpor bout indicates length of last bout prior to arousal; for torpid squirrels, number of days in continuous torpor.

* Age of wild-caught mothers was unknown and assumed to be ≥420 days; therefore, mother ages were not used in statistical analysis.

[a,b,c,d] Letters that differ within columns show significant differences ($P < 0.05$).

DNA Extraction

DNA was extracted from thawed cecal contents using the MoBio Powersoil DNA isolation kit with modifications. For each sample, the 16S rRNA gene was amplified (in triplicate) using barcoded V4 primers 515f (5′-GTGCCAGCMGC CGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′), found to be well suited to the phylogenetic analysis of pyrosequencing reads. Composite samples for pyrosequencing were prepared by pooling the triplicates of each PCR reaction back into one PCR product, which was cleaned, quantified, and then pooled into a composite sample by adding equal amounts of amplicon/DNA for each sample. The replicate PCRs were combined and cleaned with the MoBio UltraClean-htp kit. Samples were quantified using PicoGreen dsDNA reagent. Once quantified, the appropriate volumes of the cleaned PCR amplicons were combined. The composite pool was measured for final concentration, 260/280 ratio, and sent for sequencing. Controls were included in all steps to check for primer or sample DNA contamination. Samples were analyzed by the Environmental Genomics Core Facility at the University of South Carolina-Columbia for pyrosequencing on a 454 Life Sciences Genome Sequencer FLX-Titanium (Roche) machine.

Data Processing

Processing of pyrosequencing reads was performed using the QIIME 1.4.0 software package (Caporaso et al., 2010b). A total of 82,732 sequences were demultiplexed and passed the default quality filters in QIIME (minimum 497 sequences, maximum 3,772 sequences, and mean 1,798.52 sequences per sample). Demultiplexed sequences were clustered de novo into operational taxonomic units (OTUs) using 97% identity, with chimeric and singleton OTUs filtered using the USEARCH software package (Edgar, 2010). Representative sequences were assigned taxonomy via the RDP classifier (Wang et al., 2007) retrained on the Greengenes reference sequence set. A de novo alignment of these representative sequences was built using PyNast (Caporaso et al., 2010b), filtered with the Greengenes lanemask, and from this filtered alignment, a phylogenetic tree was constructed with FastTree (Price et al., 2009). Alpha diversity was calculated by first filtering the OTU table to remove any samples with less than 1,000 sequences/sample, followed by subsampling the OTU table for 50 repetitions at steps of 50 sequences/sample up to 1,000 sequences/sample. Following this, phylogenetic diversity (PD, defined as the minimum total length of all the phylogenetic branches required to span a given set of taxa on a phylogenetic tree [Faith and Baker, 2006]), Chao1, and observed species were calculated to determine alpha diversity for squirrel seasonal groups. Statistical differences among groups in PD and numbers of observed species at 1,000 sequences per sample were determined by Monte Carlo permutations to handle nonparametric data distributions (n=999, implemented in QIIME GitHub

commit 28c0020e05e4d29d9446eb3837e500e6328386bb). Beta diversity was calculated using unweighted and weighted UniFrac (Lozupone and Knight, 2005) on the evenly sampled OTU table at 436 sequences per sample. Taxonomies were grouped at the phylum, class, order, family, and genus levels. We compared sequence counts for each taxonomic level and season from samples rarefied to 436 sequences, using the Kruskal-Wallis rank sum test in R (version 2.12.0). False discovery rate (FDR) corrections were then applied.

Differences in SCFA and ammonia levels were analyzed by Kruskal-Wallis rank sum test or with t-test when only two groups were compared. Correlations between SCFAs and microbial taxa were analyzed by Pearson's correlation analysis and corrected for FDR.

Results

Cluster Analysis of Microbiotas

Comparison of individual squirrel microbiotas using principal coordinate analysis (PCoA) of the unweighted UniFrac metric showed a distinct clustering by season, with most of the variation explained by the first three coordinates (Figure A6-1). Microbiotas from active season groups (Mothers, Summer, and Spring groups) clustered together and were distinct from those in Late Winter, which were tightly clustered. Clustering of the Early Winter group was the most diffuse, and as a group, these microbiotas were intermediate between those from the active season groups and the Late Winter group. The thermal and metabolic state of hibernating squirrels at the time of sampling had little effect on microbiota clustering in either Winter group, as microbiotas from torpid squirrels (Figure A6-1, circled points) were evenly distributed among those from aroused hibernators. Because of this, in subsequent microbiota analyses, torpid and aroused hibernators were combined within the respective Early or Late Winter groups. Age of the animals had no effect on microbiota clustering; for example, Spring microbiotas clustered more closely with those from Mother and Summer squirrels than with the Late Winter group, despite Spring and Late Winter squirrels being closest in age (Figure A6-1, Table A6-1). Squirrel body mass was also not a discriminating factor in microbiota clustering, as masses of Early Winter hibernators were not different from those of Spring or Summer pups (Table A6-1) yet their microbiotas were quite distinct. Clustering of weighted Unifrac results showed a similar pattern, although the separations based on PCoA1 vs. PCoA3 were not as robust (data not shown).

Phylogenetic Diversity and Observed Species

Phylogenetic diversity and numbers of observed species (OTUs) were lowest in microbiotas from Late Winter hibernators and highest in Spring squirrels

(Figure A6-2). For PD, all groups differed significantly from each other except Early Winter and Spring, and for observed species counts, only Mother and Summer groups were not different (Figure A6-2). To better visualize microbiota diversity, we constructed phylogenetic trees using the Topiary Explorer software program (Pirrung et al., 2011). Inspection of trees constructed from each seasonal group (Figure A6-3, peripheral trees) illustrates the shift in diversity within specific taxa (see below) in Late Winter microbiotas relative to the other seasonal groups and to the entire ground squirrel microbiota data set (Figure A6-3, center tree). Although the phylogenetic trees only show presence or absence of particular OTUs and all seasonal groups have some representation across the tree, the trees suggest there is increased (or decreased) diversity within certain clades as environmental selection changes through the hibernation states.

Taxonomic Composition of Squirrel Microbiotas

Eight bacterial phyla were identified in the ground squirrel microbiota (Supplemental Table S1[27]), with the majority of sequences classified as Bacteroidetes (22–52%), Firmicutes (22–66%), or Verrucomicrobia (5–22%) (Supplemental Table S2). Other less abundant (1–5%) phyla represented were Proteobacteria, Tenericutes, and Actinobacteria, along with some very low abundance groups (<1%) that included Elusimicrobia and Cyanobacteria (Supplemental Table S1). An unclassified group of sequences (1–4%) accounted for the remainder of the bacterial diversity in the squirrel microbiota. The Archaean phylum Euryarchaeota was represented only in a few of the Mothers' microbiotas at <0.005%.

The majority of Bacteroidetes sequences within the squirrel microbiota matched to the order Bacteroidales and were represented primarily by the families Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, and Rikenellaceae. At the genus level, dominant groups were *Bacteroides*, *Prevotella*, and *Alistipes* (Supplemental Table S1). Firmicutes were dominated by Clostridiales, with most OTUs matching to the families Lachnospiracea and Ruminoccocaceae. Dominant genera included *Clostridium*, *Ruminococcus*, *Oscillospira*, and *Coprococcus*. The Lactobacillales were the next most dominant Firmicutes group, represented by Lactobacillaceae (genus *Lactobacillus*) (Supplemental Table S1).

Season strongly affected taxonomic representation of the dominant phyla. Relative to the two summer groups (Summer pups and Mothers), hibernator microbiotas had lower representation of Firmicutes and higher representation of Bacteroidetes (Figure A6-4, Supplemental Table S2). These differences were most evident between Late Winter hibernators and Summer squirrels. Spring microbiotas tended to be intermediate between Summer and Late Winter, likely reflecting the early repopulation of a "normal" microbiota once feeding resumed. The same general pattern held for seasonal changes in relative abundance

[27] The online version of this article contains supplemental material.

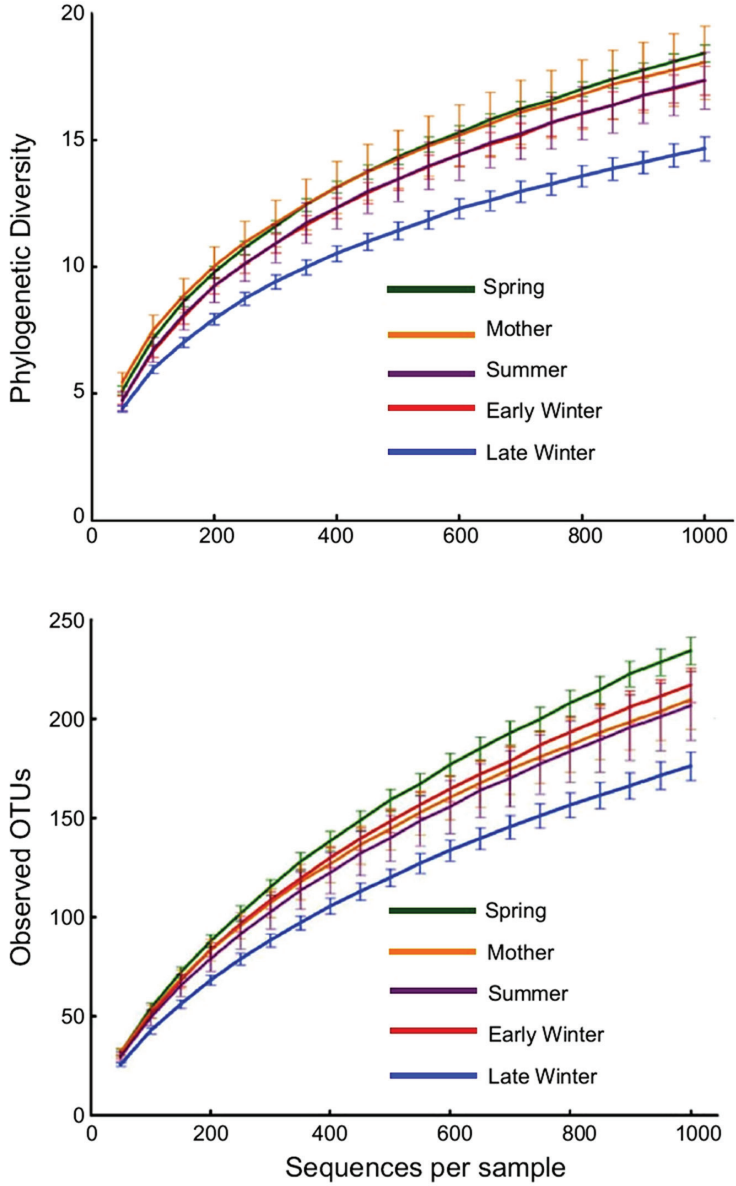


FIGURE A6-2 Alpha diversity rarefaction plots of squirrel cecal microbiotas. *Top*: phylogenetic diversity (PD). *Bottom*: number of observed OTUs based on 97% sequence identity. Points are means ± SE, with numbers of squirrels in each group shown in Table A6-1. Differences among groups were analyzed at 1,000 sequences/sample. For PD, all groups differed significantly from one another ($P < 0.05$) except for early winter and summer. For observed OTUs, all groups differed except for the mother and summer groups.

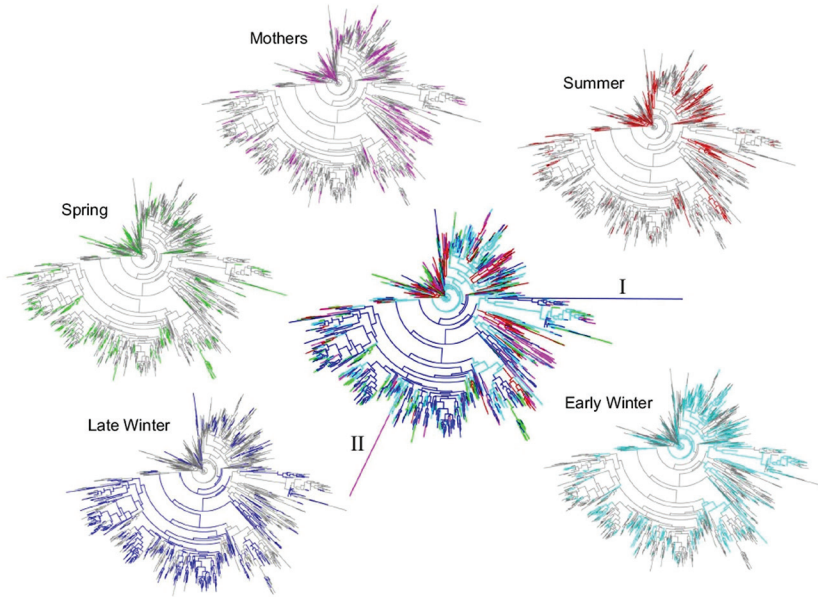


FIGURE A6-3 Phylogenetic trees of squirrel cecal microbiotas colored by seasonal taxa. Center plot includes all taxa with coloration as follows: Mothers, pink; Summer, red; Early Winter, light blue; Late Winter, dark blue; Spring, green. Branch labeled "I" represents the archeal genus Methanosphera; "II" represents a poorly defined taxa. Perimeter plots show trees for individual seasonal groups. Trees were generated and colored with Topiary Explorer (Pirrung et al., 2011).

of Verrucomicrobia (Supplemental Table S2). Sequences within this phylum matched to a single species, *Akkermansia muciniphila* (Supplemental Table S1).

Representation of the order Bacteroidales roughly doubled from Summer to Late Winter, including a six-fold rise in relative abundance of *Alistipes* (Rickenellaceae) (Figure A6-4, Supplemental Table S2). In contrast, the abundance of several Firmicute taxa fell during hibernation, including a 9-fold reduction in Lachnospiraceae from Summer to Late Winter. The family *Lactobacillaceae* was severely affected by hibernation, with only 4 of the 14 Early Winter samples containing any sequences that matched to *Lactobacillus*, and no matches within the 13 Late Winter samples (Figure A6-4, Supplemental Table S2). A preliminary report using quantitative PCR analysis also showed that hibernation depletes *Lactobacilli* in the squirrel microbiota (Carey et al., 2012). Relative abundance of Firmicutes taxa that were reduced during winter showed strong trends for partial or full reversal in Spring microbiotas. Interestingly, the only Firmicutes group that increased significantly during hibernation was one or more unclassified

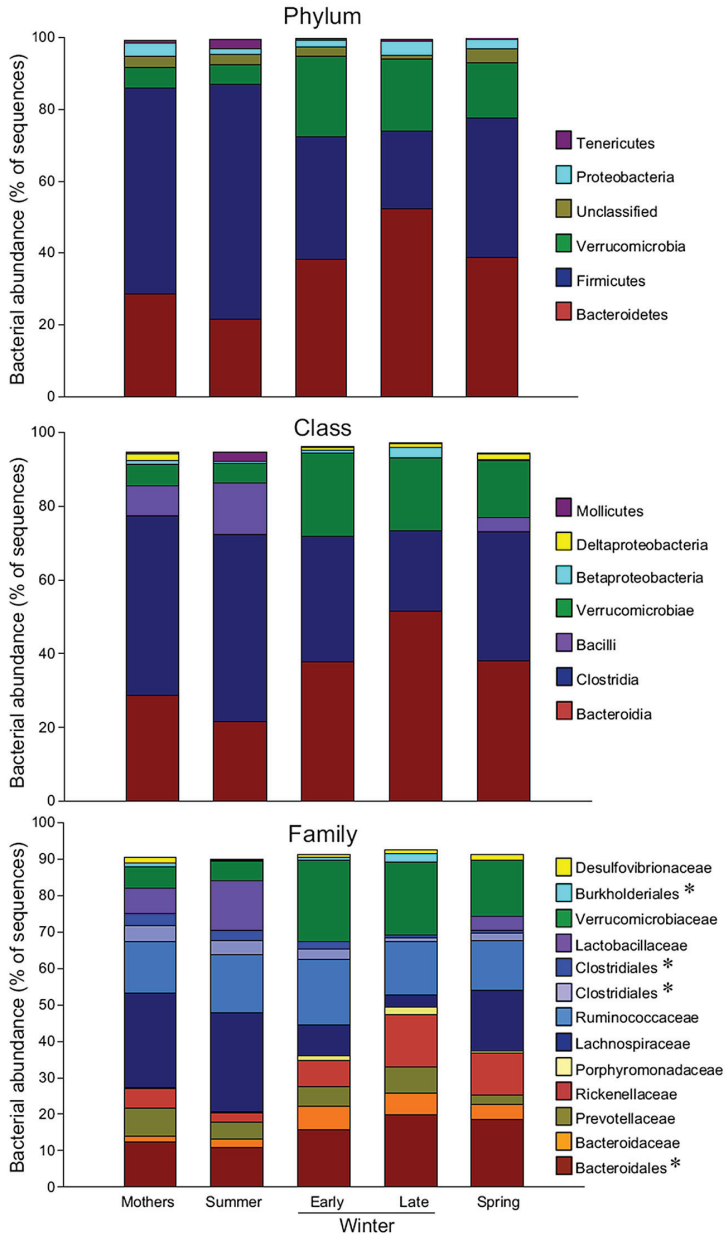


FIGURE A6-4 Relative abundance of major taxa in squirrel cecal microbiotas. Numbers of squirrels in each seasonal group are shown in Table A6-1. Asterisks indicate unclassified families within a taxonomic group. Taxa with relative abundances less than 1.5% were not included.

genera in the family Peptococcaceae, although relative abundance of these OTUs represented only 1–1.2% of all sequences.

Season also influenced relative abundance of some of the less common taxa (Supplemental Table S2). Notable among these was an unclassified genus in the *Burkholderiales* (class Betaproteobacteria) that represented <1% of OTUs in each of the seasonal groups except Late Winter, when it increased to 2.5%.

Short-Chain Fatty Acid and Ammonia Levels in Cecal Contents

We measured SCFA and ammonia concentrations in cecal contents to gain insight into how season affects the metabolic output of squirrel microbial communities. Total SCFA concentrations were highest in Summer squirrels and decreased by about 75% in aroused hibernators (whose T_bs were similar to Summer) (Table A6-2). Torpor further reduced total SCFA concentrations in Late Winter hibernators. Among the three SCFA, acetate was highest regardless of season, and all SCFA concentrations were lower in Late Winter (aroused) hibernators compared with Summer. Butyrate concentration was most affected by hibernation, falling to nearly undetectable levels in many hibernator samples and particularly those from torpid squirrels. Values from two Spring squirrels shown in Table A6-2 were not included in the statistical analysis due to low sample size; however, those data suggest that Spring SCFA concentrations remain closer to winter levels than to Summer, despite the 2-wk period of refeeding and constant homeothermy, which would stimulate rapid production of SCFA from dietary substrates. Hibernation also influenced the molar proportions of the individual SCFAs generated by the microbiota. Compared with Summer, the percent composition of acetate increased and butyrate decreased in aroused hibernators (Table A6-2), and both returned toward summer levels in spring. The proportion of propionate in cecal contents was similar in Summer squirrels and aroused hibernators. Interestingly, compared with aroused hibernators, proportions of acetate and propionate in torpid squirrels were higher and lower, respectively.

SCFA levels were correlated with relative abundances of some bacterial taxa (Table A6-3). Concentrations of all three SCFA were positively correlated with Firmicutes (specifically the Class Clostridia and Family Lachnospiraceae). Acetate and propionate concentrations were negatively correlated with Bacteroidetes and Verrucomicrobia, whereas butyrate concentration was negatively correlated only with Bacteroidetes. Concentrations of all SCFA were also positively correlated with relative abundance of the minor taxa Alphaproteobacteria and Mollicutes. The molar proportion of acetate relative to total SCFA concentration was negatively correlated with Firmicutes, and propionate was negatively correlated with Bacteroidia and Verrucomicrobiae (Table A6-3). Butyrate proportion was positively correlated with Firmicutes and negatively correlated with Bacteroidetes. Several other OTUs that were not classified to specific taxonomic groups were also significantly correlated with SCFA levels (data not shown).

TABLE A6-2 Short-Chain Fatty Acid and Ammonia Concentrations and Molar Proportions of SCFA in Ground Squirrel Cecel Contents

	Acetate	Propionate	Butyrate	Total SCFA	Ammonia
Concentration, mM					
Summer (6,6)	49.01±9.70[a]	10.93±2.71[a]	17.92±5.82[a]	81.39±18.63[a]	7.70±0.54
Early Winter (6,6)	13.55±2.16[b]	2.41±0.40[b]	0.94±0.17[b]	18.29±2.86[b]	3.50±0.81[b]
Late Winter					
Aroused (6,6)	15.64± 2.45[b]	2.53±0.66[b]	0.5±0.15b	19.50±3.31[b]	2.54±0.31[b]
Torpid (6,6)	6.26±0.93*	0.41±0.07*	0.12±0.02*	7.06±1.02*	2.25±0.18
Spring (2,5)	17.55±2.12	4.64±1.73	3.06±0.92	27.72±5.60	8.65±1.05[a]
Molar Proportions, %					
Summer (6)	63.43±2.84[a]	13.17±0.50	18.71±2.98[a]		
Early Winter (6)	74.17±1.01[b]	13.09±0.61	5.01±0.37[b]		
Late Winter					
Aroused (6)	81.30±2.58[b]	12.20±1.72	2.52±0.51[b]		
Torpid (6)	88.48±1.38*	5.85±0.57*	1.75±0.27		
Spring (2)	64.39±5.36	16.13±2.98	10.81±1.13		

Shown are means ±SE with sample sizes in parentheses [short-chain fatty acids (SCFA), ammonia]. For SCFA, groups analyzed with Kruskal-Wallis test were Summer squirrels and aroused hibernators in Early and Late Winter (no torpid Early Winter samples were available, and the two Spring squirrel samples were not included due to low sample size). For ammonia, groups analyzed were Spring and Summer squirrels and aroused hibernators in Early and Late Winter.

* Significant differences in SCFA and ammonia levels between torpid and aroused late winter hibernators, analyzed by t-tests.

[a,b] Different letters within columns show significant differences. $P < 0.05$ for all comparisons.

TABLE A6-3 Correlations Between SCFA Levels and Microbial Taxa in Squirrel Cecal Contents

	Acetate		Propionate		Butyrate	
	mM	%	mM	%	mM	%
Bacteroidetes	–		–		–	–
Bacteroidia	–			–	–	–
Firmicutes	+	–	+		+	+
Clostridia	+	–	+		+	+
Lachnospiraceae	+	–	+		+	+
Verrucomicrobia	–		–			
Verrucomicrobiae	–			–		
Verrucomicrobiaceae	–					
Proteobacteria						
Alphaproteobacteria	+		+		+	+
Tenericutes					+	
Mollicutes	+	+	+		+	

Shown are significant ($P < 0.05$) positive (+) and negative (–) correlations between SCFA concentrations (mM) or molar proportions (%) and microbial taxa.

Ammonia concentrations were higher in Spring and Summer squirrels compared with Early and Late Winter aroused hibernators (Table A6-2). Torpor had no effect on ammonia concentrations in Late Winter squirrels.

Discussion

Like other fat-storing hibernators, the 13-lined ground squirrel accumulates large adipose stores during the active season, survives solely on endogenous nutrients (primarily lipids) for the 5–6 mo hibernation season, and then begins refeeding in spring in a relatively lean condition (Carey et al., 2003). Our goal here was to determine whether this annual cycle of extreme dietary change alters the structure of the gut microbiota. Our UniFrac analysis of 16S rRNA gene sequences confirmed that season plays a dominant role in the structure of ground squirrel microbial communities, as indicated by the close clustering of microbiotas of Spring and Summer pups with those of their Mothers, and the separate clustering of microbiotas in the Winter groups. Moreover, within the hibernation season, the microbiotas of Late Winter squirrels were more tightly clustered than were Early Winter animals, indicating that the Early Winter microbiota is a transitional state that precedes the more severe contraction of the community after 4–5 mo of continuous fasting. This is further illustrated by the rarefaction curves, which show that Late Winter microbiotas had the lowest phylogenetic diversity and numbers of observed species (OTUs) among all seasons. In contrast, microbiotas of Spring squirrels had the highest diversity and numbers of observed species. Thus, emergence from hibernation appears to induce blooms of

certain microbial taxa that are adept at rapid expansion into new niches created by the constant supply of dietary substrates in an environment of sustained, high temperature. The result is a transient, diverse community that probably contains members with a range of metabolic capabilities, including those that are able to thrive on endogenous substrates alone, those that prefer (or require) substrates present only in the diet, and those that are able to degrade both substrate types. As time after refeeding progresses, the microbial community becomes more stable and less diverse (as indicated by the lower diversity and number of OTUs of Mother microbiotas compared with Spring), and it is likely dominated by species that are competitively superior in the presence of abundant and continuously available dietary substrates. These cyclical changes in structure of the ground squirrel microbiota are summarized in Figure A6-5.

The UniFrac analysis also demonstrated that maternal influences, early dietary history, and host age had minimal effects on squirrel microbiotas compared with the dominant effect of season. The microbiotas of littermates (who ate primarily laboratory chow) and their mothers (who ate laboratory chow only after capture) were similar if they were sampled in the same (active) season, and the two seasonal groups that were closest in age—Late Winter and Spring—had microbiotas that were the most diverse.

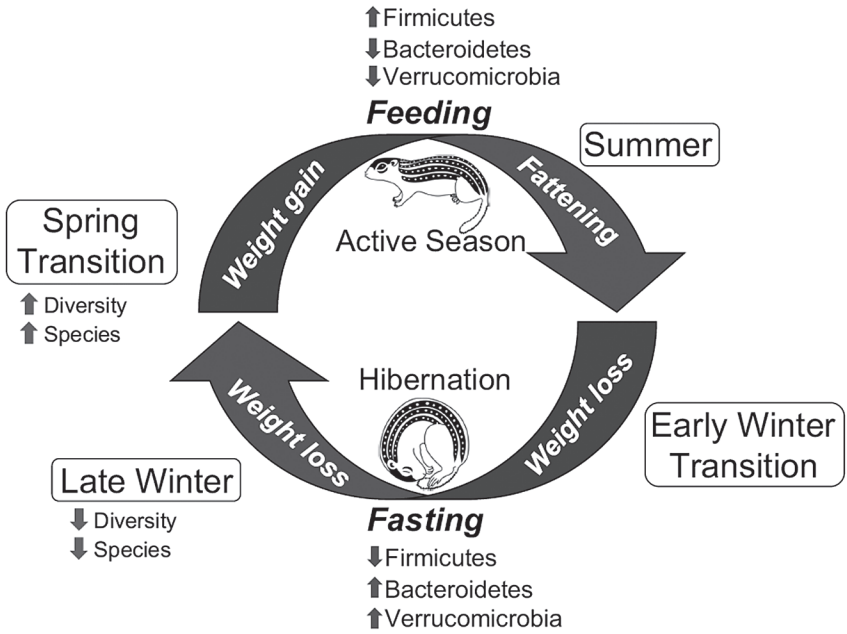


FIGURE A6-5 Schematic illustrating major changes in the ground squirrel gut microbiota over the annual hibernation.

In addition to substrate availability, other aspects of the gut environment during hibernation could influence microbial physiology, and thus microbiota structure. A major one is host T_b, which oscillates from ~36°C during interbout arousals to a few degrees above 0°C, as animals cycle into and out of torpor (Carey et al., 2003). Gut microbes may differ in their abilities to metabolize substrates and proliferate at the low T_bs of torpor (Morita, 1975), which could provide competitive advantages for some groups over others. The principal coordinates analysis revealed no effect of host T_b on microbiota structure, because samples from torpid and aroused hibernators were interspersed within the Early and Late Winter groups. That said, we may not have been able to detect subtle, but significant, changes in microbiota structure that reflect dominance of certain species that function better than others at low temperatures.

Similar to other mammals, the majority of sequences in the ground squirrel microbiota was assigned to the bacterial phyla Bacteroidetes and Firmicutes, with Verrucomicrobia comprising the next most abundant group. At the phylum level, the dominant changes from active to hibernation seasons were increased representation of Bacteroidetes and Verrucomicrobia and a reduction in Firmicutes (Figure A6-5). Thus, on the basis of known substrate preferences of gut microbes (Flint et al., 2012), the structure of the squirrel microbiota appears to be reorganized in winter in favor of taxa that are either specialists on host-derived substrates (Verrucomicrobia) (Derrien et al., 2004) or generalists that have the ability to switch their complement of carbohydrate-degrading enzymes depending on the availability of dietary vs. hostderived substrates (Bacteroidetes) (Martens et al., 2008; Slyers et al., 1977; Sonnenburg et al., 2005). Conversely, the hibernator microbiota is generally depleted in species that are known to prefer dietary polysaccharides, which include many Firmicutes (Flint et al., 2012).

The majority of host-derived substrates in the mammalian gut are gel-forming mucins, which are complex glycoproteins produced in large quantities by intestinal goblet cells and, when hydrated, form the protective mucus layer that overlies the epithelium (McGuckin et al., 2011). Only a few gut microbes have the ability to completely degrade mucins (20, 33, 45, 55, 57); one is *Akkermansia muciniphila*, the sole representative of the Verrucomicrobia present in the mammalian gut (Derrien et al., 2004), whose relative abundance increased in hibernator microbiotas. *A. muciniphila* associates with the mucus layer and is able to grow on mucin as its sole carbon and nitrogen source (Derrien et al., 2004, 2010; van Passel et al., 2011).

Competition for the limited resources in the hibernator gut is likely an important factor that shapes the microbiota, as species less able to utilize endogenous substrates or metabolites produced by primary mucin-degraders will gradually be reduced in number until dietary substrates are available in the spring. For example, relative abundance of *Lactobacillus*, which prefers simple sugars, falls to nondetectable levels once hibernation begins and does not increase until after refeeding in spring. The Lachnospiraceae, which are typically very abundant in

the mammalian gut microbiota is also negatively affected by hibernation. Although many members of this Firmicutes family prefer dietary substrates (Flint et al., 2012), some can utilize endogenous sugars such as fucose (Scott et al., 2006), which may explain the continued presence of a small subset of Lachnospiracea in the hibernator gut.

Winter fasting reduced cecal SCFA and ammonia concentrations, which is not surprising given the absence of dietary intake. Interestingly, the relative proportions of individual SCFA changed from active to hibernation seasons, with acetate rising and butyrate falling to very low levels. These shifts were likely due to the reduction in certain types of metabolizable substrates coupled with the altered microbiota composition. The absence of complex plant polysaccharides during hibernation reduces abundance of certain taxa (e.g., many Firmicutes) that specialize on these substrates. This reduces production of butyrate, because most of the major butyrate producers, including *Roseburia*, *Eubacterium*, and *Faecalibacterium* are Firmicutes (Duncan et al., 2007). The negative correlations between butyrate levels and Bacteroidetes abundance, as well as the positive correlations between butyrate and Firmicutes, support this relationship. The lower cecal butyrate concentration during winter may contribute to the changes in intestinal structure and function observed in hibernating squirrels. As the preferred fuel source for epithelial cells, butyrate stimulates epithelial proliferation and restitution, and helps maintain integrity of the intestinal barrier through modulation of apoptosis, permeability, and mucus production (Bugaut and Bentejac, 1993; Gaudier et al., 2004; Louis and Flint, 2009; Peng et al., 2007). Hibernation leads to substantial atrophy of the small intestinal mucosa in ground squirrels (Carey, 1990, 1992; Carey et al., 2012), and as reported in this study, cecal mass is also reduced. Hibernation also alters expression of several apoptosis-related proteins in the intestine (Fleck and Carey, 2005), and it increases gut permeability (Carey, 1990, 1992; Carey et al., 2012).

In contrast to butyrate, the molar proportion of acetate rose during hibernation, which may be due, in part, to the activity of mucolytic bacteria, such as *A. muciniphila*, which convert mucins to acetate (Derrien et al., 2010). Microbially derived acetate could influence hibernation physiology in several ways. After absorption, it can be metabolized in peripheral tissues to meet energy demands, or it can be converted to ketone bodies in gut epithelial cells or hepatocytes. Ketones play a particularly important role in hibernation, serving as an alternative fuel to glucose, particularly in brain and heart during torpor (Andrews et al., 2009). Although the majority of circulating ketones derive from de novo synthesis in the liver, it is possible that microbially derived acetate contributes to hepatic ketogenesis and thus cellular energetics during the winter fast, as has been shown in mice (Crawford et al., 2009). Microbially derived propionate may also play a role in hibernation, because in addition to serving as a fuel source, propionate is a substrate for gluconeogenesis, another metabolic function that is critical during hibernation to maintain blood glucose levels (Galster and Morrison, 1975).

Although the contribution of microbial SCFA to energy and nutrient balance in ground squirrels is unknown, estimates in other rodents indicate that gut-derived SCFA provide 5–20% of an animal's overall energy budget (Bergman, 1990). However, hindgut epithelial cells derive as much as 60–70% of their energy needs from luminal SCFA (Ardawl and Newsholme, 1985), which raises the possibility that through their production of SCFA from endogenous substrates, the hibernator microbiota may "recycle" some of the energy that is expended during interbout arousal periods in epithelial cell proliferation (Carey and Martin, 1996), mucus production, and maintenance of the mucosal immune system (Kurtz and Carey, 2007).

This is the first report of seasonal changes in the microbiota of a fasting hibernator based on deep sequencing of microbial 16S rRNA genes, and it expands on previous work that used only culture-based techniques (Barnes, 1970; Barnes and Burton, 1970; Cloud-Hansen et al., 2007) or quantitative PCR (Carey et al., 2012). In a culture-independent study in Syrian hamsters, a hibernating species that eats during periodic arousals, a 4-day fast in nonhibernators had a greater effect on species composition than did hibernation, relative to fed (nonhibernating) animals (Sonoyama et al., 2009). This underscores the crucial role of diet in the regulation of gut microbial communities, because periodic arousals provide ample time at high Tb for microbes to degrade and utilize newly ingested dietary substrates. In fact, several changes we noted in the microbiotas of hibernating 13-lined ground squirrels were comparable to those in fasted, but not hibernating hamsters; for example, the fasted hamster microbiota had a greater proportion of sequences represented by *A. muciniphila*, and reduced representation of Firmicutes, including Lachnospiraceae (Sonoyama et al., 2009). Our results also resemble the effects of natural fasting between meals in the Burmese python microbiota. After a more than 30-day fast, phylogenetic diversity of the python colonic microbiota is relatively low and dominated by Bacteroidetes, with reduced representation by Firmicutes (Costello et al., 2010). Feeding causes a rapid increase in phylogenetic diversity and numbers of observed OTUs, with proportional representations of Bacteroidetes and *Akkermansia* falling and Firmicutes rising, similar to our observations in Spring squirrels relative to Late Winter. Even in laboratory mice, fasting for as little as 24 h increases and decreases proportional representation of Bacteroidetes and Firmicutes, respectively (Crawford et al., 2009).

Hibernation is associated with a dramatic remodeling of the intestinal immune system, with increased numbers of intraepithelial and lamina propria lymphocytes, increased IgA expression and elevated mucosal cytokine levels (Kurtz and Carey, 2007). It is likely that seasonal reorganization of the microbiota is a major driver of these immune alterations, because the immune system is the primary sensor of gut microbes and their metabolites (Hooper et al., 2012; Van den Abbeele et al., 2011). Immune changes are often associated with altered host-microbe signaling (Hooper et al., 2012; Lee and Mazmanian, 2010;

Peterson et al., 2007; Sutherland and Fagarasan, 2012) and may function to maintain a mutually beneficial, tolerant state (Derrien et al., 2011; Maynard et al., 2012). The elevation in intestinal secretory IgA (sIgA) expression during hibernation (Kurtz and Carey, 2007) provides particularly strong evidence for a seasonal shift in host-gut microbe signaling. Gut microbiota can stimulate sIgA production (Sutherland and Fagarasan, 2012; Talham et al., 1999), and there is growing evidence that in addition to preventing bacterial adherence to epithelial cells, sIgA plays a key regulatory role in maintaining the complex, mutualistic interactions between the microbiota, the epithelium, and the immune system (Peterson et al., 2007; Sutherland and Fagarasan, 2012). Further, hibernation is accompanied by increased levels of mucosal IL-10, a potent anti-inflammatory cytokine that promotes immune tolerance of the microbiota (Kurtz and Carey, 2007; Maynard et al., 2012). Epithelial defenses are also enhanced in the hibernator gut, including increased expression of anti-apoptotic proteins (Fleck and Carey, 2005), and increased tight junction localization of occludin, which reduces intestinal barrier dysfunction induced by inflammatory cytokines (Carey et al., 2012; Marchiando et al., 2010). Because gut permeability increases during hibernation in ground squirrels (Carey, 1990, 1992; Carey et al., 2012), the benefit of heightened immune and epithelial defenses may reside in minimizing translocation of microbial products across the epithelium and in limiting inflammation if microbial translocation occurs, thus preserving intestinal homeostasis (Maynard et al., 2012).

Perspectives and Significance

The extreme dietary change that is part of the annual hibernation cycle can be viewed as a normal, recurring perturbation (Lozupone et al., 2012) that is well tolerated by both the ground squirrel host and its microbial symbionts. Our study revealed how the gut microbial community responds to this perturbation in terms of taxonomic structure but did not provide insight into functional changes in the microbiome (i.e., the microbiota and their complement of genes and gene products). Metagenomic analyses, coupled with mRNA, protein, and metabolite profiling will be required to illuminate how metabolic pathways of the community as a whole, and within specific bacterial species, are modified by winter fasting and the return of dietary substrates in spring (Lozupone et al., 2012).

Given the wide variety of effects that gut microbes can exert on mammalian hosts (Hooper et al., 2002; Nicholson et al., 2012), it is likely that the coevolution of hibernating mammals with their microbiotas has shaped not only gut physiology and immune function, but multiple aspects of the hibernation phenotype. For example, metabolic activity of gut microbes may contribute to the crucial process of prehibernation fattening, or influence energetic balance and torpor-arousal patterns during the hibernation season. Identification of which hibernation features carry a microbial imprint will ultimately require manipulation of the microbiota and analysis of host responses. Although gnotobiotic approaches are currently

not available for hibernating species, carefully designed studies that use antibiotics to deplete the microbiota or prebiotics to stimulate them may be feasible. Incorporating the potential roles of the microbiota provides a new perspective on the physiology of mammalian hibernation and will also expand our understanding of the intimate relationships that have evolved between animals and their gut symbionts.

Acknowledgments

We thank Glen Broderick and Wendy Radloff for SCFA analyses and Michael Grahn, Jessica Otis, and Amanda Pike for collection and care of animals.

Grants

This work was supported by funds from the University of Wisconsin School of Veterinary Medicine (to H. V. Carey), National Institutes of Health (NIH), and Howard Hughes Medical Institute (to R. Knight) and NIH T32 GM008759 (to W. A. Walters).

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Author contributions: H.V.C. conception and design of research; H.V.C. performed experiments; H.V.C., W.A.W., and R.K. analyzed data; H.V.C., W.A.W., and R.K. interpreted results of experiments; H.V.C. and W.A.W. prepared figures; H.V.C. and W.A.W. drafted manuscript; H.V.C., W.A.W., and R.K. edited and revised manuscript; H.V.C., W.A.W., and R.K. approved final version of manuscript.

References

Andrews MT, Russeth KP, Drewes LR, Henry PG. Adaptive mechanisms regulate preferred utilization of ketones in the heart and brain of a hibernating mammal during arousal from torpor. *Am J Physiol Regul Integr Comp Physiol* 296: R383–R393, 2009.

Ardawi MS, Newsholme EA. Fuel utilization in colonocytes of the rat. *Biochem J* 231: 713–719, 1985.

Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 101: 15718–15723, 2004.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Hostbacterial mutualism in the human intestine. *Science* 307: 1915–1920, 2005.

Barnes EM. Effect of hibernation on the intestinal flora. *Am J Clin Nutr* 23: 1519–1524, 1970.

Barnes EM, Burton GC. The effect of hibernation on the caecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). *J Appl Bacteriol* 33: 505–514, 1970.

Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* 70: 567–590, 1990.

Bugaut M, Bentejac M. Biological effects of short-chain fatty acids in nonruminant mammals. *Annu Rev Nutr* 13: 217–241, 1993.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26: 266–267, 2010a.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335–336, 2010b.

Carey HV. Effects of fasting and hibernation on ion secretion in ground squirrel intestine. *Am J Physiol Regul Integr Comp Physiol* 263: R1202– R1208, 1992.

Carey HV. Seasonal changes in mucosal structure and function in ground squirrel intestine. *Am J Physiol Regul Integr Comp Physiol* 259: R385– R392, 1990.

Carey HV, Andrews MT, Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev* 83: 1153–1181, 2003.

Carey HV, Martin SL. Preservation of intestinal gene expression during hibernation. *Am J Physiol Gastrointest Liver Physiol* 271: G804–G813, 1996.

Carey HV, Pike AC, Weber CR, Turner JL, Visser A, Beijer-Liefers SC, Bouma HR, Kroese FGM. Impact of hibernation on gut microbiota and intestinal barrier function in ground squirrels. In: *Living in a Seasonal World: Thermoregulatory and Metabolic Adaptations* , edited by Ruf T, Bieber C, Arnold A, and Millesi E. Heidelberg, Germany: Springer, 2012.

Cloud-Hansen KA, Villiard KM, Handelsman J, Carey HV. Thirteenlined ground squirrels (*Spermophilus tridecemlineatus)* harbor multiantibiotic resistant bacteria. *J Am Assoc Lab Animal Sci* 46: 17–20, 2007.

Costello EK, Gordon JI, Secor SM, Knight R. Postprandial remodeling of the gut microbiota in Burmese pythons. *ISME J* 4: 1375–1385, 2010.

Crawford PA, Crowley JR, Sambandam N, Muegge BD, Costello EK, Hamady M, Knight R, Gordon JI. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. *Proc Natl Acad Sci USA* 106: 11276–11281, 2009.

Derrien M, Van Baarlen P, Hooiveld G, Norin E, Muller M, de Vos WM. Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader *Akkermansia muciniphila*. *Front Microbiol* 2: 166, 2011.

Derrien M, van Passel MW, van de Bovenkamp JH, Schipper RG, de Vos WM, Dekker J. Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* 1: 254–268, 2010.

Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov, sp nov, a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* 54: 1469–1476, 2004.

Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ Microbiol* 73: 1073–1078, 2007.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26: 2460–2461, 2010.

Faith DP, Baker AM. Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evol Bioinform Online* 2: 121–128, 2006.

Fleck CC, Carey HV. Modulation of apoptotic pathways in intestinal mucosa during hibernation. *Am J Physiol Regul Integr Comp Physiol* 289: R586–R595, 2005.

Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3: 1–18, 2012.

Florant GL, Healy JE. The regulation of food intake in mammalian hibernators: a review. *J Comp Physiol B* 182: 451–467, 2012.

Galster W, Morrison PR. Gluconeogenesis in arctic ground squirrels between periods of hibernation. *Am J Physiol* 228: 325–330, 1975.

Gaudier E, Jarry A, Blottiere HM, de Coppet P, Buisine MP, Aubert JP, Laboisse C, Cherbut C, Hoebler C. Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose. *Am J Physiol Gastrointest Liver Physiol* 287: G1168–G1174, 2004.

Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 336: 1268–1273, 2012.

Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Ann Rev Nutr* 22: 283–307, 2002.

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291: 881–884, 2001.

Hoskins LC, Agustines M, McKee WB, Boulding ET, Kriaris M, Niedermeyer G. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J Clin Invest* 75: 944–953, 1985.

Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. *Nat Rev Microbiol* 10: 323–335, 2012.

Kurtz CC, Carey HV. Seasonal changes in the intestinal immune system of hibernating ground squirrels. *Dev Comp Immunol* 31: 415–428, 2007.

Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* 330: 1768–1773, 2010.

Leser TD, Lindecrona RH, Jensen TK, Jensen BB, Moller K. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae*. *Appl Environ Microbiol* 66: 3290–3296, 2000.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. *Science* 320: 1647–1651, 2008a.

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* 6: 776–788, 2008b.

Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett* 294: 1–8, 2009.

Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71: 8228–8235, 2005.

Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* 489: 220–230, 2012.

Lyman CP, Willis JS, Malan A, Wang LCH. *Hibernation and Torpor in Mammals and Birds*. New York: Academic, 1982.

Marchiando AM, Shen L, Graham WV, Weber CR, Schwarz BT, Austin 2nd JR, Raleigh DR, Guan Y, Watson AJ, Montrose MH, Turner JR. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* 189: 111–126, 2010.

Martens EC, Chiang HC, Gordon JI. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* 4: 447–457, 2008.

Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489: 231– 241, 2012.

McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* 9: 265–278, 2011.

Morita RY. Psychrophilic bacteria. *Bacteriol Rev* 39: 144–167, 1975.

Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, Henrissat B, Knight R, Gordon JI. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332: 970–974, 2011.

Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S. Host-gut microbiota metabolic interactions. *Science* 336: 1262–1267, 2012.

Peng L, He Z, Chen W, Holzman IR, Lin J. Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. *Pediatr Res* 61: 37–41, 2007.

Pengelley ET, Fisher KC. Rhythmical arousal from hibernation in the golden-mantled ground squirrel, Citellus lateralis tescorum. *Can J Zool* 39: 105–120, 1961.

Peterson DA, McNulty NP, Guruge JL, Gordon JI. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe* 2: 328–339, 2007.

Pirrung M, Kennedy R, Caporaso JG, Stombaugh J, Wendel D, Knight R. Topiary Explorer: visualizing large phylogenetic trees with environmental metadata. *Bioinformatics* 27: 3067–3069, 2011.

Png CW, Linden SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, McGuckin MA, Florin TH. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am J Gastroenterol* 105: 2420–2428, 2010.

Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26: 1641–1650, 2009.

Salyers AA, Vercellotti JR, West SE, Wilkins TD. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. *Appl Environ Microbiol* 33: 319–322, 1977.

Scott KP, Martin JC, Campbell G, Mayer CD, Flint HJ. Wholegenome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium "Roseburia inulinivorans". *J Bacteriol* 188: 4340–4349, 2006.

Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, Buhler JD, Gordon JI. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science* 307: 1955–1959, 2005.

Sonoyama K, Fujiwara R, Takemura N, Ogasawara T, Watanabe J, Ito H, Morita T. Response of gut microbiota to fasting and hibernation in Syrian hamsters. *Appl Environ Microbiol* 75: 6451–6456, 2009.

Sutherland DB, Fagarasan S. Iga synthesis: a form of functional immune adaptation extending beyond gut. *Curr Opin Immunol* 24: 1–8, 2012.

Talham GL, Jiang HQ, Bos NA, Cebra JJ. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun* 67: 1992–2000, 1999.

Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S. The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. *FEMS Microbiol Rev* 35: 681–1385, 2011.

van Passel MW, Kant R, Zoetendal EG, Plugge CM, Derrien M, Malfatti SA, Chain PS, Woyke T, Palva A, de Vos WM, Smidt H. The genome of *Akkermansia muciniphila*, a dedicated intestinal mucin degrader, and its use in exploring intestinal metagenomes. *PLoS One* 6: e16876, 2011.

Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu PV, Yetukuri L, Islam S, Felin J, Perkins R, Boren J, Oresic M, Backhed F. The gut microbiota modulates host energy and lipid metabolism in mice. *J Lipid Res* 51: 1101–1112, 2010.

Walter J, Ley R. The human gut microbiome: ecology and recent evolutionary changes. *Annu Rev Microbiol* 65: 411–429, 2011.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73: 5261–5267, 2007.

A7

LESSONS FROM STUDYING INSECT SYMBIOSES[28]

*Angela E. **Douglas***[29]

As in mammals, insect health is strongly influenced by the composition and activities of resident microorganisms. However, the microbiota of insects is generally less diverse than that of mammals, allowing microbial function in insects to be coupled to individual, identified microbial species. This trait of insect symbioses facilitates our understanding of the mechanisms that promote insect-microbial coexistence and the processes by which the microbiota affect insect well-being. As a result, insects are potentially ideal models to study various aspects of interactions between the host and its resident microorganisms that would be impractical or unfeasible in mammals and to generate hypotheses for subsequent testing in mammalian models.

Introduction

The common condition for animals is to be chronically infected by microorganisms, most of which are benign or beneficial. The influence of these resident microorganisms on their animal host is profound and operates at two levels: in physiological time, such that the physiology and well-being of the animal is influenced by the composition, density, and activities of colonizing microorganisms; and in evolutionary time, through selection on the magnitude and pattern of the animal response to the infecting microorganisms. We can only understand animal-microbial interactions by combining these physiological and evolutionary perspectives.

There is immense variation in the detail of the interactions between animals and their resident microbiota. A proper grasp of the nature of interactions between humans and their microbiota can only come from an awareness of the parallels and contrasts with other animals. Insects represent a superb system for comparison because, as a group, they display far greater diversity than mammals in their interactions, including some associations of remarkable morphological intimacy and molecular integration. Research on insect-microbial interactions has been invigorated in recent years by the technical advances that make it possible to identify and study unculturable microorganisms. The recent gains in mechanistic understanding of the relationships in molecular terms have immediate relevance to research on the impact of resident microbes on the physiology and health of humans.

---

[28] Reprinted from *Cell Host & Microbe*, 10(4), A. E. Douglas, Lessons from studying insect symbioses, Pp. 359-367, Copyright (2011), with permission from Elsevier.

[29] Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853, USA.

This review summarizes these recent advances by focusing on four topics: the mechanisms that promote coexistence of insect host and microbes; the contribution of microbes to insect nutrition and defense; insect dependence on their resident microbiota; and coevolutionary interactions between the partners. The impact of insect research on our understanding of associations in humans is also briefly considered.

Diversity of Insect-Microbial Symbioses

Insects and mammals share the common feature that they are inhabited by microorganisms. Even so, there are two important differences in the host-microbial relationship between the two animal groups: (1) the diversity of the microbiota tends to be an order of magnitude greater in mammals than in insects, and (2) many insects, but no known mammals, have beneficial intracellular microorganisms. To address these differences, the gut microbiota and intracellular symbioses of insects are considered in turn.

The gut lumen is very densely colonized by microbes in most mammals and insects. The animal gut can be considered a portion of the external environment in which the conditions and resources are controlled largely by the animal. Microorganisms associated with food have unrestricted access to the gut; and those that can tolerate, modulate or evade the digestive processes and immune function of the animal gut gain access to a nutrient-rich environment and a vehicle for dispersal via the feces. Nevertheless, the habitats in the insect and mammalian guts cannot be equivalent because the patterns in the composition and diversity of the gut microbiota are markedly different. The identification of bacterial operational taxonomic units (OTU) by 97% sequence identity of 16S rRNA gene sequences revealed that the gut of most insects bears < 20–30 taxa (Dillon and Dillon, 2004; Robinson et al., 2010; and Wong et al., 2011), and that of mammals (or their feces) yield 500–1000 taxa (Dethlefsen et al., 2007 and Nemergut et al., 2011). The reasons for this difference are unclear. As with many other low diversity habitats (Connell, 1978), insect guts tend to be transient and have extreme disturbance regimes. Compounding the short life span of many insects, microbes associated with the foregut and hindgut are eliminated at every insect molt (when the cuticle lining these gut regions is shed), and all microbes are shed when the larval gut is broken down and the adult gut develops during metamorphosis of higher ("holometabolous") insects, including the true flies, butterflies, and beetles. It has also been suggested that the adaptive immune system of mammals may promote microbial diversity through its greater capacity than the innate immune system to discriminate among different microorganisms, enabling the mammalian host to maintain complex multispecies consortia (McFall-Ngai, 2007).

The greater microbial diversity in individual mammals as opposed to insects is overlaid by a greater diversity across all insects relative to all mammals. Thus, the dominant microbes in all mammals are Bacteroidetes and Firmicutes, but the

gut microbiota of insects vary widely among different taxa, including Proteobacteria, Firmicutes, and Protists (Brugerolle and Radek, 2006; Dillon and Dillon, 2004; Morales-Jiménez et al., 2009; and Robinson et al., 2010). This difference could be a consequence of the greater variation of diets and gut physiology in insects as opposed to mammals, which is linked to the greater phylogenetic diversity of insects. (The class Mammalia comprises 5000 species, and the class Insecta has 900,000 known—and 2–30 million predicted—species.) For example, all mammals have an extremely acidic stomach, but an equivalent region is rare among insects and apparently restricted to the higher Diptera, including *Drosophila* (Shanbhag and Tripathi, 2009). The pH in many insects lies within the range of 6–8 units, and some, notably lepidopteran caterpillars, have a very basic midgut region, at 11–12 pH units (Wieczorek et al., 2009).

The second dominant habitat in insects utilized by microorganisms is cells. An estimated 10%–20% of insect species bear intracellular symbionts that are localized to specialized cells, known as bacteriocytes, whose sole function appears to be to house and maintain their symbionts. These associations are widespread or universal in several groups, notably cockroaches, hemipterans, ten families of beetles, and lice, and they also occur in some flies and ants (see Table 1 in Douglas, 2007). Although they have evolved independently multiple times, the microorganisms are invariably transmitted vertically (from mother to offspring), usually via the eggs in the female ovaries. The resultant perfect congruence between the phylogenies of the microbial symbiont and their insect hosts, in some insect groups over more than 100–200 million years (Dale and Moran, 2006; and Moran et al., 2005), has no parallel in mammals. Correlated with the long history and intimacy of the association, both the insect and microbial partners are dependent on the relationship, such that insects experimentally deprived of their bacteria fail to grow and reproduce (Douglas, 2007), and the symbionts generally have much-reduced genomes (<1 Mb) and are unculturable (Dale and Moran, 2006).

Bacteriocytes are not the only insect cell type that contains microorganisms. Across all insect groups, microorganisms have been reported in cells of various organs, including the fat body, gut epithelium, and gonads. Some of these taxa (e.g., *Wolbachia*, *Hamiltonella*) can occupy multiple compartments, within and between the cells of insect organs and in the blood, for example, although the location of the microorganisms may vary with host and symbiont genotype as well as the age and physiological condition of the host (Oliver et al., 2010; and Werren et al., 2008). This trait of broad and variable tissue distribution is facilitated by the open circulatory system of insects, meaning that blood is not restricted to closed vessels, but is in direct contact with various organs and moves relatively sluggishly around the body.

Bacteriocyte symbioses are unknown among mammals, and virtually all microorganisms that adopt an intracellular phase in mammals mediate chronic or acute pathogenic infections. There is no entirely satisfactory explanation for this

difference. It has been suggested repeatedly that the adaptive immune system of vertebrates poses a very high barrier to the evolution of intracellular microorganisms, which has been overcome predominantly by pathogens. Consistent with this argument, intracellular symbionts are commonplace among many invertebrates, but extremely rare across all vertebrates, with the alga *Oophila* in the embryos of a salamander (Kerney et al., 2011) as the sole documented example.

Mechanisms for Coexistence

Much more is known about the patterns than the mechanisms that promote coexistence of insects and their resident microbes. The key feature of these patterns is a relative uniformity in the location and numbers of microorganisms, suggesting that the host exerts tight controls over its symbionts. A rich literature has demonstrated that symbionts of insects are restricted to specific anatomical sites or cell types, and their abundance varies predictably with developmental age and sex of the insect host and environmental conditions. For microbes that can be either mutualistic or deleterious depending on environmental circumstance or host genotype, a key feature of the deleterious phenotype is high proliferation rates and abundance, often accompanied by an expanded distribution within the insect body. For example, the negative effect of the popcorn strain of *Wolbachia* on the longevity of *Drosophila* is strongly correlated with high bacterial numbers (McGraw et al., 2002); and the γ-proteobacterium *Hamiltonella* is harmful to its aphid host only on certain rearing plants, correlated with a 10-fold increase in bacterial abundance (Chandler et al., 2008).

Several studies indicate that the immune system plays a central role in the persistence of the microbiota. One such study concerns the gut microbiota of *Drosophila melanogaster*, dominated by *Acetobacter* and relatives, and *Lactobacillus*. The epithelial cells of the insect midgut bear receptors for the immune deficiency (IMD) signaling pathway, which is also active in other tissues, including the fat body, where it mediates the expression of various antimicrobial peptides (AMPs) against bacteria (Lemaitre and Hoffmann, 2007). The resident gut bacteria induce IMD signaling in the gut epithelial cells, resulting in localization of the NF-κB transcription factor Relish to the epithelial cell nucleus, but, contrary to expectation, the expression of AMPs is not induced (Ryu et al., 2008). The inhibition of AMP production is dependent on expression of the homeobox gene *caudal* (Figure A7-1A); when *caudal* expression is reduced by RNAi, AMP gene expression is upregulated. Importantly, the consequence of AMP production in the gut is not elimination of the gut microbiota, but a change in the microbial composition. Specifically, the relative abundance of two bacteria is dramatically altered; in the untreated fly, a bacterium of the Acetobacteriaceae (strain A911, known as *Commensalibacter intestini*) is dominant, and the abundance of *Gluconobacter morbifer* (G707) is very low. When the AMPs are expressed, the ratio of A911:G707 shifts from nearly 200:1 (favoring A911) to 1:20 (favoring

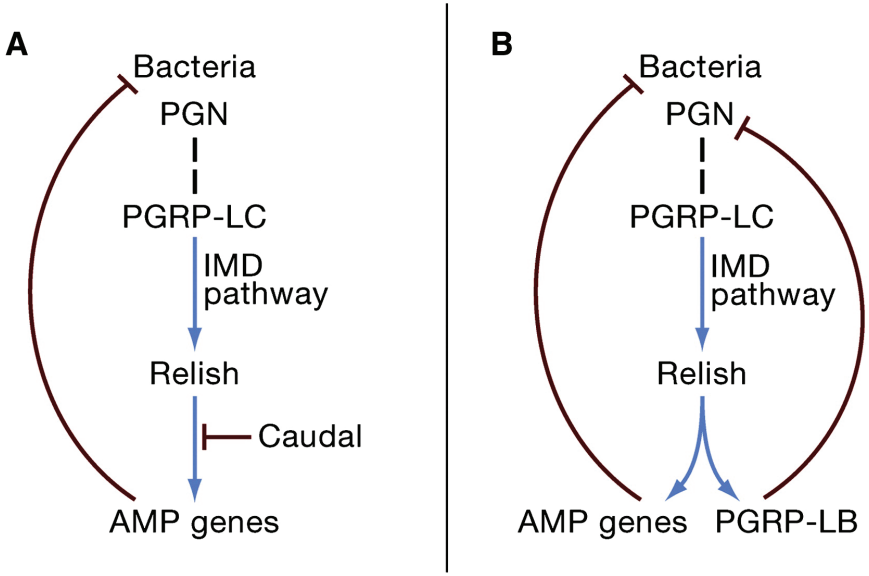


FIGURE A7-1 The insect IMD pathway and persistence of resident microorganisms. The IMD (Immune Deficiency) pathway is triggered by the binding of bacterial peptidoglycan (PGN) to the peptidoglycan recognition protein PGRP-LC (dashed vertical line). The resultant activation of the NF-κB transcription factor Relish leads to the upregulated expression of antimicrobial peptide (AMP) genes. Positive interactions (blue), negative interactions (red). (A) Resident microorganisms in the *Drosophila* gut activate the IMD pathway, but expression of AMPs is repressed by the transcription factor Caudal. (B) The IMD pathway in bacteriocytes (insect cells bearing symbiotic bacteria) of the *Glossina* tsetse fly and weevil *Sitophilus* is activated, but the immunoreactivity of bacteriocytes is dampened by the very high expression of the IMD-responsive gene PGRP-LB. PGRP-LB is a PGN-amidase that degrades the PGN ligand, thereby downregulating the IMD pathway.

EW707) (Roh et al., 2008; and Ryu et al., 2008). *G. morbifer* is deleterious to the host, resulting in severely depressed life spans of the flies with a *G. morbifer*-dominated gut community.

The implications are two-fold. First, AMPs are not exclusively antagonistic to microorganisms, but can also act to manage and regulate the microbial community. This conclusion has been reached independently for AMPs in other invertebrate animals (Fraune et al., 2010), and there is also evidence that other immune-related molecules can function in immunological management. For example, Toll-like receptor-2 (TLR2), which contributes to pathogen recognition in mammals, also suppresses the host inflammatory response to a major resident gut microorganism, *Bacteroides fragilis*; specifically, a product of *B. fragilis* (polysaccharide A) signals through TLR2 expressed by regulatory T cells to suppress

proinflammatory T_H17 cells, thereby enabling *B. fragilis* to associate closely with the intestinal mucosa without inducing a host inflammatory response (Round et al., 2011). Second, although the great flexibility of the vertebrate adaptive immune system has been suggested to facilitate immunological management of the resident microbiota (McFall-Ngai, 2007), research on *Drosophila* demonstrates that the innate immune system can also function to regulate and manage the microbiota.

The study of Ryu et al. (2008) on the interaction between the gut microbiota and immune effectors of *Drosophila* focused exclusively on the immune responses localized to the gut. Data on *Anopheles* mosquitoes suggest that the gut microbiota induces a systemic immunological response that limits the abundance and distribution of the microorganisms. RNAi-mediated silencing of AMPs and immune signaling pathways has been shown to result in increased proliferation of the gut microbiota, including *Pseudomonas* and *Novosphingobium* species, and their localization to the hemolymph (Dong et al., 2009; and Garver et al., 2008). The effect of the gut microbiota on the systemic immune response of other insects has not been investigated.

Further evidence that the innate immune system can serve to promote coexistence between insects and their resident microbiota comes from several studies on intracellular bacteria in bacteriocytes. Although they have much-reduced genomes, these bacteria are predicted to possess the molecular patterns that are recognized by the insect immune system. The host immune response is attenuated in the bacteriocytes of both the weevil *Sitophilus* and tsetse fly *Glossina*. In both cases, PGRP-LB (a peptidoglycan recognition protein with amidase activity) is strongly expressed in the bacteriocytes. Its expression is dependent on activation of the IMD pathway, and it functions to remove the peptidoglycan ligand that triggers the IMD pathway (Figure A7-1B). In this way, the responsiveness of the IMD pathway to symbiont peptidoglycan fragments is dampened, promoting persistence of the bacteria. The symbioses in *Sitophilus* and *Glossina* have separate evolutionary origins, indicating that the central role of PGRP-LB in these symbioses has arisen independently. PGRP-LB is not, however, the universal arbiter of symbiont persistence in bacteriocytes. Aphids, for example, lack both PGRPs and a functional IMD pathway, and it has been suggested that the selection pressure to accommodate their bacteriocyte symbionts (*Buchnera*) may have led to the evolutionary loss of this portion of the immune system (Gerardo et al., 2010). Taken together, these data illustrate how selection by resident microorganisms can influence the immune responsiveness of specific organs and cell types and, potentially, of the entire animal. Although parasites have been invoked repeatedly as a selection pressure for diversification of the animal immune system (Rolff, 2007; and Sackton et al., 2007), beneficial symbioses can also be important.

A recent study on *Wolbachia* in the mosquito *Aedes aegypti* has implicated insect miRNAs (18- to 25-mer nonprotein-coding RNAs) in the regulation of symbiont numbers (Hussain et al., 2011). Specifically, when either insects or

cultured insect cells are infected by *Wolbachia*, the titer of one specific miRNA (aae-miR-2940) is elevated, resulting in the increased expression of an insect metalloprotease gene. When either the miRNA was inhibited or the metalloprotease gene expression silenced, *Wolbachia* numbers in the insect cells were reduced. Further research is required to establish the generality of miRNA-mediated promotion of insect-microbial coexistence and to determine how these mechanisms interact with the humoral immune system.

Overlaying these mechanisms acting in physiological time are selection pressures for coexistence operating in evolutionary time. In this regard, mode of transmission of the microorganisms plays a key role (Ewald, 1994). The selective interest of microorganisms that require a live host, and especially depend on a live host for transmission, overlaps with that of the host, and these microorganisms tend to be more benign that those that are transmitted efficiently from dead or dying hosts. Obligate vertical transmission generates an especially great overlap in selective interest between the microbial and host partners because the fitness of the microorganism is critically dependent on the fecundity of the host. Obligate vertical transmission is the norm in bacteriocyte symbioses of insects and is assured by behavioral mechanisms for various gut associations. The transmission of the γ-proteobacterium *Ishikawaella* in the distal midgut of various stinkbugs provides a particularly vivid example. The female insect deposits a fecal pellet containing *Ishikawaella* alongside an egg, the offspring feed on the pellet immediately after emerging from the egg, and the larval gut then undergoes dramatic changes involving the effective separation of the proximal blind-ended gut region for digestion and the distal symbiotic organ in the distal region (Fukatsu and Hosokawa, 2002; and Hosokawa et al., 2007).

Benefits of the Resident Microbiota to the Host

The contribution of the resident microbiota to the well-being of an animal, and the underlying mechanisms, can most readily be investigated by comparing animals experimentally deprived of their microbiota with untreated animals bearing their natural microbial complement. Useful supplementary approaches to identifying the contribution of specific microorganisms and the underlying mechanisms include the analysis of animals with modified microbial contents, e.g., associations with single taxa, microorganisms with known genetic mutations or microbiota of different host genotypes or species. These manipulations come under the umbrella term of "gnotobiotics," meaning the rearing of animals under germ-free conditions, either continuously through life or with experimental administration of specific microorganisms. Gnotobiotics are especially important for investigating potentially important functions of microbial taxa that are at low abundance in unmanipulated associations. Various insect associations are very amenable to gnotobiotics because the treatments can be administered easily and cheaply to hundreds to thousands of individual insects. It is generally

straightforward to attribute function to individual microbial taxa because many insects bear a microbiota of low diversity (see above). The equivalent experiments using gnotobiotic mammals are technically demanding and, in some instances, extremely difficult to interpret because many microorganisms are members of complex, interdependent consortia with much functional redundancy, such that one host may bear multiple taxa with similar traits and the dominant taxa mediating a function of interest may vary among different host individuals.

Because of their experimental tractability, insect symbioses can provide valuable lessons about the services that resident microbes provide to animal hosts. Analyses of both gut and intracellular symbioses in many insects have revealed two core microbial functions: nutrition and defense. In many instances, it has been straightforward to link function to individual microbial taxa.

Particular insights are being obtained from studying insects that feed through the life cycle on diets of extremely unbalanced composition or low overall nutritional value, e.g., vertebrate blood (deficient in B vitamins), plant sap (for which essential amino acids are in short supply), and sound wood (grossly deficient in nitrogen and various essential nutrients). These insects all possess symbiotic microorganisms that provide specific nutrients in short dietary supply (Douglas, 2009). Strong indications that blood-feeding insects, including tsetse flies, lice, and bedbugs, derive B vitamins from their symbionts come from experiments comparing the performance of insects bearing and lacking their symbionts on blood diets that are untreated or supplemented with B vitamins. The same experimental approach, together with complementary metabolic analysis, has been applied to plant sap feeders, revealing that these symbionts provide essential amino acids.

The most detailed information is available for the association between the plant phloem sap-feeding pea aphid *Acyrthosiphon pisum* and its bacteriocyte symbiont *Buchnera*, for which both genomes are sequenced and annotated (International Aphid Genomics Consortium, 2010; and Shigenobu et al., 2000). Up to 50% of the essential amino acids synthesized by *Buchnera* cells are released to the surrounding host cell contents (Akman Gündüz and Douglas, 2009). The genome of this bacterium has a dearth of recognizable regulatory sequences, its gene expression is remarkably unresponsive to dietary perturbation (Reymond et al., 2006; and Wilson et al., 2006), and variation in aphid requirements for essential amino acids cannot generally be attributed to SNPs or other sequence variants in the *Buchnera* genome of the different aphids (MacDonald et al., 2011; and Vogel and Moran, 2011). Taken together, these data suggest that the insect host plays a major role in shaping the composition and quantity of essential amino acids released from *Buchnera*. Analysis of both the transcriptome and proteome of bacteriocytes has demonstrated that the bacteriocytes are enriched in enzymes mediating the synthesis of precursors required for *Buchnera*-mediated essential amino acid synthesis (Hansen and Moran, 2011; and Poliakov et al., 2011). Although the size and turnover of the precursor pools in the host cell

remain to be established, these data raise the possibility that *Buchnera* metabolism is poised for maximal production of essential amino acids and the realized synthesis rate is determined by precursor supply from the host cell.

Most research on the contribution of microorganisms resident in the gut to insect nutrition has focused on termites, which feed on various diets rich in plant fiber (wood, soil, humus). Historically—and erroneously—these associations were described as "miniature cows," in which cellulose-rich plant material was degraded exclusively by the hindgut microbiota to short-chain fatty acids (SCFAs) that were utilized by the termite. It is now realized that the termites are more complex and diverse than originally envisaged. The guts of termites (unlike mammals) have considerable intrinsic cellulase activity, which is partly or entirely responsible for cellulose degradation, varying among termite groups (Watanabe and Tokuda, 2010). Members of the gut microbiota additionally contribute to the nitrogen economy of the insect by recycling insect waste nitrogen or fixing atmospheric nitrogen, again varying among termite taxa (Burnum et al., 2011; and Warnecke et al., 2007).

As the examples above illustrate, most research on the nutrition of insect-microbial symbioses has focused on animals with diets that are extremely nutrient-poor or nutritionally unbalanced and are not utilized generally by mammals. Even so, the nutritional significance of the resident gut microbiota in a few insects utilizing less extreme diets has been studied. Notably, elimination of the microbiota from *Drosophila melanogaster* has been reported to extend development time and shorten life span (Bakula, 1969; and Brummel et al., 2004). The indication that the latter effect appears to be diet-dependent (Ren et al., 2007) suggests that the microbiota may have a nutritional role in this insect, but this has not been demonstrated definitively.

There is unambiguous evidence that specific resident microorganisms promote the resistance of insects to certain natural enemies, including viruses, bacteria, fungi, nematodes, and parasitic wasps. Examples include: the α-proteobacterium *Wolbachia*, which protects *Drosophila melanogaster* against various viruses (Hedges et al., 2008); *Spiroplasma* bacteria, which confer resistance in *Drosophila neotestacea* against the nematode parasite *Howardula aoronymphium* (Jaenike et al., 2010) and in *Drosophila hydei* against the parasitic wasp *Leptopilina heterotoma* (Xie et al., 2010); and the γ-proteobacterium *Regiella insecticola*, which reduces the mortality of pea aphids infected with entomopathogenic fungi (Scarborough et al., 2005). In these and many other interactions, the underlying mechanisms are not known, but for a few instances an outline understanding of the mechanisms has been obtained. The following three examples illustrate the diversity of interactions mediated by defensive microbes.

The first example concerns actinobacteria with protective function. Beewolf digger wasps house cultures of actinobacteria "Candidatus *Streptomyces* philanthi" in antennal glands and smear the bacteria onto cocoons deposited in their humid, microbe-rich burrows (Kaltenpoth et al., 2005). The complex mix of

antibiotics synthesized by the bacteria confers a generalized protection against microbial attack (Kroiss et al., 2010). The thorax of leafcutting ants is enveloped in a multispecies actinobacterial mat (Mueller et al., 2008) that produces antibiotics with activity against fungi, including a major fungal pathogen, *Escovopsis* (Oh et al., 2009). Actinobacteria in a similar relationship with *Dendronoctus* beetles provide analogous protection (Scott et al., 2008). In each of these systems, the low diversity of the actinobacterial partners has facilitated the identification of the actinobacterial partner, its function, and the chemical nature of its antimicrobials.

The second example relates to parasitic wasps, many of which exploit various insects by depositing an egg in the body cavity of their victim. The resultant wasp larva consumes tissues of the living insect over some days, and following the death of the insect, the adult wasp emerges from the dead insect "mummy." The resistance of pea aphids to the parasitic wasp *Aphidius ervi* is heightened by the presence of the γ-proteobacterium *Hamiltonella defensa*, which occurs in the body cavity of some pea aphids (the prevalence of *H. defensa* varies widely among different pea aphid populations). Importantly, this defensive role is evident only for *H. defensa* isolates that contain the virus APSE bearing the genes for toxins, including homologs of cytolethal toxins (Oliver et al., 2009). The reasonable inference is that resistance against the parasitic wasp is mediated by these toxins, but neither this interpretation nor how the aphid tissues avoid toxin-incurred damage has been demonstrated.

The resident microbiota in *Anopheles* mosquitoes reduces infection of the mosquitoes by the malaria parasite *Plasmodium* ingested with the blood meal. Specifically, the invasion of the midgut epithelium by the *Plasmodium* ookinetes is inhibited. This effect is mediated by bacteria established in the gut, and cofeeding *Anopheles* with bacteria and *Plasmodium* or coinjecting bacteria into the insect body cavity with *Plasmodium* feeding mediates these effects, indicating that it is not caused by a direct interaction between the bacteria and *Plasmodium* (Dong et al., 2009). Instead, the insect immune system is involved, including immune effectors that are active against both bacteria and *Plasmodium*. The precise mechanism of protection is not fully established (Cirimotich et al., 2010). After feeding on blood, the mosquito may mount a vigorous immune response against its resident bacteria, which proliferate rapidly when blood is ingested, or against hemozoin, which is an immunogenic breakdown product of the blood with an indirect effect on the *Plasmodium*. Alternatively or additionally, the mechanical disruption of the midgut epithelium by the invading *Plasmodium* ookinetes may introduce bacteria into the insect blood, triggering a systemic immune response. There is evidence for some specificity in this effect, with gram-negative bacteria providing greater protection than gram-positives (Cirimotich et al., 2010).

Dependence and Addiction

An important lesson from insect symbioses comes from the parasitic wasp *Asobara tabida*. In nature, every individual of this insect bears the bacterium *Wolbachia*, which is transmitted vertically via the eggs of the wasp. When *Wolbachia* is eliminated by antibiotic treatment, *A. tabida* is reproductively sterile with egg production halted by massive apoptosis of the nurse cells in the ovary. A limited incidence of apoptosis occurs during normal oogenesis in *A. tabida* bearing *Wolbachia*. A reasonable inference from these data is that *Wolbachia* inhibits apoptosis, and there is strong correlative evidence that Wolbachia perturbs iron metabolism in its host, resulting in increased oxidative stress that disrupts cellular physiology, including apoptosis (Kremer et al., 2009). This interaction has been argued to select for increased apoptotic signaling in the host to compensate for the otherwise harmful effects of *Wolbachia*on host physiology (Pannebakker et al., 2007). The putative compensatory response of *A. tabida* is constitutive, such that apoptosis is excessive in the absence of the inhibitory signal from *Wolbachia*. In this way, *A. tabida* has become dependent on *Wolbachia* without deriving any discernible benefit, a condition that is defined as addiction (Aanen and Hoekstra, 2007).

Dependence without benefit (i.e., addiction) has received little attention, partly because dependence is often interpreted as evidence for benefit, even in the absence of evidence for any service (e.g., nutrient provisioning, defensive role), but also because interactions between animal hosts and their resident microbiota are often investigated without considering their evolutionary context. Even so, there are candidate instances of dependence without benefit in various symbioses, including those between mammals and their resident microbiota. As one specific example, the capillary network in the small intestine of the mouse develops normally only in the presence of the gut microbiota (Stappenbeck et al., 2002). When mice are reared under aseptic conditions, the small intestine is poorly vascularized and the mice have reduced capacity to assimilate nutrients from the gut. Capillary growth is not induced by the bacteria directly, but by antibacterial peptides produced by the Paneth cells of the intestine in response to the presence of the microorganisms. In this way, the bacteria have become integrated into the animal signaling pathway required for normal development of the intestinal capillary bed, even though the capillaries function independently of the microbiota. There is no selection on the host to be independent of the microbiota because they are invariably infected by gut microorganisms soon after birth. Further candidate instances of dependence without benefit come from the central role of the gut microbiota in the development of the gut-associated lymph tissue and various populations of intestinal immune cells (Duan et al., 2010; Falk et al., 1998; Niess et al., 2008; and Pollard and Sharon, 1970).

How can dependence without benefit evolve? One possibility is that these many instances are animal compensatory responses to microbial perturbations of the host immune system or metabolism. As in *A. tabida*, the host responses may

be constitutive because there is no direct selection for function in the absence of the microbiota (because the microbiota are never absent under natural conditions). An alternative scenario is that certain signaling networks are truly symbiotic: that the resident microbiota in the ancestors of modern animals may have provided elements of the signaling networks regulating developmental processes, including angiogenesis and immune function, and that these elements have been retained in modern animals (Lee and Mazmanian, 2010; and McFall-Ngai, 2002). Whatever the evolutionary origin of dependence, the consequent addiction of animals for their resident microbiota is evident only under the unnatural experimental conditions of axenic (germ-free) rearings or large-scale perturbation of the microbiota, as in various immunological diseases or grossly unsuitable diets.

Coevolution Between Insects and Their Microbiota

Insect symbioses offer spectacular examples of coevolution with major consequences for the health and well-being of the animal host. For example, coevolutionary changes may include increased microbial production of nutrients valuable to the host and correlated changes in host metabolism, transporters, etc., that promote the host processing of microbial nutrients.

Coevolutionary interactions have been inferred from genome sequencing projects on plant sap-feeding insects and their microbial symbionts, including several instances of perfect complementarity in amino acid metabolism through differential gene loss in the partners in the association. For example, the genomes of both the pea aphid *Acyrthosiphon pisum* and its *Buchnera* symbiont have been sequenced (International Aphid Genomics Consortium, 2010; and Shigenobu et al., 2000), revealing a large-scale loss in the capacity of *Buchnera* to synthesize nonessential amino acids (which can be synthesized by the aphid host) and loss of the capacity to synthesize one amino acid, arginine, in the aphid host (Figure A7-2A). Unlike aphids, which have a single obligate symbiont, plant sap-feeding insects of a related suborder (the Auchenorrhyncha) possess dual symbioses, comprising a Bacteroidetes *Sulcia muelleri* and a second auxiliary bacterium, the identity of which varies with the insect group (Figure A7-2B). Remarkably, *Sulcia* has the capacity to synthesize 7 or 8 of the 10 essential amino acids (varying with insect group), and the auxiliary symbiont can synthesize the remaining essential amino acids (McCutcheon et al., 2009; McCutcheon and Moran, 2007; and McCutcheon and Moran, 2010). As a consequence, each symbiont supplies the required nutrients for both the other bacterium and the host (Figure A7-2B).

The "perfect" metabolic complementarity in these insect symbioses is a product of the reciprocal coevolutionary changes in the host and symbiont(s), accompanied by genome reduction of the symbionts. Both of these latter traits are facilitated by the persistent obligate vertical transmission of the symbionts via the egg, for example, over an estimated 160 million years for *Buchnera* and

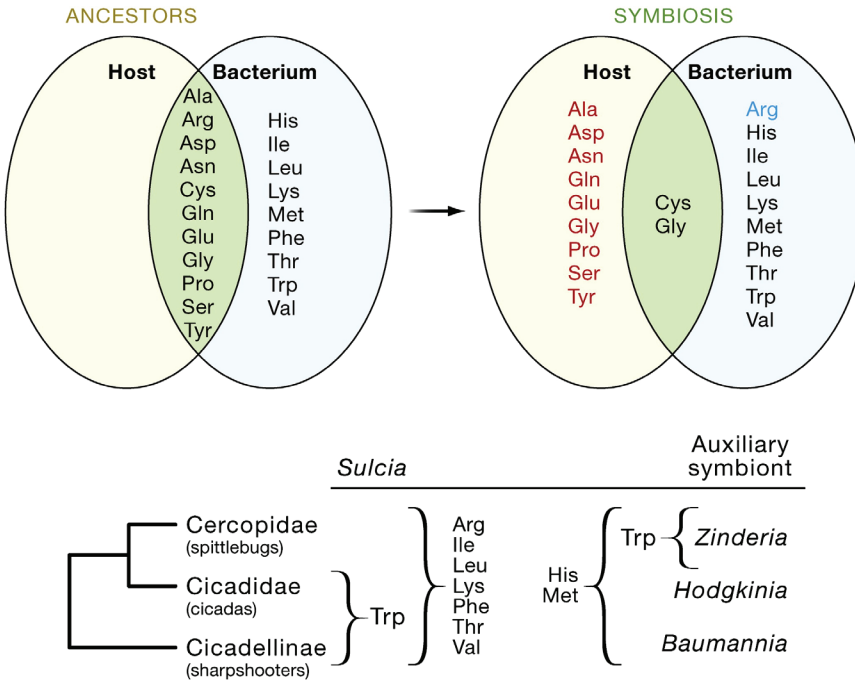


FIGURE A7-2 Coevolution of amino acid biosynthesis in plant sap–feeding insects and their bacterial symbionts. (A) In the symbiosis between the pea aphid and *Buchnera* bacteria, the *Buchnera* (but not aphid) has lost the capacity to synthesize eight amino acids (red), and the aphid (but not *Buchnera*) has lost the capacity to synthesize arginine (blue) relative to the ancestral condition (*E. coli*, a relative of *Buchnera*, and a generalized insect, respectively). The one-reaction synthesis of glycine from serine is retained by both partners. Cysteine is synthesized by different pathways in the two partners (from methionine in the aphid, from serine in *Buchnera*). (B) Spittlebugs, cicadas, and sharpshooters bear two bacterial symbionts, *Sulcia* and an auxiliary symbiont with complementary amino acid biosynthetic capabilities. The amino acids produced by each bacterium are made available to both the insect host and the alternative bacterium. Both bacteria require the ten nonessential amino acids from the host.

Note: ala: alanine; arg: arginine; asn: asparagine; asp: aspartate; cys: cysteine; gln: glutamaine; glu: glutamate; gly: glycine; his: histidine; ile: isoleucine; leu: leucine; lys: lysine; met: methionine; phe: phenylalanine; pro: proline; ser: serine; thr: threonine; trp: tryptophan; tyr: tyrosine; val: valine.

260 million years for *Sulcia* (Moran et al., 1993; and Moran et al., 2005). The continuous interaction between individual lineages of host and symbiont provides the basis for both genome reduction of the symbiont (by relaxed selection and genome deterioration) and strict reciprocal coevolution.

These associations provide an important lesson in the power of coevolutionary interactions in shaping the functional traits of animals and their resident

microbes. Nevertheless, we should not necessarily anticipate precisely equivalent relationships in mammals because no resident microorganisms of mammals are known to be obligately vertically transmitted and the coevolutionary interactions are more diffuse, i.e., likely to involve guilds of microbes (Oh et al., 2010).

Current and Future Lessons from Insect Symbioses

Insect symbioses offer various clear-cut exemplars of processes underlying interactions between animals and their resident microbiota. Most of these interactions are unlikely to be replicated precisely in mammalian systems, simply because symbioses in mammals and insects are different. Their principal value to researchers investigating mammalian systems is conceptual, demonstrating, for example, how the persistence of microorganisms can be shaped by modulation of the innate immune system (Figure A7-1) or how the metabolic capabilities of hosts and multiple microorganisms can coevolve to perfect complementarity (Figure A7-2). The low diversity of microorganisms in most insects, compared to mammals, is important for the clarity of these exemplars. For insects, it is generally possible to couple microbial function to one (or several) well-defined microbial taxa, and functional redundancy is minimal.

The key traits of low diversity and minimal functional redundancy in the microbiota of many insects provide the opportunity to exploit insect systems to investigate fundamental problems in animal-microbial interactions that are impractical or unfeasible to investigate in mammalian systems. From insect-based research, specific hypotheses can be generated for subsequent analysis in biomedical rodent models or in human trials. The opportunities relate especially to the link between the gut microbiota and various human diseases, including metabolic syndrome (obesity, type 2 diabetes, and cardiovascular disease), Crohn's disease, and related immunological dysfunction (Lee and Mazmanian, 2010; and Wang et al., 2011). The causal networks underpinning these diseases are difficult to disentangle because the mammalian microbiota is complex and variable. Various insect systems have potential value. *Drosophila melanogaster* offers a particularly strong model because the low diversity of its gut microbiota (Cox and Gilmore, 2007; Roh et al., 2008; and Wong et al., 2011) can be harnessed with the unparalleled set of *Drosophila* genetic resources and tools. Insect symbioses can certainly provide many valuable lessons to be learned, but their greatest value is not yet realized. We should anticipate a shift in the curriculum toward the explicit exploitation of insect symbioses as models for biomedical research on the role of the resident microbiota in health and disease.

Acknowledgments

This work was supported by NIH grant 1R01GM095372-01, NSF grant IOS-0919765, and the Sarkaria Institute for Insect Physiology and Toxicology.

References

Aanen, D.K., and Hoekstra, R.F. (2007). The evolution of obligate mutualism: if you can't beat 'em, join 'em. Trends Ecol. Evol. (Amst.) 22, 506–509.

Akman Gündüz, E., and Douglas, A.E. (2009). Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc. Biol. Sci. 276, 987–991.

Bakula, M. (1969). The persistence of a microbial flora during postembryogenesis of Drosophila melanogaster. J. Invertebr. Pathol. 14, 365–374.

Brugerolle, G., and Radek, R. (2006). Symbiotic protozoa of termites. In *Intestinal Microorganisms of Soil Invertebrates*, H. König and A. Varma, eds. (Berlin: Springer-Verlag), pp. 243–269.

Brummel, T., Ching, A., Seroude, L., Simon, A.F., and Benzer, S. (2004). Drosophila lifespan enhancement by exogenous bacteria. Proc. Natl. Acad. Sci. USA 101, 12974–12979.

Burnum, K.E., Callister, S.J., Nicora, C.D., Purvine, S.O., Hugenholtz, P., Warnecke, F., Scheffrahn, R.H., Smith, R.D., and Lipton, M.S. (2011). Proteome insights into the symbiotic relationship between a captive colony of *Nasutitermes corniger* and its hindgut microbiome. ISME J. 5, 161–164.

Chandler, S.M., Wilkinson, T.L., and Douglas, A.E. (2008). Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. Proc. Biol. Sci. 275, 565–570.

Cirimotich, C.M., Dong, Y., Garver, L.S., Sim, S., and Dimopoulos, G. (2010). Mosquito immune defenses against Plasmodium infection. Dev. Comp. Immunol. 34, 387–395.

Connell, J.H. (1978). Diversity in tropical rain forests and coral reefs. Science 199, 1302–1310.

Cox, C.R., and Gilmore, M.S. (2007). Native microbial colonization of Drosophila melanogaster and its use as a model of Enterococcus faecalis pathogenesis. Infect. Immun. 75, 1565–1576.

Dale, C., and Moran, N.A. (2006). Molecular interactions between bacterial symbionts and their hosts. Cell 126, 453–465.

Dethlefsen, L., McFall-Ngai, M., and Relman, D.A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811–818.

Dillon, R.J., and Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49, 71–92.

Dong, Y., Manfredini, F., and Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 5, e1000423.

Douglas, A.E. (2007). Symbiotic microorganisms: untapped resources for insect pest control. Trends Biotechnol. 25, 338–342.

Douglas, A.E. (2009). The microbial dimension in insect nutritional ecology. Funct. Ecol. 23, 38–47.

Duan, J., Chung, H., Troy, E., and Kasper, D.L. (2010). Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. Cell Host Microbe 7, 140–150.

Ewald, P.W. (1994). Evolution of Infectious Diseases (Oxford, UK: Oxford University Press).

Falk, P.G., Hooper, L.V., Midtvedt, T., and Gordon, J.I. (1998). Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. Microbiol. Mol. Biol. Rev. 62, 1157–1170.

Fraune, S., Augustin, R., Anton-Erxleben, F., Wittlieb, J., Gelhaus, C., Klimovich, V.B., Samoilovich, M.P., and Bosch, T.C. (2010). In an early branching metazoan, bacterial colonization of the embryo is controlled by maternal antimicrobial peptides. Proc. Natl. Acad. Sci. USA 107, 18067–18072.

Fukatsu, T., and Hosokawa, T. (2002). Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. 68, 389–396.

Garver, L.S., Xi, Z., and Dimopoulos, G. (2008). Immunoglobulin superfamily members play an important role in the mosquito immune system. Dev. Comp. Immunol. 32, 519–531.

Gerardo, N.M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S.M., de Vos, M., Duncan, E.J., Evans, J.D., Gabaldón, T., Ghanim, M., et al. (2010). Immunity and other defenses in pea aphids, Acyrthosiphon pisum. Genome Biol. 11, R21.

Hansen, A.K., and Moran, N.A. (2011). Aphid genome expression reveals hostsymbiont cooperation in the production of amino acids. Proc. Natl. Acad. Sci. USA 108, 2849–2854.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2008). Wolbachia and virus protection in insects. Science 322, 702.

Hosokawa, T., Kikuchi, Y., and Fukatsu, T. (2007). How many symbionts are provided by mothers, acquired by offspring, and needed for successful vertical transmission in an obligate insect-bacterium mutualism? Mol. Ecol. 16, 5316–5325.

Hussain, M., Frentiu, F.D., Moreira, L.A., O'Neill, S.L., and Asgari, S. (2011). Wolbachia uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector Aedes aegypti. Proc. Natl. Acad. Sci. USA 108, 9250–9255.

International Aphid Genomics Consortium. (2010). Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol. 8, e1000313.

Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M., and Perlman, S.J. (2010). Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. Science 329, 212–215.

Kaltenpoth, M., Göttler, W., Herzner, G., and Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. Curr. Biol. 15, 475–479.

Kerney, R., Kim, E., Hangarter, R.P., Heiss, A.A., Bishop, C.D., and Hall, B.K. (2011). Intracellular invasion of green algae in a salamander host. Proc. Natl.Acad. Sci. USA 108, 6497–6502.

Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B., and Vavre, F. (2009). Wolbachia interferes with ferritin expression and iron metabolism ininsects. PLoS Pathog. 5, e1000630.

Kroiss, J., Kaltenpoth, M., Schneider, B., Schwinger, M.G., Hertweck, C., Maddula, R.K., Strohm, E., and Svatos, A. (2010). Symbiotic Streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nat. Chem.Biol. 6, 261–263.

Lee, Y.K., and Mazmanian, S.K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330, 1768–1773.

Lemaitre, B., and Hoffmann, J. (2007). The host defense of Drosophila melanogaster. Annu. Rev. Immunol. 25, 697–743.

MacDonald, S.J., Thomas, G.H., and Douglas, A.E. (2011). Genetic and metabolic determinants of nutritional phenotype in an insect-bacterial symbiosis. Mol. Ecol. 20, 2073–2084.

McCutcheon, J.P., and Moran, N.A. (2007). Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc. Natl. Acad. Sci. USA 104, 19392–19397.

McCutcheon, J.P., and Moran, N.A. (2010). Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. Genome Biol Evol 2, 708–718.

McCutcheon, J.P., McDonald, B.R., and Moran, N.A. (2009). Convergent evolution of metabolic roles in bacterial co-symbionts of insects. Proc. Natl. Acad. Sci. USA 106, 15394–15399.

McFall-Ngai, M.J. (2002). Unseen forces: the influence of bacteria on animal development. Dev. Biol. 242, 1–14.

McFall-Ngai, M. (2007). Adaptive immunity: care for the community. Nature 445, 153.

McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2002). Wolbachia density and virulence attenuation after transfer into a novel host. Proc. Natl. Acad. Sci. USA 99, 2918–2923.

Morales-Jiménez, J., Zùñiga, G., Villa-Tanaca, L., and Hernández-Rodríguez, C. (2009). Bacterial community and nitrogen fixation in the red turpentine beetle, Dendroctonus valens LeConte (Coleoptera: Curculionidae: Scolytinae). Microb. Ecol. 58, 879–891.

Moran, N.A., Munson, M.A., Baumann, P., and Ishikawa, H. (1993). A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. Biol. Sci. 253, 167–171.

Moran, N.A., Tran, P., and Gerardo, N.M. (2005). Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Appl. Environ. Microbiol. 71, 8802–8810.

Mueller, U.G., Dash, D., Rabeling, C., and Rodrigues, A. (2008). Coevolution between attine ants and actinomycete bacteria: a reevaluation. Evolution 62, 2894–2912.

Nemergut, D.R., Costello, E.K., Hamady, M., Lozupone, C., Jiang, L., Schmidt, S.K., Fierer, N., Townsend, A.R., Cleveland, C.C., Stanish, L., and Knight, R. (2011). Global patterns in the biogeography of bacterial taxa. Environ. Microbiol. 13, 135–144.

Niess, J.H., Leithäuser, F., Adler, G., and Reimann, J. (2008). Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. J. Immunol. 180, 559–568.

Oh, D.C., Poulsen, M., Currie, C.R., and Clardy, J. (2009). Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nat. Chem. Biol. 5, 391–393.

Oh, P.L., Benson, A.K., Peterson, D.A., Patil, P.B., Moriyama, E.N., Roos, S., and Walter, J. (2010). Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. ISME J. 4, 377–387.

Oliver, K.M., Degnan, P.H., Hunter, M.S., and Moran, N.A. (2009). Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325, 992–994.

Oliver, K.M., Degnan, P.H., Burke, G.R., and Moran, N.A. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu. Rev. Entomol. 55, 247–266.

Pannebakker, B.A., Loppin, B., Elemans, C.P., Humblot, L., and Vavre, F. (2007). Parasitic inhibition of cell death facilitates symbiosis. Proc. Natl. Acad. Sci. USA 104, 213–215.

Poliakov, A., Russell, C.W., Ponnala, L., Hoops, H.J., Sun, Q., Douglas, A.E., and van Wijk, K.J. (2011). Large-scale label-free quantitative proteomics of the pea aphid-Buchnera symbiosis. Mol. Cell Proteomics 10, M110.007039.

Pollard, M., and Sharon, N. (1970). Responses of the Peyer's Patches in Germ-Free Mice to Antigenic Stimulation. Infect. Immun. 2, 96–100.

Ren, C., Webster, P., Finkel, S.E., and Tower, J. (2007). Increased internal and external bacterial load during Drosophila aging without life-span trade-off. Cell Metab. 6, 144–152.

Reymond, N., Calevro, F., Viñuelas, J., Morin, N., Rahbé, Y., Febvay, G., Laugier, C., Douglas, A.E., Fayard, J.-M., and Charles, H. (2006). Different levels of transcriptional regulation due to trophic constraints in the reduced genome of Buchnera aphidicola APS. Appl. Environ. Microbiol. 72, 7760–7766.

Robinson, C.J., Schloss, P., Ramos, Y., Raffa, K., and Handelsman, J. (2010). Robustness of the bacterial community in the cabbage white butterfly larval midgut. Microb. Ecol. 59, 199–211.

Roh, S.W., Nam, Y.D., Chang, H.W., Kim, K.H., Kim, M.S., Ryu, J.H., Kim, S.H., Lee, W.J., and Bae, J.W. (2008). Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in Drosophila melanogaster. Appl. Environ. Microbiol. 74, 6171–6177.

Rolff, J. (2007). Why did the acquired immune system of vertebrates evolve? Dev. Comp. Immunol. 31, 476–482.

Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A., and Mazmanian, S.K. (2011). The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science 332, 974–977.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science 319, 777–782.

Sackton, T.B., Lazzaro, B.P., Schlenke, T.A., Evans, J.D., Hultmark, D., and Clark, A.G. (2007). Dynamic evolution of the innate immune system in Drosophila. Nat. Genet. 39, 1461–1468.

Scarborough, C.L., Ferrari, J., and Godfray, H.C. (2005). Aphid protected from pathogen by endosymbiont. Science 310, 1781.

Scott, J.J., Oh, D.C., Yuceer, M.C., Klepzig, K.D., Clardy, J., and Currie, C.R. (2008). Bacterial protection of beetle-fungus mutualism. Science 322, 63.

Shanbhag, S., and Tripathi, S. (2009). Epithelial ultrastructure and cellular mechanisms of acid and base transport in the Drosophila midgut. J. Exp. Biol. 212, 1731–1744.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature 407, 81–86.

Stappenbeck, T.S., Hooper, L.V., and Gordon, J.I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc. Natl. Acad. Sci. USA 99, 15451–15455.

Vogel, K.J., and Moran, N.A. (2011). Sources of variation in dietary requirements in an obligate nutritional symbiosis. Proc. Biol. Sci. 278, 115–121.

Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., Dugar, B., Feldstein, A.E., Britt, E.B., Fu, X., Chung, Y.M., et al. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472, 57–63.

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T.H., Stege, J.T., Cayouette, M., McHardy, A.C., Djordjevic, G., Aboushadi, N., et al. (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450, 560–565.

Watanabe, H., and Tokuda, G. (2010). Cellulolytic systems in insects. Annu. Rev. Entomol. 55, 609–632.

Werren, J.H., Baldo, L., and Clark, M.E. (2008). Wolbachia: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.

Wieczorek, H., Beyenbach, K.W., Huss, M., and Vitavska, O. (2009). Vacuolartype proton pumps in insect epithelia. J. Exp. Biol. 212, 1611–1619.

Wilson, A.C., Dunbar, H.E., Davis, G.K., Hunter, W.B., Stern, D.L., and Moran, N.A. (2006). A dual-genome microarray for the pea aphid, Acyrthosiphon pisum, and its obligate bacterial symbiont, Buchnera aphidicola. BMC Genomics 7, 50.

Wong, C.N., Ng, P., and Douglas, A.E. (2011). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. 13, 1889–1900.

Xie, J., Vilchez, I., and Mateos, M. (2010). Spiroplasma bacteria enhance survival of Drosophila hydei attacked by the parasitic wasp Leptopilina heterotoma. PLoS ONE 5, e12149.

A8

A NEW VISION OF IMMUNITY: HOMEOSTASIS OF THE SUPERORGANISM[30]

*Gérard **Eberl***[31,32]

Abstract

The immune system is commonly perceived as an army of organs, tissues, cells, and molecules that protect from disease by eliminating pathogens. However, as in human society, a clear definition of good and evil might be sometimes difficult to achieve. Not only do we live in contact with a multitude of microbes, but we also live *with* billions of symbionts that span all

[30] Eberl, G. 2010. A new vision of immunity: Homeostasis of the super organism. *Mucosal Immunology* 3:450–460. Reprinted with kind permission from Nature Publishing Group.

[31] Department of Immunology, Institut Pasteur, Lymphoid Tissue Development Unit, Paris, France

[32] CNRS URA1961, Paris, France.

Correspondence: G Eberl (gerard.eberl@pasteur.fr).

the shades from mutualists to potential killers. Together, we compose a superorganism that is capable of optimal living. In that context, the immune system is not a killer, but rather a force that shapes homeostasis within the superorganism.

Introduction

The capacity to discriminate between good and evil is crucial for survival. Our senses inform us on the nature of the environment and generate negative feelings of fear and disgust, or positive feelings of comfort and love. Accordingly, decisions are taken to follow a secure path. Societies have developed vast cultures to define good and evil to guarantee the survival of the system, be it a religion, a nation, or a political party. An extreme version of such a culture is Manichaeism, created during the third century AD in Babylon by the prophet Mani, and successfully spread within decades to all corners of the known world. Manichaeism describes the cosmological struggle between the good spiritual world of light and the evil material world of darkness, and prescribes how human society should guide this struggle to its resolution by separating good from evil. As esoteric as Manichaeism theology might sound, its dualistic principle seems nevertheless to pervade our usual perception and description of the world, and thereby to shape microbiology and immunology alike.

At the heart of the dualistic view in microbiology and immunology is the "germ theory of disease" or "pathogenic theory of medicine," stating that microbes are the cause of a range of diseases. The first mention of such a theory goes back to 36 BC when Marcus Terentius Varro, a Roman scholar, wrote that "certain minute creatures, which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose, and there cause serious diseases" (Cato and Varro, 1934). Following up in 1020, the Persian polymath Avicenna stated in *The Canon of Medicine* that bodily secretions are contaminated by "foul foreign earthly bodies" before a person becomes infected (Syed, 2002). The formal demonstration of this theory by Robert Koch and Louis Pasteur in the late 19th century marks the birth of microbiology, hygiene, and vaccine development (Baxter, 2001). However, in the early 20th century, Ilya Metchnikoff proposed that bacteria are also agents of good. He suggested that lactic acid-producing bacteria prolong life by inhibiting the growth of putrefactive (proteolytic) bacteria, such as common gut microbiota members of the Clostridia class, which produce toxic substances and might provoke intestinal "auto-intoxication" (Metchnikoff, 2004). The term "*probiotic,*" defined as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance," was coined in 1953 by Werner Kollath to contrast with antibiotics (Vergin, 1954). The use of probiotics in a number of ailments, including inflammatory bowel disease (IBD) and cancer, has shown some level of protective effects by strains

of the *Lactobacillus*, *Bifidobacteria*, *Bacteroides*, *Escherichia*, and *Faecalibacterium* genera (Round and Mazmanian, 2009).

The discovery of innate immune receptors or pattern-recognition receptors (PRRs) in the late 1990s marks a fundamental turn in immunology (Lemaitre et al., 1996; Medzhitov and Janway, 2000). PRRs recognize microbe-associated molecular patterns (MAMPs), thereby allowing the immune system to detect microbes and act accordingly. They are believed to universally elicit rejection and lead to antimicrobial immunity. Reflecting this view, MAMPs are in general termed PAMPs for pathogen-associated molecular patterns (Vance et al., 2009). Thereby, immunology has assigned to the immune system a dualistic perception of the world, aiming it at the detection and destruction of microbes. The inevitable corollary of this view posits that activation of the immune system leads or at least aims at destruction of the activator, and thus defines the activator as a pathogen. However, immune responses come in different blends, best characterized by generation of proinflammatory interferon-γ-producing Th1 cells and interleukin-17 (IL-17)-producing Th17 cells; IgE- and allergy-promoting Th2 cells; and anti-inflammatory IL-10-producing or $Foxp3^+$ regulatory T cells (Weaver et al., 2007). Therefore, the current view holds that pathogens elicit proinflammatory responses, whereas innocuous microbes, such as probiotics, elicit anti-inflammatory responses if detected at all (Sansonetti, 2004). Considerable efforts are made to understand the distinction between pathogens and innocuous microbes, which point to differences in the structure and expression of PAMPs, virulence factors, and invasive properties (Vance et al., 2009).

Here, I challenge this dualistic view of microbes and of the immune system. I propose instead that microbes navigate between shades of good and evil, a position that is determined during interaction with the host, and a position that can change with host, tissue, and time. Facing the microbes, the immune system does not react to combat evil, but merely shapes the microbial environment to allow the organism to live with the microbes. It is not a fight between good and evil, it is rather an equilibrium between microbes and host that generates a superorganism (Gill et al., 2006).

The Superorganism

The Role of the Symbiotic Microbiota

The human intestine hosts an astronomical 10^{14} bacteria, roughly 100 times the number of cells in our body, and close to 1,000 distinct species (Backhed et al., 2005), not taking in account archea, fungi, and viruses. This microbiota is usually termed commensal, even though there is a considerable degree of mutualism with the host (Box A8-1). The microbes benefit from a selective environment that is regularly flooded with nutrients, and the host benefits from microbial activity that complements its digestive pathways, degrades xenobiotics, regulates epithelial homeostasis (Buchon et al., 2009), and provides a barrier

against potential pathogens (Benson et al., 2009; Brandl et al., 2008; Duerkop et al., 2009; Turnbaugh et al., 2007). Microbial communities reside on all body surfaces, including the entire length of the digestive tract, the vagina, and the skin. Altogether, the partnership of the host with its microbiota can be described as a new functional entity termed a *superorganism* (Figure A8-1). This superorganism encodes ~2×10^4 host genes and an estimated ~10^6 microbial genes (Backhed et al., 2005; Gill et al., 2006), and in addition to the mammalian metabolic pathways, operates a plethora of microbial metabolic pathways collectively termed the metabolome (Turnbaugh and Gordon, 2008).

The effect of the microbiota extends beyond complementation of the host. In germfree mice, the intestinal immune system is underdeveloped (Round and Mazmanian, 2009). Lymphoid tissues such as Peyer's patches (PPs), mesenteric lymph nodes (LNs), and the splenic white pulp remain small, and isolated lymphoid follicles (ILFs), present in hundreds in the lamina propria of normal mice, fail to develop (Bouskra et al., 2008). Furthermore, intestinal lymphocyte populations are markedly reduced, including intra-epithelial T cells, lamina propria T cells, and IgA-producing B cells, and pro-inflammatory Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Niess et al., 2008) and innate IL-22-producing natural killer (NK)-like cells fail to be recruited or generated (Sanos et al., 2009; Satoh-Takayama et al., 2008). Expression of antibacterial peptides by Paneth cells is also reduced (Cash et al., 2006). Thus, the intestinal microbiota, mostly studied at the bacterial level, has an important role in the development and maturation of the immune system. Evidence accumulates that the collection of viruses, or virome, that stably infects the host, also has a major role in shaping the immune system, as discussed recently by Virgin et al. (2009).

Evolution of the Superorganism

Organisms are selected by their fitness within a particular environment. Traditionally, an organism is defined by its genome, and inter-individual competition sorts out the fittest genome. However, selection might also operate at higher levels of organization, such as the group where the social abilities of individuals may provide a collective advantage to the group and hence to each individual of the group (Wynne-Edwards, 1962). At a lower level, the mutualistic partnership between a host and its microbiota provides digestive, protective, and immune advantages to the superorganism. Selection can operate both at the individual host and microbial levels to evolve the best partnership, and the best partnership can be selected to evolve the fittest superorganism. Reflecting this selection process, only few bacterial phyla successfully colonize the human intestine, and the majority of bacterial species are members of either the *Firmicutes* or the *Bacteroidetes* phyla (Dethlefsen et al., 2007). On the host's side, a restricted number (~50 known) of PRRs define the universe of MAMPs that are sensed by the innate immune system (Brown, 2006; Fritz et al., 2006; Kawai and Akira, 2009), a limited reactivity that is nevertheless expected to provide a broad vision of the microbial world to

BOX A8-1
Definitions

The recent literature is relatively confusing regarding its usage of the terms "*symbiosis*" and "*commensals*." Symbiosis is generally understood as a relationship between two organisms from which both organisms benefit. In that definition, symbiosis is synonymous with mutualism (Wilkinson, 2001).Furthermore, qualified as commensals are microbes living within a host. However, a number of such commensals are actually mutualists, helping for example in digestion and others are opportunistic pathogens, such as *Staphylococcus aureus* on the skin. Thus, the usage of the term "*commensal*" in that context may not be adequate. In this review, I use the original definitions of these terms to categorize the different types of microbes in their relationship with the host.

Symbiosis

Originated from the Greek words *syn* and *biosis*, meaning "with" and "living." The original meaning of symbiosis is the "living together of unlike organisms," first coined in 1879 by the mycologist Heinrich Anton de Bary (Wilkinson, 2001). The common usage of the term "*commensal*" should therefore be replaced by "*symbiont*" to designate the microbiota living within a host. The symbiotic relationships can be formally categorized as mutualistic, commensal, or parasitic, even though parasites are rarely considered symbionts.

Mutualism

Originated from the Latin word *mutuus*, meaning lent, borrowed, or mutual. A relationship between two organisms where both organisms benefit. For example,

the host. Beyond innate immunity, a virtually unlimited number of receptors are generated by lymphocytes to fill any potential gaps, a diversity suggested to allow vertebrate organisms to host a more complex microbiota (McFall-Ngai, 2007). Furthermore, the immune system must have co-evolved with the microbiota to adjust its reactivity and maintain homeostasis of the superorganism. In conclusion, the dualistic view that separates the host from its microbiota is of course valid in terms of separation of the genomes, but appears to be overruled at the functional level in the superorganism (Box A8-2).

Microbes and Immunity

The Nature of Microbes

Given the proximity with so many microbes, it is expected that we are in constant danger of invasion, and that lowering our guard should be dangerous. As

bacteria that expand in the intestinal niche and provide metabolic pathways complementing the digestive functions of the host.

Commensalism

Originated from the Latin word *cum mensa*, meaning "sharing a table." A relationship between two organisms where one organism benefits but the other is unaffected. Microbes that expand in the intestine obviously benefit from the intestinal niche. However, it is more difficult to ascertain that microbes have no effect on the host, so as to be described as commensals.

Parasitism

Originated from the Greek words *para* and *sitos*, meaning "beside" and "food," or one who eats at another's table. A relationship between two organisms where one organism benefits at the expense of the other. Defines the behavior of a pathogen.

Superorganism

Originated from the Latin word *supra*, meaning "above," and the Greek word *organon*, meaning "organ, instrument, tool." In biology, an organism is a living system capable of autonomous metabolism and reproduction. A superorganism is a living system of a superior degree of complexity, consisting of many organisms. It may be defined more generally as a "collection of agents that can act in concert to produce phenomena governed by the collective." Examples of superorganisms include ants and termite societies.

a matter of fact, immunosuppressed individuals are prone to opportunistic infections by ubiquitous and otherwise symbiotic microbes (Slack et al., 2009; Virgin et al., 2009). It seems, therefore, safe to state that the immune system protects us from microbe invasion. However, it is an inaccurate leap forward to state that the immune system protects us from pathogens. How could it define a pathogen? Does a microbe express markers of pathogens before attack? This fundamental question is at the root of the current vision to develop immunotherapies and vaccines (Sansonetti and Medzhitov, 2009; Vance et al., 2009). The discovery of PRRs suggested indeed that expression of PAMPs defines a pathogen, but this view is problematic. Toll-like receptor-4 (TLR-4), which recognizes lipopolysaccharides (LPS), a component of the outer membrane of gram-negative bacteria, best illustrates this problem. Given its wide expression, LPS can hardly be defined as a PAMP. Nevertheless, it has been shown that LPS expressed by prominent symbionts, such as *Bacteroides*, is modified and poorly recognized by TLR-4 (Sansonetti, 2004; Weintraub et al., 1989). However, potential pathogens such as

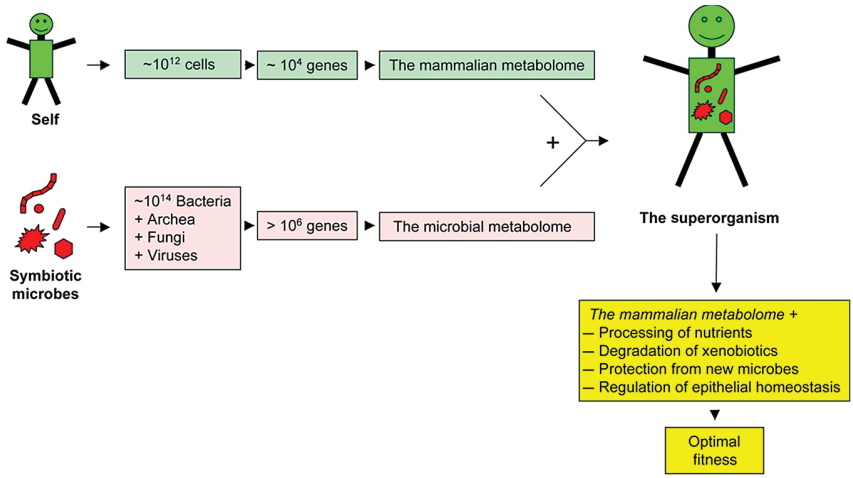


FIGURE A8-1 The superorganism. In the context discussed here, the superorganism is the composite of a host with its symbiotic microbiota. These two worlds have evolved to live together and establish an equilibrium that optimizes the fitness of the superorganism, and thereby the fitness of both the host and the members of the microbiota. The microbial metabolome (Turnbaugh and Gordon, 2008) complements the mammalian metabolome in a number of functions best described in the intestine (Turnbaugh et al., 2007), whereas the mammalian metabolome forms a niche that allows survival of selected microbes.

Helicobacter pylori (Tran et al., 2005) and *Porpyromonas gingivalis* (Darveau et al., 2004) also express variant forms of LPS that evade efficient recognition by TLR-4. Therefore, the term MAMPs seems to better describe the nature of bacterial moieties recognized by PRRs (Mackey and McFall, 2006; Vance et al., 2009), and cannot be the basis of pathogen recognition.

Recently, it was suggested that pathogenic microbes are recognized by "patterns of pathogenesis" (Vance et al., 2009), or POPs. Rather than by its structure, a pathogen would be defined by its characteristic behavior. A first POP is growth, as pathogens are able to grow in their host upon invasion. A second POP is cytosolic invasion, as many pathogens deliver active proteins, or virulence factors, into the cell host through syringe-like secretion systems or pores, which interfere with activation of the immune system (Sansonetti and Di Santo, 2007). A third proposed POP is disruption of the normal function of the host cell cytoskeleton, as bacterial pathogens and viruses have been shown to exploit the actin network to move within and between cells. During such pathogenic behaviors, MAMPs access forbidden compartments of host tissues and cells. In such a context, MAMPs would be recognized by PRRs as genuine PAMPs and elicit an appropriate inflammatory immune response. In accordance with this view, many PRRs are segregated to react only within a POP context (Barton and Kagan, 2009). In

BOX A8-2
Predictions

The value of a concept or a model is measured by its predictive power. Therefore, what is the predictive power of the *superorganism* concept? What does the continuum model add to the classical dualistic models of immunity? Some leads are proposed here.

The Superorganism

The host and its symbiotic microbiota constitute a superorganism with superior efficiency in digestion, defense, and detoxification, to list a few items, as compared with the bare host (Figure A8-1). Many systems are actually affected in germfree mice (Smith et al., 2007), and therefore, the concept of superorganism is largely validated. Importantly the intestinal microbiota not only affects the intestine but also more distant organs, such as the pancreas (Wen et al., 2008), and the hypothalamic–pituitary–adrenal axis during stress response in mice (Sudo et al., 2004). The mechanism leading to such effects is still a matter of speculation, but a recent paper shows that microbiota-derived peptidoglycans are present in the serum and bone marrow where they enhance neutrophil function (Clarke et al., 2010). Thus, it is predicted, and testable, that homeostasis of the superorganism not only requires an extensive local crosstalk between symbionts and the immune system, but also a more general systemic effect of microbiota based on invaders or circulating MAMPs.

The Continuum Model

The continuum model questions the dualistic view of the immune system based on self/non-self discrimination or physiologic versus pathological inflammation (Figure A8-2). Instead, it proposes that triggers of immunity, microbes for that matter, and the immune responses they elicit are best described by a continuum of properties. The type of microbes and immune responses are defined by their position on this continuum, and the properties of individual microbes can vary between the two extremes depending on their interaction with the host. Thus it is predicted, and already documented (Barton et al., 2007; Brandl et al., 2008; Virgin, 2009), that microbes perceived as pathogens may become mutualists and that microbes perceived as mutualists may become pathogens, depending on the context. Another consequence of the continuum model is that proinflammatory immunity, which is viewed as elicited during a pathological situation, is also required to maintain steady state. Similarly, regulatory immunity is required to maintain steady state but is also involved during microbial infection to establish long-term resistance (Belkaid et al., 2002). It is predicted that blocking proinflammatory immunity during steady state may lead to loss of homeostasis and, conversely, that blocking regulatory immunity during pathology may lead to loss of homeostasis during steady state.

the intestine, TLR-5 is expressed mainly on the basolateral side of the intestinal epithelial cells (Gewirtz et al., 2001) and on dendritic cells in the intestinal lamina propria (Uematsu et al., 2006). Thus TLR-5 is not contacted by its flagellin ligand that is ubiquitous in the intestinal lumen, but by bacteria that cross the epithelial barrier and invade the forbidden compartment of the lamina propria. Some PRRs are only expressed within the cytosol, such as NOD-1 and NOD-2, as well as a number of components of the inflammasome and nucleic acid-recognizing molecules, and can only react to MAMPs that penetrate the cell (Vance et al., 2009). The definition of PAMPs in that context is nevertheless complicated by the existence of membrane transporters encoded by the host, as in the case of NOD-2 (Vavricka et al., 2004), or endosome portals for both NOD-1 and NOD-2 (Lee et al., 2009) that can translocate MAMPs into the cytosol.

The "patterns of pathogenesis" POP hypothesis was proposed with the aim to understand how the immune system recognizes pathogens, and thus was framed within a dualistic view of microbes and immunity (Vance et al., 2009). It can be taken a step further to suggest that a POP does not define a pathogen, but merely a pathogenic behavior that can be adopted by any microbe. The distinction may seem subtle, but allows a particular microbe to be considered a mutualist or a pathogen, depending on time and context. In that view, a pathogenic microbe is not a pathogen *per se*. Its pathogenicity is determined by its interaction with the host, and can change with host, location in the host, and the immune state of the host. The interaction between a microbe and its host determines the position of the microbe on the scale of mutualists to pathogens, and its position on the scale can change continuously. Many examples exist of bacteria and viruses (Virgin et al., 2009) that can change sides, such as the symbiotic intestinal bacteria that become a source of pathogens during colitis (Sartor, 2008), and pathogenic herpesviruses that cause an acute infection of the host before turning into a helpful ally against bacterial infection during latency (Barton et al., 2007). These and other examples will be discussed in detail below.

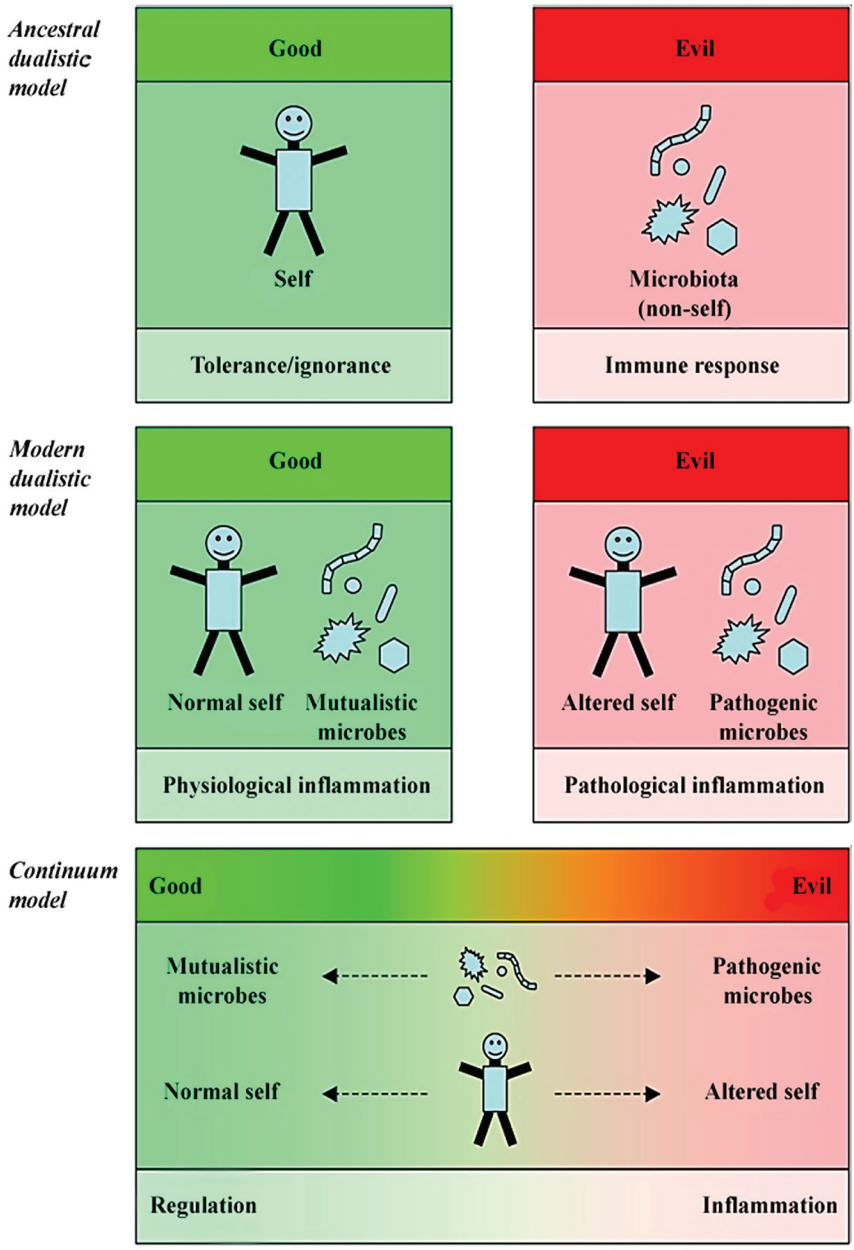
The Nature of Immunity

Microbes, be it bacteria, fungi, or viruses, grow and move. However, the niches offered by the host to the microbes in the gastrointestinal tract, the skin, and elsewhere considerably restrict the type of microbes able to colonize (Dethlefsen et al., 2007). For example, most bacteria colonizing the human intestine are members of only two phyla, the *Firmicutes* and the *Bacteroidetes*. In addition, containment of the microbiota is enforced by multiple epithelial cell layers, such as in the skin, or thin layers of epithelial cells in the digestive tract and lungs, which produce antibacterial peptides and are protected by mucus produced by Goblet cells. In addition, B cells in mucosal surfaces produce large amounts of IgA that are transported across the epithelial barrier and released into the lumen. There, IgA contribute to the containment of microbes beyond and

within the mucus, and to opsonization of microbes for sampling or destruction by phagocytes (Duerkop et al., 2009).

However, the quality of the containment can change, due for example to environmental variations, exposure to chemicals, and injury. Breaches might be generated and microbes might penetrate the forbidden compartments of host tissues and cells. When this happens, the immune system is triggered through PRRs, such as by TLR-5 on the basolateral side of epithelial cells and on phagocytes in the intestinal lamina propria, which activate cascades of proinflammatory pathways (Gewirtz et al., 2001; Uematsu et al., 2006). The consequence is recruitment and activation of polymorphonuclear cells, macrophages, and lymphocytes; elimination of the intrusive microbes; and eventual wound healing. Ideally, containment and homeostasis is re-established. The rules of engagement of proinflammatory immunity are best described by the "danger model," which posits that modification of self, through invasive microbes and injury of tissues and cells, is the ultimate trigger (Matzinger, 1994, 2002). Thus, another dualistic view of immunity has emerged: one arm of immunity is involved in every day's containment of well-behaving symbiotic microbes, whereas another arm of immunity is involved in the elimination of invasive bad-behaving pathogenic microbes that breach containment (Figure A8-2) (Duerkop et al., 2007; Sansonetti, 2004; Sansonetti et al., 2007).

It might be inferred from this view that homeostasis of the superorganism commands the immune system to fight breaches of containment *at all cost*. However, such a conclusion is a dubious leap forward on the role of proinflammatory immunity and the nature of homeostasis in the superorganism. Even though proinflammatory immunity may follow dualistic rules of engagement to fight the pathogenic behavior of microbes, it cannot avoid breaches of containment. Such breaches seem to occur constantly and are really part of homeostasis. Direct evidence of such breaches is difficult to obtain, but the elevated proinflammatory state of the intestinal immune system is well documented. In specific pathogen-free mice, the proportion of proinflammatory Th17 cells producing IL-17, which induces granulopoiesis and recruitment of neutrophils (Fossiez et al., 1996; Jones and Chan, 2002), is significantly higher in the intestine and skin than in any other tissues (Ivanov et al., 2006; Lochner et al., 2008). This state is induced by the intestinal microbiota (Gaboriau-Rothiau et al., 2009; Ivanov et al., 2009) and is regulated by IL-10 to avoid overdrive of intestinal proinflammatory immunity; IL-10-deficient mice suffer from severe colitis (Kuhn et al., 1993). The notion of "physiologic inflammation" has been suggested a hundred years ago by Metchnikoff in the context of homeostatic removal of dead cells (Metchnikoff, 1989), and was more recently discussed by Sansonetti and Di Santo (Sansonetti and Di Santo, 2007) in reference to immunity induced for containment of symbionts, distinct from proinflammatory immunity to pathogens. However, physiologic inflammation, which might be defined as immunity required for maintaining



homeostasis in the superorganism, appears also to include proinflammatory immunity (Box A8-2).

Homeostasis in the Superorganism

Activation of the Immune System During Homeostasis

Homeostasis in the superorganism is defined by the optimal cohabitation of the host with its microbiota. It is a dynamic equilibrium between host and microbes, where growth and movement of the microbes are constantly kept in check by mechanical, chemical, and immunological containment by the host. The dynamic nature of this equilibrium results from variations in the composition of the microbiota, caused by changes in environmental factors such as chemistry, food, and incoming microbes, and variations in the state of the host through mutations, injury, and other forms of stress. The host must have developed a flexible system that adapts to these variations and maintains homeostasis of the superorganism. The immune system perfectly matches this function: it contributes to containment and equilibrium through for example expression of IL-13 that induces production of mucus by Goblet cells (Wills-Karp et al., 1998), expression of IL-22

FIGURE A8-2 The dualistic and the continuum models of the microbial and immunological worlds. The ancestral view of immunology states that the immune system is educated not to react to self and to react to non-self. In that view, self is good and induces tolerance during lymphocyte maturation and selection, or anergy of self-reacting mature lymphocytes. Non-self, including microbes, as well as allotypic and xenotypic tissues, are evil and elicit an immune response aimed at destroying non-self. All can be boiled down to self/non-self discrimination (Moller, 1977; Nossal, 1991). The modern view of immunology states that the immune system reacts primarily to danger signals (Matzinger, 1994, 2002). The immune system is still educated to be tolerant to self, but the notion of self is modified. Good includes normal self and mutualistic microbes, whereas evil includes altered self such as dead cells releasing danger signals and pathogenic microbes that alter the antigenic landscape of normal self. In that context, the normal self induces a physiological level of inflammation that contributes to homeostasis through for example containment of the intestinal microbiota, whereas injury and pathogens induce pathological inflammation that leads to "full-blown" inflammation (Sansonetti and Di Santo, 2007). The continuum model states that the perceived duality of mutualistic and pathogenic microbes, normal and altered self, and regulatory or inflammatory immunity, represents extremes of a continuous reality. Microbes can express different levels of mutualistic or pathogenic properties and these levels can vary during interaction with the host. Similarly, the state of self and of immune responses can navigate between well-described extremes, and the most likely states are combination of these extremes.

that induces the production of antibacterial peptides by epithelial cells (Wolk et al., 2004), and generation of IgA-producing B cells (Duerkop et al., 2009). Furthermore, it can be activated to a higher state of inflammation by breaches in containment that provoke deviations from the equilibrium. Thus, the immune system emerges as a crucial force to maintain homeostasis (Figure A8-3).

In the superorganism, the immune system is never at rest. It is like a spring: the more microbes colonize the host niches or behave like pathogens, the stronger they pull the spring of immunity, and the stronger the spring of immunity pushes the microbes back. In germfree animals, the immune spring is close to rest, but in animals grown in a normal microbial world, the immune spring is always under tension, the tension required to maintain homeostasis. This tension also contributes to defining the niche for symbionts, and thus to select incoming microbes and preserve homeostasis. This principle is nicely illustrated by the following experiment: when mice, and humans, were treated with a mix of antibiotics to eradicate most of their intestinal microbiota, they became highly sensitive to infection by an antibiotic-resistant strain of *Enterococcus* (Brandl et al., 2008). Because of the lack of symbionts, intestinal epithelial cells expressed low levels of antibacterial peptides that normally provide selection against the *Enterococcus* strain. However, resistance was re-established by concomitant treatment of mice with LPS, which binds TLR-4 and re-induces the expression of antibacterial peptides. Thus, the symbiotic microbiota pulls the string of immunity and induces the production of antibacterial peptides that contribute to defining an intestinal niche permissive for symbionts, but mostly toxic for potential pathogens such as antibiotic-resistant *Enterococcus*.

Development of the Immune System for Homeostasis

In the absence of microbiota, the immune system does not fully develop. The set of lymphoid tissues is incomplete and immature, and lymphocytes populations in the intestine are reduced in numbers or fail to develop (Vergin, 1954). Particular members of the intestinal microbiota, such as segmented filamentous bacteria, are potent activators of intestinal T-helper cells, including Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009) and in germfree mice, Th17 cells and NKp46+ cells that produce IL-17 and/or IL-22 are markedly reduced (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Niess et al., 2008; Sanos et al., 2009; Satoh-Takayama et al., 2008). These cytokines have an important role in the containment of microbiota, as IL-17 induces the recruitment of neutrophils (Fossiez et al., 1996) and both cytokines induce the production of antibacterial peptides by epithelial and Paneth cells, such as β-defensins, RegIIIγ, and S100 family members (Ishigame et al., 2009; Liang et al., 2006; Wolk et al., 2004).

The microbiota-induced development of lymphoid tissues is particularly instructive. Secondary lymphoid tissues, such as LNs and PPs, are programmed to develop in the sterile environment of the fetus (Mebius et al., 2003). Lymphoid

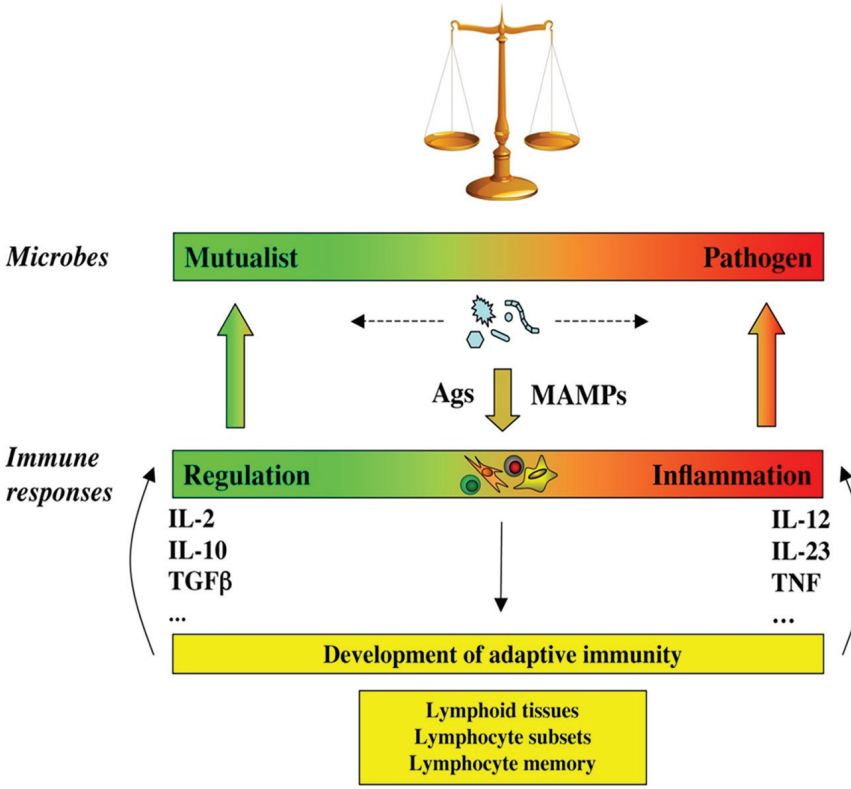


FIGURE A8-3 The continuum of microbial states and immune responses: a dynamic equilibrium. Microbes, including members of the symbiotic microbiota, are not inherently mutualistic or pathogen, but navigate between shades of mutualism and parasitism. Facing the microbes, the immune system is not designed to discriminate between mutualist or pathogens, but merely to react to signals, including MAMPs and antigens. The nature of the immune response is not purely regulatory or inflammatory, but more generally adjusts to the nature of the trigger it faces, like a spring that is pulled by the intensity of the microbial challenge. Furthermore, the immune system has the capacity to evolve when challenged, through generation of different types of lymphocyte subsets such as Th1, Th2, Th17, Treg, Th22 cells (Weaver et al., 2007), and follicular T helper cells (Nurieva et al., 2008), and generation of memory lymphocytes and lymphoid tissues (Round and Mazmanian, 2009). This adds another level of adaptability to the immune system and provides it with the necessary flexibility to maintain homeostasis of the superorganism. MAMP, microbe-associated molecular pattern.

tissue inducer cells are recruited to LN and PP anlagen where they activate specialized stromal cells to produce chemokines and cytokines for subsequent recruitment of B and T cells (Yoshida et al., 1999). After birth, bacterial colonization induces the enlargement of mesenteric LNs and PPs, but does not alter their basic organization, nor are microbes required to maintain this organization. By contrast, ILFs, present in the adult intestinal lamina propria as hundreds of small B-cell follicles, are not programmed during ontogeny, but induced by colonizing microbiota (Hamada et al., 2002). Peptidoglycans shed by dividing bacteria are recognized by NOD-1 in epithelial cells, which then upregulate the expression of β-defensin-3 and of the chemokine CCL20. Both are ligands of CCR6 expressed by lymphoid tissue inducer cells clustered in cryptopatches of the lamina propria. Lymphoid tissue inducer cells in cryptopatches then initiate local lymphoid tissue development and recruitment of CCR6+ B cells (Bouskra et al., 2008). Importantly, ILFs generate IgA-producing B cells that contribute to the containment of the intestinal microbiota, thus establishing a negative feedback loop on their own development (Tsuji et al., 2008). Therefore, ILFs seem to be an optimal system to maintain intestinal homeostasis, as the more bacteria are present in the intestine, the more ILFs are generated that produce IgA, but the more IgA is produced, the less bacteria will be capable of inducing the formation of ILFs. It remains to be established whether a "breach in containment" is required to activate NOD-1 and formation of ILFs, or whether a mechanism of active sampling of luminal content by epithelial cells and transfer to cytosolic NOD-1 is sufficient to activate this pathway (Lee et al., 2009).

Unexpected Mutualists for a Changing Homeostasis

The interaction between a microbe and its host determines the position of the microbe on the scale of mutualists to pathogens, and its position on the scale can change continuously. It may be added that not only the host but also the environment decides the position of the microbe on the scale. Environmental factors may include climate, chemicals, microbes (such as phages), or anything that conditions the fitness of the superorganism. In a changing context, new combinations of microbes and host may yield a competitive advantage to the superorganism, and old foes might become new mutualists. Conversely, old friends may turn bad.

A striking example has been reported in mice infected with a γ-herpesvirus (Barton t al., 2007). Early after infection, γHV68 undergoes lytic replication in a number of cell types, before establishing lifelong latency in memory B cells, macrophages, and dendritic cells. Such a latent infection confers mice resistance to infection with *Listeria monocytogenes* and *Yersinia pestis* through a state of increased and persistent immune activation. It was thus proposed that viral latency can define a mutualistic relationship between the host and the virus. In this case, the superorganism includes a former pathogen that eventually has been brought under control: its status within the superorganism changed from an invasive

pathogen to a contained mutualist. Homeostasis is modified as the proinflammatory state of the immune system is increased, providing an advantage to the superorganism in the face of *Listeria* infection.

Another example is immunological memory. Being exposed constantly to microbes that breach containment, the host develops a collection of memory lymphocytes. The host becomes thereby more reactive to such microbes and alters the niche available to the microbial community. As in the previous example, this evolution of the superorganism is expected to make homeostasis more resistant to pathogenic invasion and thus more robust. Two non-mutually exclusive mechanisms generate and maintain immunological memory: differentiation of long-lived memory lymphocytes during a primary immune response, and latent infection by the microbe or long-lasting antigen depots to uphold specific T- and B-cell activation (Antia et al., 2005; Sprent and Surh, 2002; Welsh et al., 2004; Zinkernagel, 2002). In another case of herpesvirus infection, cytomegalovirus establishes latency after systemic infection in both mouse and man (Snyder et al., 2008). In infected individuals, accumulation of cytomegalovirus-specific CD8+ T cells can reach the exceptional proportion of 10% of total CD8+ T cells, in a phenomenon known as memory inflation. It was shown that this high proportion of cytomegalovirus-specific CD8+ T cells was maintained by long-lived memory cells as well as by continuous generation of short-lived and functional T cells by the latent infection. Such latent infections are thus expected to protect the superorganism from secondary infections, and may operate upon childhood infections and vaccination with other microbes. In this case, homeostasis of the superorganism is modified as immunological memory supported in part by the latent cytomegalovirus infection increases resistance to new infections by itself or related viruses. The old foe remains a foe, but its "domestication" can make it a member of the superorganism to fight its wild relatives.

It has recently been discussed that the host is in a situation of metastable equilibrium with such old foes or pathogens, as a decrease in immunosurveillance, or progressive erosion of this equilibrium, may turn latent microbes into a life-threatening pathogen (Virgin et al., 2009). This view can be applied to the whole symbiotic microbiota, and is the basis of ruptures of homeostasis discussed in the next chapter.

Rupture of Homeostasis

A rupture in homeostasis can be caused by the environment, invading microbes, symbiotic microbes, or the host, and can have severe consequences if the superorganism cannot re-establish equilibrium between the microbiota and the host. Here, we will examine ruptures of homeostasis caused by alterations in the immune system, as well as two examples involving subtle alterations in the host metabolic and tissue regulation. Examples of progressive or abrupt rupture of homeostasis involving the host virome (Virgin et al., 2009), or strong pathogens

such as *Shigella* or Influenza A virus, are well described (Kobasa et al., 2007; Sansonetti, 2004) and will not be discussed here.

Weak Immunity

Too weak an immune system exposes the superorganism to a conversion of mutualists and commensals to pathogens. The immune forces that normally contain symbionts and establish equilibrium are at risk to be overwhelmed, and microbes that are not normally behaving as pathogens may invade forbidden areas and become pathogens. Immunocompromised people, such as patients suffering from AIDS, treated in intensive care units, receiving chemotherapy, or organ transplants, are exposed to life-threatening opportunistic infections by microbes that are otherwise harmless or ubiquitous symbionts. The latter include the yeast *Candida albicans* (d'Enfert, 2009), a member of the human microbiota of the skin, intestine, and vagina, and *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Nordmann et al., 2007), both present in the skin microbiota (Gao et al., 2007). Recently, it has been reported that lipoteichoic acid derived from *Staphylococcus* strains inhibits inflammatory cytokine release from keratinocytes through a TLR-2-dependent mechanism (Lai et al., 2009), suggesting a possible mutualistic role of *Staphylococci* in skin homeostasis. Additional benefits these symbionts may provide to the host include exclusion of related microbes from the niche.

A number of examples have been reported on the loss of containment of the intestinal microbiota in mice deficient for components of the innate or the adaptive immunity. When specific pathogen-free mice are fed orally with the symbiont *Enterobacter cloacae*, dendritic cells collect bacteria in the PPs sub-epithelial dome before migrating to the draining mesenteric LN where live bacteria can be recovered (Macpherson and Uhr, 2004). In the absence of immunoglobulins in J_H-deficient mice, containment of the symbionts is less efficient and significantly more bacteria are recovered from the mesenteric LNs. A similar loss of containment is found in mice deficient for the Toll-like receptor-signaling molecules Myd88 and Trif, or deficient in the phagocyte oxidative burst (Slack et al., 2009). When specific pathogen-free mutant mice are fed with the symbiont *Escherichia coli* K-12, live bacteria are recovered from the spleen, and serum IgG are produced against members of the symbiotic microbiota, indicating bacterial leakage into the bloodstream. Similar findings were reported earlier in mice deficient for Myd88 or TLR-4 (Fukata et al., 2005). The consequence of decreased containment of the intestinal microbiota is increased morbidity in Myd88- and J_H- double-deficient mice (Slack et al., 2009), and increased susceptibility of TLR-4- or Myd88-deficient mice to IBD induced by sodium dextran sulfate (Fukata et al., 2005).

Strong Immunity

Too strong an immune system destabilizes the microbiota and causes dysbiosis, or alteration in the symbiotic microbial community. In the case of *Drosophila* flies, immune hyperactivity against intestinal symbionts can be fatal (Ryu et al., 2008). Wild-type flies harbor an intestinal community dominated by five bacterial species, including *Gluconobacter* strain EW707 and *Acetobacter* strain EW911. Mutant flies that show uncontrolled, bacteria-induced nuclear factor-κB activation, due to knockdown of the regulatory Caudal transcription factor, suffer excessive apoptosis of epithelial cells and progressed to death. It was shown that the mutant flies harbor an altered intestinal bacterial community, where EW911 becomes a minor population and EW707 expands. EW707 alone is pathogenic to germfree flies, but EW911 protect the flies from the damaging effect of EW707. Thus, even though EW707 is a "regular" member of the symbiotic microbiota, its population size has to be kept in check by the presence of other symbionts to maintain homeostasis in wild-type flies. A beneficial role of EW707 for the host during homeostasis remains to be established.

Furthermore, given the nearly unlimited source of antigens generated by the intestinal microbiota, an aggressive immune system can progress into overdrive, generate acute and chronic pathological inflammation, and cause severe damage to the tissue. Such as scenario unfolds during Crohn's disease and mutations that map to the *CARD15* locus significantly increase disease susceptibility in patients (Lesage et al., 2002). *CARD15* codes for the innate receptor NOD-2, which recognizes muramyldipeptides shed mostly by Gram-positive bacteria (Giardin et al., 2003). Contradictory data have been reported on the effect of such mutations on the activity of NOD-2. It was found that mutations either increase NOD-2 activity and induce elevated nuclear factor-κB activity and IL-1β secretion (Maeda et al., 2005), or abolish NOD-2 activity and decrease containment through antibacterial peptides (Kobayashi et al., 2005). Decreased or lack of NOD-2 activity, reflecting the mutations found in Crohn's patients, leads to increased stimulation in response to TLR-2 stimulation and consequent hyperactivity toward the microbiota (Strober et al., 2008).

Dysbiosis

Dysbiosis can be caused by dysregulated immunity and have life-threatening consequences as we just discussed. But dysbiosis can also be induced by subtle shifts in immunity or by non-immunological factors and elicit dramatic consequences.

Propionibacterium acnes is a major constituent of the microbiota on human skin and feeds on fatty acids produced by sebaceous glands (Gao et al., 2007). However, disruption of homeostasis can lead to skin disease caused by *P. acnes* such as acne. Acne unfolds as hair follicles undergo hyperkeratinization, which blocks sebum egress and causes its accumulation within the follicle

(Noble, 1984). Under such conditions, *P. acnes* expands and damages the follicle and local containment through increased production of bacterial enzymes. The immune system reacts to occasional breaches in containment and invasion of *P. acnes* and generates local inflammation and folliculitis. The situation worsens when other skin symbionts, such as *Staphyloccocus aureus*, penetrate the breaches and cause more severe infections.

Another, much publicized, example of dysbiosis is shown to lead to obesity. The intestinal microbiota of obese ob/ob mice and patients was sequenced and compared with that of lean mice and patients (Ley et al., 2006; Turnbaugh et al., 2006). The microbiome and its inferred metabolome, the collection of metabolic pathways encoded by the microbiota, show that the microbiota of obese individuals is more efficient than that of control individuals in harvesting energy from nutrients, in particular through enhanced catabolism of dietary polysaccharides (Turnbaugh et al., 2006). As a result, the consumable energy remaining in feces is lower in obese individuals. Remarkably, transfer of the cecal microbiota of ob/ob mice to wild-type controls induces a significant increase in body fat as compared with control transfers, showing the dominant effect of the dysbiosis present in ob/ob mice. These data also suggest that obesity can be inherited from the mother not only through genes and behavior, but also through symbiotic bacteria. It was further shown that ob/ob mice and obese patients have an increased proportion of *Firmicutes* versus *Bacteroidetes* symbionts, the two dominant phyla in the mouse and human intestine (Ley et al., 2005, 2006). However, the primary cause of dysbiosis in ob/ob mice and obese patients remains a fundamental and fascinating problem (Turnbaugh et al., 2008).

A striking effect of dysbiosis was also reported in the susceptibility of NOD mice to type-1 diabetes (Wen et al., 2008). A series of experiments was designed to assess whether progression to disease was dependent on proinflammatory signals delivered through innate receptors such as TLRs. Apparently confirming this hypothesis, Myd88-deficient NOD mice show marked resistance to development of the disease. T cells reactive to diabetes-associated peptides are found in the spleen and in the mesenteric LN of such mice, yet are markedly reduced in the pancreatic LNs. These data indicated that Myd88-deficient NOD mice do not suffer from systemic immunosuppression but rather from localized immunosuppression in the pancreatic LNs that drain both the pancreas and the intestine. Surprisingly, germfree Myd88-deficient NOD mice develop diabetes to the same extent as germfree or normally colonized Myd88-sufficient NOD mice, indicating a role for the intestinal microbiota in protection from disease progression in the absence of Myd88. A key experiment showed that Myd88-deficient mice develop an altered intestinal microbiota that somehow suppresses the generation of diabetogneic T cells in the pancreatic LN: when NOD newborn mice were raised with Myd88-deficient mothers, disease progression was mitigated. Myd88-deficient mice harbor a decreased ratio of Firmicutes versus Bacteroidetes symbionts as compared with Myd88-sufficient mice, as well as increased

proportions of Lactobacillaceae. A cause-to-consequence relationship has not been established yet in this model, but a probiotic mix containing four species of Lactobacillaceae has been previously shown to protect NOD mice from diabetes and induce a concomitant increase in IL-10 production (Calcinaro et al., 2005).

Restoration of Homeostasis for Prevention of Disease and Therapy

Prevention and therapy of a rupture in homeostasis, leading to disease, might be directed against the primary cause in the host or the microbiota, such as immune dysregulation or potential pathogens. It might also aim at restoring homeostasis through engineering of the symbiotic microbiota.

In cases of immunodeficiencies, the key is to maintain equilibrium between host and microbiota through restoration of normal immunity or a decrease in the microbial load through chemotherapy. In cases of pathological immune hyperactivity, unfolding for example during Crohn's disease, the most widely used therapy is immunosuppression through administration of anti-tumor necrosis factor-α antibodies. Decreasing the bacterial load through antibiotic therapy would in principle also be a valid approach during acute inflammatory disease, but in the long run exposes the host to invasion by antibiotic-resistant microbes that take advantage of empty niches, and deprives the host of microbial metabolic pathways involved in protection, digestion, and catabolism of xenobiotics.

The study by Pamer and co-workers proposes a new strategy to combine antibiotic treatment with replacement of microbiota (Brandl et al., 2008). In their study, susceptibility of antibiotic-treated mice to an antibiotic-resistant *Enterococcus* could be mitigated through concomitant administration of LPS. The LPS induced TLR-4-mediated production of antibacterial peptides targeting the pathogenic bacteria, an effect normally induced by the symbiotic microbiota. Thus, in patients treated with antibody cocktails, some degree of homeostasis could be restored by complementing the dwarfed microbiota with selected microbiota-derived compounds. Therefore, deciphering the activity of microbial components on the immune system and beyond the immune system might lead to an extension of such replacement strategies. As microbial metabolic pathways would still be missing, such strategies would have to be complemented with specific diets.

IBD, including Crohn's disease and ulcerative colitis, is associated with dysbiosis (Packey and Sartor, 2009; Tamboli et al., 2004). It has therefore been proposed that dysbiosis is a potential target for the treatment of IBD. Potential treatments are based on the use of antibiotics targeting specific types of bacteria associated with IBD, or on the use of pre- and probiotics that would favor the restoration of a normal microbiota. Nevertheless, a cause-to-consequence relationship between dysbiotic microbiota and IBD remains to be clearly established. An alternative strategy is based on the use of bacterial strains that exert an immunomodulatory effect on the immune system. The Firmicutes *Faecalibacterium prausnitzii* is significantly reduced in patients suffering from Crohn's

disease (Sokol et al., 2008). In vitro, *F. prausnitzii* induces lower IL-12 and interferon-γ and higher IL-10 production by mononuclear cells, and in vivo, modulated TNBS (2,4,6-trinitro benzene sulfonic acid)-induced colitis. Similar anti-inflammatory effects and protection from IBD have been shown for different species of *Lactobacilli*, *Bifidobacteria*, *Bacteroides*, and *E. coli* (Round and Mazmanian, 2009). Thus, homeostasis might be restored through modifications of the microbial community through administration of probiotics or prebiotics that favor the emergence of such probiotics. This modified microbiota would exert an anti-inflammatory effect on the hyperactive immune system found in IBD patients and establish a new and potentially viable homeostasis. An important issue however is the stability of such modified homeostasis with time and its robustness in pathological settings.

We have discussed how stable dysbiosis, transmitted by the mother to her offspring or maintained by mutations in the immune system, promotes obesity (Turnbaugh et al., 2006) or prevents the progression of type-1 diabetes (Wen et al., 2008), respectively. These examples show how altered but stable associations between microbiota and host influence the metabolic and inflammatory state of the superorganism. It is therefore expected that a fine understanding of the cross-talk and equilibrium between microbiota and host, and eventually of the rules that govern the superorganism, should lead to preventive and therapeutic strategies in numbers of ailments that are characterized by disrupted homeostasis.

Conclusions

The superorganism is discussed here as the association of the mammalian host with its symbiotic microbiota. This concept offers a novel perspective of self and microbiota, where self is embedded in the microbial world and depends on it for full development and optimal survival. The superorganism requires forces, such as the immune system, to maintain homeostasis. During pathogenesis, the equilibrium between the microbiota and the immune system can become dangerously unstable and glide toward collapse and death of the superorganism. In that context, it appears important to decipher the cellular and molecular cross-talk between the symbiotic microbiota, the host, and its immune system. The molecular messengers of this crosstalk may lead to a new generation of preventive and therapeutic avenues: it will be possible to diagnose and understand ruptures of homeostasis, elaborate strategies to prevent progression to disease, and design therapies for return to homeostasis.

Conflict of Interest

The authors declared no conflict of interest.

Acknowledgments

I thank the following co-workers for discussions, suggestions, and critical reading of the paper: Marc Daëron, Ivo Gomperts-Boneca, Nadine Cerf-Bensussan, Matthew Albert, Pascale Cossart, and Jim Di Santo.

References

Antia, R., Ganusov, V.V. & Ahmed, R. The role of models in understanding CD8+ T-cell memory. *Nat. Rev. Immunol*. 5, 101–111 (2005).

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. & Gordon, J.I. Host–bacterial mutualism in the human intestine. *Science* 307, 1915–1920 (2005).

Barton, E.S. *et al*. Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447, 326–329 (2007).

Barton, G.M. & Kagan, J.C. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat. Rev. Immunol*. 9,535–542 (2009).

Baxter, A.G. Louis Pasteur's beer of revenge. *Nat. Rev. Immunol*. 1, 229–232 (2001).

Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M. & Sacks, D.L. $CD4^+CD25^+$ regulatory T cells control *Leishmania* major persistence and immunity. *Nature* 420, 502–507 (2002).

Benson, A., Pifer, R., Behrendt, C.L., Hooper, L.V. & Yarovinsky, F. Gut commensal bacteria direct a protective immune response against*Toxoplasma gondii*. *Cell Host Microbe*. 6, 187–196 (2009).

Bouskra, D. *et al*. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456, 507–510 (2008).

Brandl, K. *et al*. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455, 804–807 (2008).

Brown, G.D. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol*. 6, 33–43 (2006).

Buchon, N., Broderick, N.A., Chakrabarti, S. & Lemaitre, B. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev*. 23, 2333–2344 (2009).

Calcinaro, F. *et al*. Oral probiotic administration induces interleukin-10 production and prevents spontaneous autoimmune diabetes in the non-obese diabetic mouse. *Diabetologia* 48, 1565–1575 (2005).

Cash, H.L., Whitham, C.V., Behrendt, C.L. & Hooper, L.V. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313,1126–1130 (2006).

Cato, M.P. &Varro M.T. *Cato and Varro: On Agriculture* (Loeb Classical Library, 1934).

Clarke, T.B. *et al*. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med*. 16, 228–231.

Darveau, R.P. *et al*. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect. Immun*. 72, 5041–5051 (2004).

d'Enfert, C. Hidden killers: persistence of opportunistic fungal pathogens in the human host. *Curr. Opin. Microbiol*. 12, 358–364 (2009).

Dethlefsen, L., McFall-Ngai, M. & Relman, D.A. An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* 449, 811–818 (2007).

Duerkop, B.A., Vaishnava, S. & Hooper, L.V. Immune responses to the microbiota at the intestinal mucosal surface. *Immunity* 31, 368–376 (2009).

Fossiez, F. *et al*. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med*. 183, 2593–2603 (1996).

Fritz, J.H., Ferrero, R.L., Philpott, D.J. & Girardin, S.E. Nod-like proteins in immunity, inflammation and disease. *Nat. Immunol*. 7, 1250–1257 (2006).

Fukata, M. *et al*. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am. J. Physiol. Gastrointest. Liver Physiol*. 288, 1055–1065 (2005).

Gaboriau-Routhiau, V. *et al*. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses.*Immunity* 31, 677–689 (2009).

Gao, Z., Tseng, C.H., Pei, Z. & Blaser, M.J. Molecular analysis of human forearm superficial skin bacterial biota. *Proc. Natl. Acad. Sci. USA* 104,2927–2932 (2007).

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J. & Madara, J.L. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol*. 167,1882–1885 (2001).

Gill, S.R. *et al*. Metagenomic analysis of the human distal gut microbiome.*Science* 312, 1355–1359 (2006).

Girardin, S.E. *et al*. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem*. 278, 8869–8872 (2003).

Hamada, H. *et al*. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol*. 168,57–64 (2002).

Ishigame, H. *et al*. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* 30, 108–119 (2009).

Ivanov, I.I. *et al*. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* (2009).

Ivanov, I.I. *et al*. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17^{+} T helper cells. *Cell* 126,1121–1133 (2006).

Jones, C.E. & Chan, K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol*. 26, 748–753 (2002).

Kawai, T. & Akira, S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol*. 21, 317–337 (2009).

Kobasa, D. *et al*. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445, 319–323 (2007).

Kobayashi, K.S. *et al*. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307, 731–734 (2005).

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263–274 (1993).

Lai, Y. *et al*. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat. Med*. 15, 1377–1382 (2009).

Lee, J. *et al*. pH-dependent internalization of muramyl peptides from early endosomes enables Nod1 and Nod2 signaling. *J. Biol. Chem*. 284,23818–23829 (2009).

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. & Hoffmann, J.A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86, 973–983 (1996).

Lesage, S. *et al*. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am. J. Hum. Genet*. 70, 845–857 (2002).

Ley, R.E. *et al*. Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. USA* 102, 11070–11075 (2005).

Ley, R.E., Turnbaugh, P.J., Klein, S. & Gordon, J.I. Microbial ecology: human gut microbes associated with obesity. *Nature* 444, 1022–1023 (2006).

Liang, S.C. *et al*. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med*. 203, 2271–2279 (2006).

Lochner, M. *et al*. *In vivo* equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J. Exp. Med*. 205, 1381–1393 (2008).

Mackey, D. & McFall, A.J. MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Mol. Microbiol*. 61, 1365–1371 (2006).

Macpherson, A.J. & Uhr, T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303, 1662–1665 (2004).

Maeda, S. *et al*. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307, 734–738 (2005).

Matzinger, P. The danger model: a renewed sense of self. *Science* 296,301–305 (2002).

Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol*. 12, 991–1045 (1994).

McFall-Ngai, M. Adaptive immunity: care for the community. *Nature* 445,153 (2007).

Mebius, R.E. Organogenesis of lymphoid tissues. *Nat. Rev. Immunol*. 3,292–303 (2003).

Medzhitov, R. & Janeway, C. Jr Innate immune recognition: mechanisms and pathways. *Immunol. Rev*. 173, 89–97 (2000).

Metchnikoff, I.I. *Lectures on the Comparative Pathology of Inflammation Delivered at Pasteur Institute in 1891* (Dover Press, New York, 1989).

Metchnikoff, I.I. *The Prolongation of Life: Optimistic Studies* (Springer Publishing Company, New York, 2004).

Moller, G. Mechanism of B-cell activation and self-non-self discrimination. *Cold Spring Harb. Symp. Quant. Biol*. 41 (Part 1), 217–226 (1977).

Niess, J.H., Leithauser, F., Adler, G. & Reimann, J. Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *J. Immunol*. 180,559–568 (2008).

Noble, W.C. Skin microbiology: coming of age. *J. Med. Microbiol*. 17, 1–12 (1984).

Nordmann, P., Naas, T., Fortineau, N. & Poirel, L. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. *Curr. Opin. Microbiol*. 10, 436–440 (2007).

Nossal, G.J. Molecular and cellular aspects of immunologic tolerance. *Eur. J. Biochem*. 202, 729–737 (1991).

Nurieva, R.I. *et al*. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages.*Immunity* 29, 138–149 (2008).

Packey, C.D. & Sartor, R.B. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Curr. Opin. Infect. Dis*. 22, 292–301 (2009).

Round, J.L. & Mazmanian, S.K. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol*. 9,313–323 (2009).

Ryu, J.H. *et al*. Innate immune homeostasis by the homeobox gene caudal and commensal–gut mutualism in *Drosophila*. *Science* 319, 777–782 (2008).

Sanos, S.L. *et al*. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat. Immunol*. 10, 83–91 (2009).

Sansonetti, P.J. & Di Santo, J.P. Debugging how bacteria manipulate the immune response. *Immunity* 26, 149–161 (2007).

Sansonetti, P.J. & Medzhitov, R. Learning tolerance while fighting ignorance. *Cell* 138, 416–420 (2009).

Sansonetti, P.J. War and peace at mucosal surfaces. *Nat. Rev. Immunol*.4, 953–964 (2004).

Sartor, R.B. Microbial influences in inflammatory bowel diseases.*Gastroenterology* 134, 577–594 (2008).

Satoh-Takayama, N. *et al*. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense.*Immunity* 29, 958–970 (2008).

Slack, E. *et al*. Innate and adaptive immunity cooperate flexibly to maintain host–microbiota mutualism. *Science* 325, 617–620 (2009).

Smith, K., McCoy, K.D. & Macpherson, A.J. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol*. 19, 59–69 (2007).

Snyder, C.M. *et al*. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells.*Immunity* 29, 650–659 (2008).

Sokol, H. *et al*. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. USA* 105, 16731–16736 (2008).

Sprent, J. & Surh, C.D. T cell memory. *Annu. Rev. Immunol*. 20, 551–579 (2002).

Strober, W., Kitani, A., Fuss, I., Asano, N. & Watanabe, T. The molecular basis of NOD2 susceptibility mutations in Crohn's disease. *Mucosal. Immunol*. 1 (Suppl 1), S5–9 (2008).

Sudo, N. *et al*. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. Physiol*. 558, 263–275 (2004).

Syed, I.B. Islamic medicine: 1000 years ahead of its times. *J. Islam. Med. Assoc*. 2, 2–9 (2002).

Tamboli, C.P., Neut, C., Desreumaux, P. & Colombel, J.F. Dysbiosis in inflammatory bowel disease. *Gut* 53, 1–4 (2004).

Tran, A.X., Stead, C.M. & Trent, M.S. Remodeling of *Helicobacter pylori* lipopolysaccharide. *J. Endotoxin. Res*. 11, 161–166 (2005).

Tsuji, M. *et al*. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin a generation in the gut. *Immunity* 29, 261–271 (2008).

Turnbaugh, P.J. & Gordon, J.I. An invitation to the marriage of metagenomics and metabolomics. *Cell* 134, 708–713 (2008).

Turnbaugh, P.J. *et al*. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031 (2006).

Turnbaugh, P.J. *et al*. The human microbiome project. *Nature* 449, 804–810 (2007).

Turnbaugh, P.J., Backhed, F., Fulton, L. & Gordon, J.I. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe*. 3, 213–223 (2008).

Uematsu, S. *et al*. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat. Immunol*. 7,868–874 (2006).

Vance, R.E., Isberg, R.R. & Portnoy, D.A. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe*. 6, 10–21 (2009).

Vavricka, S.R. *et al*. hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells.*Gastroenterology* 127, 1401–1409 (2004).

Vergin, F. Antibiotics and probiotics. *Hippokrates* 25, 116–119 (1954).

Virgin, H.W., Wherry, E.J. & Ahmed, R. Redefining chronic viral infection.*Cell* 138, 30–50 (2009).

Weaver, C.T., Hatton, R.D., Mangan, P.R. & Harrington, L.E. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol*. 25, 821–852 (2007).

Weintraub, A., Zahringer, U., Wollenweber, H.W., Seydel, U. & Rietschel, E.T. Structural characterization of the lipid A component of *Bacteroides fragilis* strain NCTC 9343 lipopolysaccharide. *Eur. J. Biochem*. 183, 425–431 (1989).

Welsh, R.M., Selin, L.K. & Szomolanyi-Tsuda, E. Immunological memory to viral infections. *Annu. Rev. Immunol*. 22, 711–743 (2004).

Wen, L. *et al*. Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* 455, 1109–1113 (2008).

Wilkinson, D.M. At cross purposes. *Nature* 412, 485 (2001).

Wills-Karp, M. *et al*. Interleukin-13: central mediator of allergic asthma.*Science* 282, 2258–2261 (1998).

Wolk, K. *et al*. IL-22 increases the innate immunity of tissues. *Immunity*21, 241–254 (2004).

Wynne-Edwards, V.C. *Animal Dispersion in Relation to Social Behavior* (Oliver & Boyd, London, 1962).

Yoshida, H. *et al*. IL-7 receptor α^+ $CD3^-$ cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int. Immunol*. 11,643–655 (1999).

Zinkernagel, R.M. On differences between immunity and immunological memory. *Curr. Opin. Immunol*. 14, 523–536 (2002).

A9

HOST DEFENSE AND IMMUNOMODULATION OF MUCOSAL CANDIDIASIS

*Paul L. **Fidel**, Jr.*,[33,†] *and Mairi C. Noverr*[33,‡]

Introduction

Candida albicans is a commensal organism of the oral cavity and gastrointestinal tract of most human adults as well as the genital tract of adult females. Point prevalence studies show asymptomatic colonization by *C. albicans* in the vagina of 15–25 percent of healthy women and in the mouth and gastrointestinal tracts of up to 60 and 90 percent of healthy adults, respectively (reviewed in Fidel, 2002). However, *C. albicans* can also be a pathogen of these same mucosal tissues and produce infections in both immunocompetent and immunocompromised conditions. Oropharyngeal candidiasis (OPC) encompasses infections of the hard and soft palate, tongue, buccal mucosa, and floor of the mouth, and can present as reddened patches (erythematous) or white curdlike lesions (pseudomembranous). Chewing and swallowing can be difficult and painful under these conditions. Infections can be acute or recurrent, and are common in immunocompromised patients, especially those infected with HIV. *Candida*-associated denture stomatitis (DS) is the most prevalent form of oral candidiasis, affecting up to 65 percent of otherwise healthy denture wearers (Budtz-Jorgensen et al., 1975; Daniluk et al., 2006). DS is characterized by inflamed oral mucosa, halitosis, and burning or bleeding of the infected mucosa (reviewed in Webb et al., 1998). Vulvovaginal candidiasis (VVC) affects a significant number of women predominantly in their reproductive years (Kent, 1991; Sobel, 1988, 1992). An estimated 75 percent of all women will experience at least one episode of acute VVC in their lifetime with another 5–10 percent developing recurrent VVC (RVVC) (Sobel, 1988, 1992). VVC involves infections of the vaginal lumen as well as the vulva. Symptoms include burning, itching, soreness, an abnormal discharge, and dyspareunia. Signs include vaginal and vulvar erythema and edema. Acute VVC has several known predisposing factors including antibiotic and oral contraceptive usage, hormone replacement therapy, pregnancy, and uncontrolled diabetes mellitus (Kent, 1991; Sobel, 1988, 1992). RVVC is multifactorial in etiology, but it is usually defined as idiopathic with no known predisposing factors in the majority of those affected (Sobel, 1988, 1992).

[33] Center of Excellence in Oral and Craniofacial Biology, Louisiana State University Health Sciences Center School of Dentistry, New Orleans, LA 70119.

† Email: pfidel@lsuhsc.edu

‡ Email: mnover@lsuhsc.edu

Prior to the HIV epidemic, host defense against *C. albicans* at these mucosal sites was largely considered one-dimensional. Although general anatomical distinctions were always understood in some capacity, the concept that "all mucosa are equal" was often implied for mucosal candidiasis whereby discussions of host defense at one mucosal site were generally applied to other mucosal sites as well. This was primarily due to the lack of large populations of individuals with mucosal candidiasis at any one geographical site that could be studied in-depth. OPC was relatively uncommon until transplantation and other forms of therapeutic or disease-associated immunosuppression (i.e., HIV/AIDS) became more evident. Yet numbers of patients to study at any one place remained quite small. VVC has always been common, but never taken very seriously as a mucosal fungal infection due to anecdotal causes and treatment regimens. *Candida*-associated DS that occurs in immunocompetent denture wearers was always known, but not well studied from an immunological or host-response perspective. This lack of research interest in DS was extended further as OPC became more prominent in HIV disease.

Prior to in-depth analyses of specific patient populations the majority of mucosal *Candida* infections were thought to be associated with some form of IgA antibody or humoral immune deficiency. However, antibody deficiency was difficult to demonstrate (Kirkpatrick et al., 1971; Mathur et al., 1977; Odds, 1988); therefore, it was the syndrome of chronic mucocutaneous candidiasis (CMC) that stimulated the next series of explanations. Because CMC was considered to be due to a deficiency in peripheral (blood) T cell–mediated immunity (Kirkpatrick, 1984; Kirkpatrick and Sohnle, 1981; Kirkpatrick et al., 1970, 1971), most other mucosal *Candida* infections were also considered to be caused by a similar T cell deficiency despite site specificity and individual prevalence.

The HIV epidemic created large numbers of individuals with severe immunosuppression and a significant increase in mucosal candidiasis. Accordingly, mucosal candidiasis became synonymous with OPC or esophageal candidiasis because they were being diagnosed most often. In fact, esophageal candidiasis is considered an AIDS-defining illness (Klein et al., 1984; Macher, 1988; Phelan et al., 1987). As much as VVC was considered common in HIV-infected women and a possible AIDS defining illness at one point (Burns et al., 1997; Duerr et al., 1997; Spinillo et al., 1994), it soon became clear that vaginal candidiasis, while common, was really no more common in HIV-positive women than in HIV-negative women (Clark et al., 1995; Imam et al., 1990; Leigh et al., 2001; Rhoads et al., 1987; Schuman et al., 1998b; White, 1996). This finding together with basic immunology research that was becoming of age for VVC (reviewed in Fidel and Sobel, 1996) began to suggest that factors associated with susceptibility to oral and vaginal candidiasis were different. Following some additional studies in HIV-infected persons with OPC, immunological data too began to follow the physiological anatomical distinctions and revealed that host defenses against *Candida* at the oral and vaginal mucosa were unique, distinct, and independent

(Leigh et al., 2001). As a result, it is now considered that all mucosa are *not* equal for mucosal *Candida* infections and that host defenses at the various mucosal sites need to be studied independently and exclusively at the local level with respect to cells and soluble immune factors.

The vast data accumulated over the past 15 years showing distinct T cell percentages or composition at different mucosal sites (e.g., vagina, gastrointestinal tract), especially in the intraepithelial layers, antigen presenting cells (e.g., dendritic cells, macrophages, Langerhans cells), or B cells (Fidel et al., 1996b; Hladik et al., 1999; Ibraghimov et al., 1995; Inghirami et al., 1990; Itohara et al., 1990; Johansson et al., 2000; Nandi and Allison, 1991) have wholly supported the concept that mucosal sites are indeed independent and unique relative to host immune reactivity. Thus, a clear divergence of immunity at mucosal sites has become evident. There is perhaps more dogma with reference to host defense against OPC (protection by $CD4^+$ Th1-type responses), yet other nonconventional host defenses are also critically important. On the other hand, natural host defense against VVC does not appear to include T cells or any type of adaptive immunity (reviewed in Fidel Jr., 2007), but relies on innate immunity for both protection and susceptibility to infection (reviewed in Fidel and Noverr, 2012).

Host Defense Against Oropharyngeal Candidiasis

The majority of data suggest that $CD4^+$ Th1-type cells are critical for host defense against OPC. Clinically, OPC is most common in HIV^+ persons when $CD4^+$ cell numbers drop below 200 cells/μl (Greenspan et al., 2000; Nielsen et al., 1994; Rabeneck et al., 1993; Schuman et al., 1998a). Within vitro immune analyses, peripheral blood mononuclear cells (PBMC) from most individuals, including HIV^+ persons, respond to *Candida* antigens with Th1-type cytokines (Kunkl et al., 1998; Leigh et al., 2001). Together these results suggested that the *Candida*-specific T cells themselves were not becoming defective with immunosuppression, but that a threshold number of $CD4^+$ T cells is required to protect the oral cavity against infection by this commensal organism. Below this threshold number of cells, other systemic and/or local immune mechanisms must function exclusively. Resistance and susceptibility to OPC then depends on the status of these alternative immune mechanisms.

Several aspects of local immunity have been evaluated clinically. In support of the Th1/Th2 dichotomy concept, it was reported that HIV^- individuals had Th1/Th0 cytokines in their saliva, whereas HIV^+ individuals had primarily Th2-type cytokines, which was more profound in those patients with OPC (Leigh et al., 1998). More recently, a distinct protective role for Th17 cells and associated cytokines has been identified for OPC (Conti et al., 2009). In fact, Th17-type responses may be more critical to normal protection than Th1-type responses. Yet like Th1-type responses, Th17-type responses, that are largely also CD4 T cell dependent, will be lost during HIV disease.

Lymphocytes have also been examined specifically in the OPC lesions. While both $CD4^+$ and $CD8^+$ cells have been identified (Romagnoli et al., 1997), we reported an accumulation of $CD8^+$ T cells at a considerable distance from *Candida* that is located superficially at the outer epithelium (Myers et al., 2003), suggesting a role for $CD8^+$ T cells against infection, but with a potential dysfunction in cell trafficking that promotes susceptibility to OPC. Increases in mRNA for several CD8 cell-associated cytokines (IL-2 and IL-15) and chemokines (IP-10, RANTES, and MCP-1) (Lilly et al., 2004) supported a role for reactivity by CD8 T cells. A murine AIDS model (MAIDS) also showed a rate of 30 percent recurrent OPC with a predominance of $CD8^+$ T cells recruited into the oral tissues (Deslauriers et al., 1997). The tissue-associated $CD8^+$ T cells primarily possess the $\alpha\beta$ T cell receptor (TCR) (McNulty et al., 2005). The CD8 T cells present in the lesions are activated memory T cells as evidenced by cell surface activation markers (CD69, CD45R0) that are transitioning between central and effector memory status ($CD27^{hi}$, $CCR7^{Lo}$, $CD62L^{Lo}$) (Leigh et al., 2006). Hence, the CD8 T cells appear as normal activated memory cells recruited into the oral mucosa but are inhibited or challenged from migrating through the tissue otherwise.

The issue of cellular migration was addressed through the study of homing receptor/adhesion molecules and chemokine receptors. Chemokine receptor expression was similar in HIV^+OPC^+ persons and HIV^+OPC^- persons (Lilly et al., 2006). Cellular migration is controlled by chemokine receptors as well as cell-associated heterodimeric homing receptors (integrins) and reciprocal tissue-associated adhesion molecules. Some homing receptor/adhesion molecule interactions ($\alpha_4\beta_7$/MAdCAM; $\alpha_4\beta_1$/VCAM-1) govern migration of cells out of blood and into mucosal tissues, while others govern migration through mucosal tissues ($\alpha_e\beta_7$/E-Cadherin). The $CD8^+$ T cells present in both OPC^- and OPC^+ tissue had positive integrin expression with varying combinations, but no discernible difference in OPC^- versus OPC^+ tissue. In contrast, MAdCAM expression was significantly increased in OPC^+ tissue in support of the increased presence of T cells, while E-cadherin was significantly decreased in OPC^+ tissue (McNulty et al., 2005). The increase in MAdCAM is consistent with the migration of $CD8^+$ T cells into the oral mucosal tissue. Likewise, the reduced E-Cadherin provides an explanation for the inability of the $CD8^+$ T cells to migrate to the outer epithelium and represents a putative dysfunction in those with OPC lending to the susceptibility to OPC.

Based on the cross-sectional data with the CD8 T cells and E-cadherin (reviewed in Fidel, 2006), an important next step was to conduct a longitudinal analysis of $CD8^+$ T cells in the tissue of those HIV^+ patients who have not acquired OPC, have had sporadic cases of OPC, or who have recurrent OPC. The main objective was to confirm the cross-sectional findings and determine whether the reduction in E-cadherin in those with OPC was permanent or transient. Oral swabs were taken every 2 weeks and biopsies every 2 months to track changes in the $CD8^+$ T cells and adhesion molecule expression before, during, and after

OPC episodes. In total, the results showed that while OPC- patients with low oral fungal burden revealed an unremarkable presence of $CD8^+$ T cells and normal E-cadherin expression, OPC- patients who had increased oral *Candida* colonization (indicative of a potential preclinical OPC condition), had higher numbers of $CD8^+$ T cells throughout the tissue with normal E-cadherin expression. In patients with an acute episode of OPC where $CD8^+$ T cells were accumulated at the epithelial-lamina propria interface together with reduced E-cadherin expression, evaluation of E-cadherin following successful antifungal treatment revealed a return to normal expression (Quimby et al., 2011). These results suggested that under conditions of $CD4^+$ T cell deficiency, $CD8^+$ T cells typically migrate to the site of a preclinical infection via normal expression of E-cadherin and that reduced E-cadherin expression in those with OPC is not permanent. The reduction in E-cadherin that we have observed in patients with OPC is consistent with two independent studies showing that *Candida* can cleave or degrade E-cadherin through secretory aspartyl proteases (SAPs) (Frank and Hostetter, 2007; Villar et al., 2007). Hence, the reduction in E-cadherin may be a virulence mechanism for *Candida* that when adherent to the epithelium, promotes subsequent invasion. We postulate that increases in *Candida* levels lead to increased SAP-mediated degradation of E-cadherin, which creates an environment more conducive to infection and invasion and the onset of OPC.

The metabolic state of the epithelial cells is important in understanding the interaction of Candida with E-cadherin, as is the cellular localization of the epithelial cells. Filler and coworkers have shown that an adhesin (Als3) on *C. albicans* hyphae can bind E-cadherin on metabolically active epithelial cells, which induces endocytosis (Phan et al., 2007). Thus, it appears that *Candida* can either degrade or use E-cadherin. We hypothesize that these two conditions can and do occur depending on the location of the epithelial cells. If *Candida* gains access to basal epithelial cells that are metabolically active it will use E-cadherin to gain intracellular access through endocytosis. But if *Candida* remains on the apical epithelium it will likely degrade E-cadherin to occupy and superficially attach to the outer epithelium rather than be released from the epithelium inside metabolically inactive sloughing cells.

In any event, it appears that *Candida* has the ability to modulate E-cadherin rather than it being modulated (transient or permanent) by a host-dependent mechanism. If so, immunotherapeutic strategies directed towards restoring normal E-cadherin expression would allow $CD8^+$ T cells to migrate to the outer epithelium for optimal effector function. Current studies are focusing on the mechanism of action by $CD8^+$ T cells. Interestingly, treatment with IFN-γ (Bodasing et al., 2002) or the presence of protease inhibitors (PIs) in antiretroviral therapies (ART) (Cassone et al., 2002) have been successful for reducing/eliminating OPC. Additionally, IFN-γ has recently been shown to inhibit the *Candida*-mediated degradation of E-cadherin in vitro (Rouabhia et al., 2012). Thus, it is possible that PI and IFN-γ functioned in part by directly (IFN-γ) or indirectly (PI via inhibition of

SAPs) allowing restoration of E-cadherin and normal migration of CD8 T cells for effector function.

In contrast to cell-mediated immunity by T cells, humoral immunity does not appear to play a role in protection against or susceptibility to OPC. There is no evidence to date that a deficiency in *Candida*-specific antibodies is present in HIV^+ persons that could account for the increased prevalence of OPC (Millon et al., 2001; Wozniak et al., 2002; Wray et al., 1990).

Innate cellular defenses also play a role in host defense. Our laboratory has studied the role of epithelial cells for a number of years. These studies show that oral epithelial cells can inhibit up to 80 percent of the *Candida* growth in vitro by a fungistatic mechanism via cell contact by intact, but not necessarily live epithelial cells, through an acid-labile protein receptor (Nomanbhoy et al., 2002; Steele et al., 1999, 2000, 2001; Yano et al., 2005). Vaginal epithelial cells have an identical antifungal property, although weaker (~40 percent inhibition) compared to oral epithelial cells (Nomanbhoy et al., 2002; Steele et al., 2000). Analysis of oral epithelial cells in HIV^+ persons showed significantly reduced activity by cells from patients with OPC compared to that from patients without OPC, providing support as an innate protective mechanism against infection (Steele et al., 2000). Additionally, epithelial cells produce both cytokines and chemokines in response to *Candida*, which may contribute to the innate and/or adaptive immune response (Dongari-Bagtzoglou and Kashleva, 2003a,b; Rouabhia et al., 2002; Schaller et al., 2002; Steele and Fidel, 2002).

In subsequent studies a primary objective was to identify the antifungal effector moiety on oral epithelial cells. Accordingly, the acid treatment (that abrogates the antifungal activity) was used as a tool to accomplish this objective. The strategy involved extracting surface associated glycoproteins from the epithelial cells treated with and without periodic acid (PA). The extracted epithelial cell surface proteins were evaluated for antifungal activity. Results demonstrated that proteins extracted from PBS-treated, but not PA treated cells, were capable of inhibiting the growth of *Candida* similar to intact epithelial cells. These data are consistent with the fact that live epithelial cells are not required despite the need for cell contact. For identification of the effector moiety, the extracted glycoproteins were incubated with *Candida*, and any bound epithelial proteins were then eluted from *Candida* and separated by SDS-PAGE. Because this method could potentially result in contamination of samples with *Candida* cell surface-associated proteins, we additionally labeled the epithelial cell surface proteins with biotin prior to the acid treatment and protein extraction. This allowed for the use of Western blots to focus only on epithelial cell proteins. Results revealed two protein bands (33 and 45 kDa) eluted from *Candida* blastoconidia or hyphae that are present in PBS-treated, but not periodic acid (PA)-treated epithelial cells. Proteomic analysis showed that the ~45kDa protein for the PBS-treated cells was an actin molecule, whereas the ~33 kDa protein was annexin-A1. Annexin-A1 is a viable candidate for the effector molecule as it functions through signaling

cascades to inhibit cellular processes including growth (Croxtall et al., 2003; Liu et al., 2007). Further studies showed that annexin-A1, but not actin (as expected) was present on the epithelial cell surface and immunoprecipitation of annexin-A1 from the extracted proteins abrogated the antifungal activity (Lilly et al., 2010). Thus, annexin-A1 is considered to be the primary effector moiety responsible for the epithelial cell antifungal activity. Current studies are focused on the mechanism of action.

The static antifungal activity by mucosal epithelial cells can be considered an interesting symbiotic relationship. The host benefits by the lack of inflammation in the presence of the yeast, or significant invasion of tissue. Commensalism is maintained via annexin-A1 activity that we speculate signals the yeast to shut down its growth. The benefit for *Candida* is that it sacrifices its growth for protection against other immune responses that might otherwise kill it. This is representative of a form of immune evasion where no "danger signals" are elicited by the presence of *Candida*.

Based on these data, we propose that that several secondary lines of defense are important for protection against OPC when the primary defense by $CD4^+$ cells are below the protective threshold (summarized in Figure A9-1). These include CD8 T cells and oral epithelial cells. In HIV^+ persons with <200 CD4 cells/μl and OPC^-, it is postulated that although the $CD4^+$ T cells are below the protective threshold, epithelial cells have activity against *Candida* to hold it in check, and $CD8^+$ T cells are able to migrate to the outer epithelium to aid in protection. On the other hand, in those susceptible to OPC, the $CD8^+$ T cells are inhibited from migrating to the outer epithelium because of reduced E-cadherin together with reduced levels of epithelial cell anti-*Candida* activity resulting in bouts of OPC. When the mechanisms surrounding E-cadherin degradation/restoration, $CD8^+$ T cell activity, and annexin-A1 antifungal activity are fully understood, each could be exploited to reverse susceptibility and/or enhance resistance or protection against OPC.

Host Defense Against Vulvovaginal Candidiasis

For VVC/RVVC, studies from a mouse model of vaginal candidiasis and many clinical studies evaluating women with RVVC over two decades have revealed a general lack of protection by local or systemic adaptive immunity (reviewed in Fidel and Noverr, 2012). It is postulated that this diversion from dogma is the result of the commensal relationship evolving to avoid frequent inflammatory responses at a reproductive site. Instead, resistance and susceptibility to RVVC and VVC is now considered to involve innate immunity acting at the vaginal mucosa.

The general lack of a role for systemic and/or local adaptive immunity against vaginal candidiasis is considered to be due to multiple putative immunoregulatory mechanisms. These include significant constitutive concentrations of

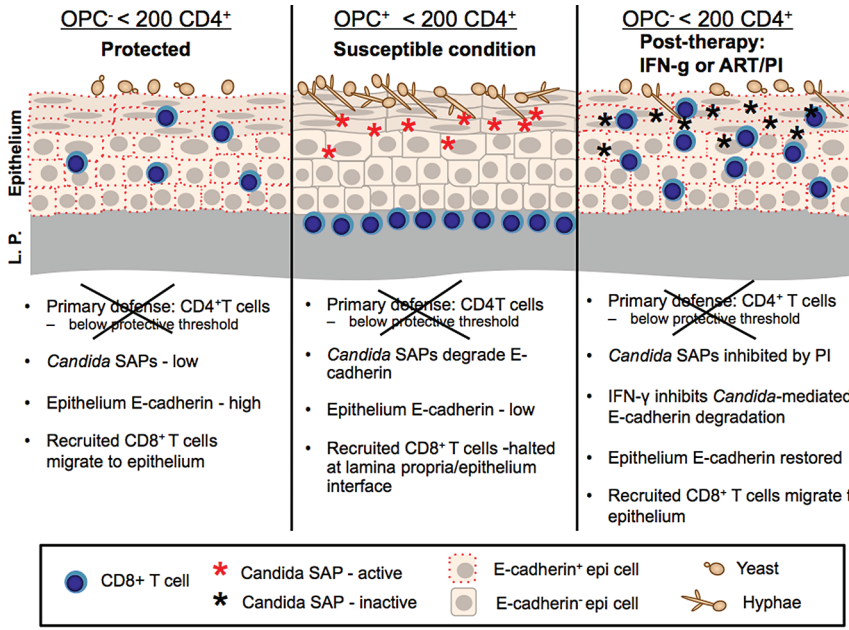


FIGURE A9-1 OPC: Protection, susceptibility, and results of treatment with ART/PI or IFN-g. Proposed immune function of the oral mucosa in HIV^+ individuals with and without OPC and < 200 CD4 cells/ml. Left frame depicts the protective mechanisms associated with the HIV^+ $OPC^–$ individual colonized with *Candida albicans* when the primary defense of $CD4^+$ T cells is below the protective threshold (200 cells/µl). The secondary defense consists of $CD8^+$ T cells migrating to the outer epithelium under normal expression of E-cadherin and functional oral epithelial cell anti-*Candida* activity that together keep *Candida* in check and prevent symptomatic infection. Th1 cytokines in saliva may also act in protection against infection, along with cytokines in tissue. Center frame depicts the condition of OPC. In this scenario, when $CD4^+$ T cells are below the protective threshold, $CD8^+$ T cells are inhibited from migrating to *Candida* for effector function due to a transient reduction in E-cadherin via *Candida*-associated SAPs, and the epithelial cells have reduced capacity to inhibit *Candida*, resulting in *Candida* overgrowth and symptomatic infection. Reduced Th1 cytokines in saliva may also contribute to the susceptibility. Right frame depicts potential treatment strategies that reduce/eliminate OPC via the restoration of E-cadherin followed by the normal migration of $CD8^+$ T cells for effector function. This can be done directly by IFN-γ or indirectly by PI in ART that inhibits *Candida*-associated SAPs.

SOURCE: Yano, J., M. C. Noverr, and P. L. Fidel, Jr. 2012. Cytokines in the host response to *Candida* vaginitis: Identifying a role for non-classical immune mediators, S100 alarmins. Special issue: Cytokines in fungal immunity. *Cytokine* 58:118-128, Fig. 2, 124.

TGF-β (Taylor et al., 2000), resident γ/δ T cells (Wormley et al., 2001), reduced homing receptors on activated T cells in the draining lymph nodes to promote T cell entry (Wormley et al., 2001), lack of effector function by infiltrating polymorphonuclear neutrophils (PMNs) (Black et al., 1998; Fidel et al., 1999), presence of $CD25^+$ Treg cells (Wormley et al., 2001), and a strong presence of plasmacytoid dentritic cells (LeBlanc et al., 2006).

In more recent years dramatic progress in our understanding of host susceptibility to VVC has been attributed to the development of a human live challenge system. In the live challenge study, resistant (no previous VVC episode) or susceptible (with infrequent VVC episodes) women were evaluated for the natural history of infection following intravaginal inoculation with live *C. albicans* (Fidel et al., 2004). Results revealed that protection, as evidenced by asymptomatic vaginal colonization with *Candida*, occurred in the absence of any inflammatory response consistent with the antifungal activity by epithelial cell annexin-A1 (Lilly et al., 2010). In contrast, symptomatic infection was accompanied by a heavy vaginal cellular infiltrate consisting of PMNs. In addition, PMN infiltration scores assessed from these women positively correlated with vaginal fungal burden although the hyphal (pathogenic) form of *Candida* could be present in both asymptomatic and symptomatic conditions (Fidel et al., 2004). Finally, when tested in an in vitro PMN migration assay, vaginal lavage fluid from women with symptomatic infection showed increased PMN chemotactic activity compared to lavage fluid from those with asymptomatic colonization, suggesting a chemotactic factor or factors (e.g., cytokines, chemokines) had been produced and secreted into the vaginal cavity in response to the *Candida* challenge (Fidel et al., 2004). Notably, evaluation of common proinflammatory cytokines and chemokines (G-CSF, TNF-α, IL-1, IL-6, IL-8, and IL-17) in lavage fluids from the study failed to identify candidates for the PMN chemotactic factors (reported in Yano et al., 2010). Moreover, *Candida* itself fails to directly induce PMN chemotaxis or through any organism-derived factors (Fidel, unpublished observations). Hence, organism virulence or virulence factors alone do not appear to be a major player in the PMN response. These important data from the live challenge study reshaped our knowledge of the immunopathogenesis of VVC/RVVC. Accordingly, based on the evidence showing the strong involvement of innate components of host immune responses during VVC, the paradigm has shifted into the current concept that both resistance and susceptibility to VVC are associated with innate immunity.

To explain the relationship between host susceptibility to VVC and the vaginal presence of *Candida*¸ the hypothesis was proposed that symptomatic infection is acquired when the number of *Candida* organisms achieves a threshold that varies between women depending on the sensitivity of vaginal epithelial cells to *Candida* and may ultimately determine the clinical outcome (reviewed in Fidel, 2007). Based on the hypothesis, we postulate the following:

1. In women with RVVC, vaginal epithelial cells are extremely sensitive to *Candida* and respond by secreting a danger signal (rather than typical antifungal activity via annexin-A1) that promote PMN infiltration after exposure to low numbers of *Candida*. These women are susceptible to recurrence by small increases in organism numbers (i.e., shortly following disruption or cessation of maintenance antifungal therapy).
2. In women with infrequent history of VVC due to any of the known predisposing factors (e.g., oral contraceptives, hormone replacement therapy, antibiotic usage), vaginal epithelial cells are less sensitive to *Candida* and have a higher threshold for *Candida*. These women remain asymptomatic following exposure to moderate numbers of *Candida* (with typical antifungal activity by epithelial cell annexin-A1), but if the numbers rise following the use of antibiotics or estrogen, the threshold will be breached and a similar danger response will result in a symptomatic condition.
3. In women with no history of VVC, their vaginal epithelial cells are insensitive to even large numbers of *Candida* and hence the threshold is rarely breached. Rather, these women likely benefit greatly from the annexin-A1-mediated antifungal activity via epithelial cells for a continued commensal state of *Candida*. In any event, the PMN response rarely, if ever, occurs and these women remain asymptomatic. The results of the live challenge study clearly support this hypothesis. In women with no history of vaginitis, 90 percent remained asymptomatic following inoculation. In contrast, 55 percent of women with infrequent episodes of vaginal candidiasis due to oral contraceptives, hormone replacement therapy, or antibiotic usage became symptomatic following inoculation. Moreover, if those women were tested in their susceptible state (e.g., using oral contraceptives, hormone replacement therapy, or antibiotic usage), 90 percent became symptomatic (Fidel et al., 2004). Clinical cross-sectional and the live challenge natural history study also supports the protective effects of epithelial cell annexin-A1; women with RVVC or who were susceptible to symptomatic VVC following inoculation had lower epithelial cell antifungal activity compared to women without RVVC or who simply became asymptomatically colonized following inoculation (Barousse et al., 2001, 2005). At present, it is unknown what factors are involved in establishing the level of epithelial cell sensitivity to *Candida*, but is presumed to be a genetic predisposition. Indeed, some polymorphisms have been identified in women with RVVC (Babula et al., 2005; Ferwerda et al., 2009). On the other hand, differences in susceptibility to infection could not be demonstrated for various haplotypic strains of mice (Black et al., 1999; Calderon et al., 2003; Clemons et al., 2004; Yano et al., 2010).

In parallel with the clinical studies examining innate immune factors against VVC, the mouse model also showed an erratic presence of vaginal PMNs during

infection (Black et al., 1998; Fidel et al., 1999; Yano et al., 2010). Although PMNs were identified to be the most predominant leukocyte population in vaginal lavage fluid irrespective of time postinoculation, their presence never resulted in correlates to reduced vaginal fungal burden and thus was presumed to be due to the pseudo-estrus condition (Fidel and Sobel, 1999; Fidel et al., 2000). In fact, the presence of the PMNs was quite variable with 60–70 percent of inoculated mice having substantial PMNs in the vagina, while the remainder had little to no vaginal PMNs.

Consequently, a series of studies were conducted to formally classify the PMN response in various conditions. Indeed, similar to clinical observations, data showed that a heavy vaginal PMN presence occurred consistently in a high percentage of inoculated animals irrespective of the haplotype or the duration of infection without affecting fungal burden (Yano et al., 2010). Of note, previous studies testing effects of estrogen concentrations, inocula and strains of *C. albicans* also showed similar PMN infiltration patterns (Fidel and Sobel, 1999; Fidel et al., 1996a, 2000). In light of symptomatic/asymptomatic conditions observed in inoculated women from the live challenge study, the high PMN and low PMN responses in mice were classified to be simulating the symptomatic and asymptomatic conditions of VVC, respectively (Yano et al., 2010). Although previous attempts to quantify clinical signs and symptoms of vaginitis in mice (e.g., redness, swelling, irritation, scratching) were unsuccessful and therefore could not be used as correlates to the PMN infiltration, vaginal PMNs levels appear to be rigid criteria based on the association with the clinical symptomatology of VVC. Accordingly, when tested in an in vitro PMN migration assay, results showed, similar to clinical studies, that vaginal lavage fluids from mice with high PMNs (symptomatic) had increased PMN chemotactic activity compared to those from a low PMN (asymptomatic) condition, suggesting the presence of a similar chemotactic factor or factors secreted in response to *Candida* (Yano et al., 2010). Taking into account previous reports showing a lack of strong vaginal cytokine/chemokine responses in humans and mice in VVC, identification of the putative PMN chemotactic factors was crucial to further uncovering immune processes associated with susceptibility to symptomatic disease. The mouse model of VVC was then exploited to further dissect the innate mechanisms associated with the immunopathology of VVC.

Subsequent animal studies by Yano et al. incorporated proteomic approaches in search of the putative factors responsible for the inflammatory response during the symptomatic condition (Yano et al., 2010). First, vaginal lavage fluid from mice inoculated with *C. albicans* was evaluated for protein expression patterns. Based on the PMN-associated symptomatology criteria, two distinct proteins at 10 kDa and 14 kDa were identified by differential expression that positively correlated with the levels of vaginal PMNs. These proteins were further analyzed by mass spectrometry for protein identification and showed a significant match for S100A8 (10 kDa) and S100A9 (14 kDa) calcium-binding proteins. Confirming

the initial protein identification, detection by Western blots and ELISA showed high abundance of S100A8 and S100A9 in vaginal lavage fluid from inoculated mice with high PMNs, but results were extremely low in fluids from those with low PMNs. Consistently, evaluation of vaginal tissues by immunohistochemistry showed elevated S100 protein expression localized at the apical surface of vaginal epithelium of mice with high PMNs, while the expression was dramatically reduced in tissues from those with low PMNs. This observation was further supported by increased S100 expression in vaginal epithelial cells at the mRNA levels, confirming epithelial cells as a primary source of S100 proteins produced under the symptomatic condition. Finally, neutralization of S100A8, but not S100A9, reduced the PMN chemotactic activity of vaginal lavage fluid in an in vitro migration assay, suggesting a role for S100A8 in mediating PMN migration during vaginal infection. Hence, at least in the mouse model, S100A8 appears to be produced by vaginal epithelial cells following interaction with *Candida*, and may play a key role as a PMN chemotactic factor that initiate the inflammatory response during symptomatic VVC.

S100A8 and S100A9 are small molecular weight calcium- and zinc-binding proteins of the S100 family and are also known as calgranulin A, myeloid-related protein (MRP) 8, chemotactic protein (CP) 10, and calgranulin B, MRP-14, respectively. S100A8 and S100A9 are found as monomers or heterodimeric complex called calprotectin, which has been shown to elicit antimicrobial properties to various microbial pathogens including *C. albicans* (Sohnle et al., 1996; Urban et al., 2009; Zimmer et al., 1995). These proteins also serve as potent chemoattractants for PMNs and participate in inflammatory processes. Due to their involvement as endogenous danger-signaling chemoattractant molecules, S100A8 and S100A9 have been termed "alarmins", a subgroup of endogenous damage-associated molecular patterns (DAMPs) eliciting host immune responses in similar mechanisms as exogenous pathogen-associated molecular patterns (PAMPs) (Ehrchen et al., 2009). S100 alarmins are produced by phagocytes, monocytes, epithelial cells, and endothelial cells and are released at sites of inflammation (Foell et al., 2008; Gebhardt et al., 2006; Kumar et al., 2001; Ross and Herzberg, 2001; Ryckman et al., 2003). In contrast to other mucosal sites where S100 alarmins are readily detected as calprotectin (Sohnle et al., 1996; Urban et al., 2009; Zimmer et al., 1995), data from the mouse study showed that S100A8 and S100A9 were mostly present as monomers in the vaginal secretions (Yano et al., 2010). In addition, vaginal S100 alarmins exhibited no antimicrobial effect on *C. albicans* as evidenced by the fact that vaginal fungal burden remained unaffected by the levels of S100 alarmins detected in the vagina. However, the magnitude of PMN infiltration positively correlated with the amount of vaginal S100 alarmins (Yano et al., 2010). Recognizing the dichotomous PMN migration in inoculated animals, together with the lack of a genetic disposition of inbred mice to explain the dichotomy, other explanations were sought. Accordingly, studies showed that the alarmin trigger is based on early adherence where higher

rates of adherence are presumed to be sensed as danger by the epithelial cells and result in the triggering of the alarmin response.

Although vaginal epithelial cells appear to be a primary source of S100 alarmins during experimental VVC, PMNs also likely contribute to the S100 production once recruited into the vaginal mucosa. A proposed hypothesis is that PMNs recruited in response to the initial epithelial cell-derived S100 alarmins may initiate a second wave of S100 production, amplifying the PMN response as part of positive feedback mechanism. Figure A9-2 illustrates the complete sequence of events considered to be critical in the immunopathogenesis of VVC/RVVC. Furthermore, although the Th17 response is involved in protection against, or susceptibility to, other forms of mucosal candidiasis (Conti et al., 2009; Eyerich et al., 2008; Huang et al., 2004; Urban et al., 2009), and is a strong contributor to S100 alarmin production (Liang et al., 2006), a recent study using IL-23p19$^{-/-}$, IL-17RA$^{-/-}$, and IL-22$^{-/-}$ mice found no role for the cells or cytokines of the Th17 lineage in the S100 alarmin response during VVC (Yano et al., 2012).

Taken together, the more complete understanding of the immunopathogenesis of VVC/RVVC has important implications for diagnostics and treatment. Current diagnostics for VVC are limited. The challenge has been that *Candida* is commensal and present in an asymptomatic state. Positive diagnosis requires the presence of symptoms and a positive yeast culture. However, current diagnostic tests are based on the presence of organism alone. Hence, an improved diagnostic could include testing for the presence of *Candida* as well as symptoms using alarmins as the key biomarker for the inflammatory event. Additionally, alarmins could be key targets for immunotherapeutic strategies. One can envision blocking or neutralizing the S100 alarmins to reduce or eliminate the symptoms of VVC/RVVC. The end result would be an asymptomatic condition with *Candida* relegated back to a commensal state. Current studies are focused on testing the immunopathogenesis/alarmin hypothesis in clinical VVC/RVVC to provide the necessary evidence to embark on these diagnostic and immunotherapeutic strategies.

The Emerging Role of Mucosal *Candida* Biofilms and Host Immunity

Recently there has been a tremendous interest in the role of biofilms in infectious diseases. It is estimated that 80 percent of human infections result from pathogenic biofilms (Costerton et al., 1999). Biofilms are communities of microorganisms that are embedded in extracellular matrix (ECM), forming a complex microbial community. A unique feature of *C. albicans* biofilms is the morphological heterogeneity of the biofilm cells, which results in a complex 3-dimensional biofilm architecture (Chandra et al., 2001). Biofilm formation is dependent on the ability to undergo morphogenesis; mutants defective in hyphal formation in vitro are also defective in biofilm formation (Nobile et al., 2006; Ramage et al., 2002). *C. albicans* biofilms have a unique gene expression pattern (Yeater et al.,

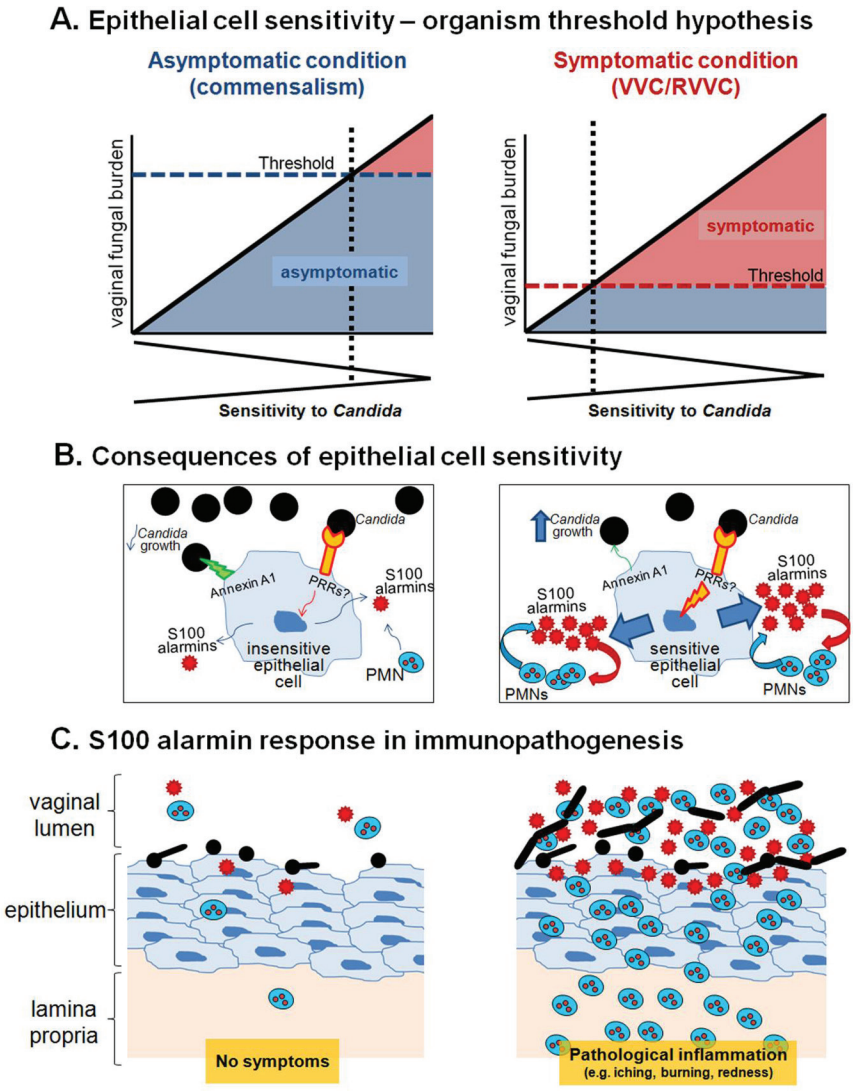


FIGURE A9-2 Schematic diagrams representing the mechanism for the effects of vaginal S100 alarmins on PMN migration during VVC. (A) Epithelial cell sensitivity–organism threshold hypothesis. In women with no history of VVC (left panel), their vaginal epithelial cells are insensitive to *Candida*. These women remain asymptomatic as even in the presence of high numbers for *Candida* (threshold number to initiate pathological response rarely breached). In women with RVVC (right panel), vaginal epithelial cells are extremely sensitive to *Candida*. These women are susceptible to symptomatic infection following exposure to even small numbers of *Candida* (low organism threshold). The thresholds represent an arbitrary organism number of the upper limit for vaginal fungal burden that

2007) and are more resistant to antifungal treatment than planktonic cells (Kojic and Darouiche, 2004; Ramage et al., 2002). There, in a clinical setting, biofilms represent a significant risk and are difficult to eradicate.

Candida biofilms have been studied primarily on abiotic surfaces (Blankenship and Mitchell, 2006; Ramage et al., 2006). A large proportion of *C. albicans* infections involve biofilms, which can form on a variety of synthetic polymers used in medical devices (Dominic et al., 2007; Douglas, 2003; Kojic and Darouiche, 2004). Significant attention has been given to *Candida* biofilm formation of indwelling catheters, which can lead to life-threatening systemic infections (Crump and Collignon, 2000; Dominic et al., 2007; Kojic and Darouiche, 2004). *Candida albicans* is the fourth leading cause of bloodstream infections and the third most commonly isolated organism from intravascular catheters and is associated with the highest incidence of mortality (Crump and Collignon, 2000; Wisplinghoff et al., 2004). *Candida* biofilm development on abiotic surfaces can be divided into several growth stages, including early, intermediate, and mature (Chandra et al., 2001). During early biofilm formation, yeast cells adhere to an appropriate surface and initiate germ tube formation. The intermediate phase is characterized by continued hyphal elongation and ECM production, which consists of cell wall polysaccharides and protein (Baillie and Douglas, 2000; Nett et al., 2007). Mature biofilms consist of a yeast base, with hyphal elements encased

---

would initiate symptomatic infection. (B) Consequences of epithelial cell sensitivity. Under asymptomatic condition (left panel), vaginal epithelial cells are insensitive to *Candida* and remain unstimulated following interaction with *Candida*. In turn, PMN migration does not occur in the absence of S100 alarmin production. Strong cell-surface annexin A1-dependent (proposed based on oral epithelial cells) antifungal activity provides noninflammatory means to maintain *Candida* at the commensal state. Under symptomatic conditions (right panel), vaginal epithelial cells are extremely sensitive to *Candida* and exert weak antifungal activity through annexin A1. Epithelial cells become activated upon recognition of *Candida* via unidentified PRRs. S100 alarmins are secreted as danger signals toward which vaginal PMNs migrate through vaginal epithelium. Once in the vaginal epithelium, recruited PMNs also produce S100 alarmins as part of positive feedback mechanism to further amplify the PMN response. (C) S100 alarmin response in immunopathogenesis. PMN infiltration remains minimal in the absence of S100 alarmin production by vaginal epithelial cells, therefore, no symptom occurs (left panel). In contrast, high concentrations of S100 alarmins in vaginal epithelium trigger PMN migration to the vaginal cavity, resulting in pathological inflammation associated with the symptoms of infection. The inflammatory process enhances *Candida* growth and hyphal formation (right panel).
SOURCE: Adapted from: Fidel, P. L., Jr. 2011. *Candida*-host interactions in HIV disease: Implications for oropharyngeal candidiasis. Proceedings from the 6th World Workshop on Oral HIV and AIDS. *Adv Dent Res*. 23:45-49, Fig. 1, p. 47.

in ECM extending away from the surface. In addition, persister cells are present, which are a multidrug tolerant subpopulation of the biofilm (LaFleur et al., 2006). Newly formed daughter yeast cells grow out of hyphal elements and are released (dispersal), seeding new niches for biofilm formation or infection.

Candida biofilm formation on biotic surfaces has not been investigated until recently. *Candida* resides at mucosal surfaces as a normal inhabitant of the microbiota. Generally, mucosal disease is associated with a shift towards hyphal growth during vaginitis, oral candidiasis, and invasive GI tract infections (Bilhan et al., 2009; Cantorna and Balish, 1990; Sobel et al., 1984). This provides some evidence that biofilm formation could occur on mucosal tissues and may be associated with disease. Alternatively, biofilm growth could represent a reservoir of chronic and/or persistent colonizing organisms that serve as a source of opportunistic infection. Several animal models of infection have recently been used/established to study *Candida* mucosal biofilm formation including OPC (Dongari-Bagtzoglou et al., 2009), vaginitis (Harriott et al., 2010), and denture stomatitis (Johnson et al., 2010).

For VVC, in vivo and ex vivo models were recently used to examine mucosal biofilm formation by scanning electron and confocal microscopy. Wild-type *C. albicans* strains formed biofilms on the vaginal mucosa in vivo and ex vivo as indicated by high fungal burden and microscopic analysis demonstrating typical biofilm architecture and ECM that co-localized with the presence of fungi. In contrast, mutants in a regulator of hyphal formation (*efg1*/*efg1*) and biofilm formation (*bcr1*/*bcr1*) exhibited weak to no biofilm formation and ECM production in both models despite comparable colonization levels. This raised an interesting question: does the presence of a biofilm determine whether *C. albicans* behaves as a pathogen and allows the switch from commensalism and whether a biofilm influences the host response (i.e., alarmin-dependent PMN migration to the vagina)? This was recently addressed in the experimental VVC model. Unexpectedly, the presence or absence of mucosal *Candida* biofilm had no effect on the alarmin response. The animals, irrespective of the *Candida* isolate used to inoculate, had similar vaginal fungal burden, vaginal S100 alarmin concentrations, and vaginal PMNs (Lilly et al. Submitted). Hence, it does not appear that mucosal biofilm formation is critical for the immunopathogenesis of VVC/RVVC. This may be explained by the fact that symptomatic episodes of VVC/RVVC are acute and initiated more by the adherence and sensitivity to the epithelial cells than the presence of biofilm. In the general pathogenesis though, biofilm is probably more critical to treatment and clearance via pharmacologic drugs or the immune response. One might envision difficulty of drugs or immune cells/mediators (i.e., cytokines, antibodies) to penetrate the biofilm effectively to function optimally.

In the area of *Candida*-associated denture stomatitis, a novel contemporary rat model of DS was recently developed using a custom-fitted denture system composed of both fixed and magnetic removable plates (Lee et al., 2010). The denture system was installed against the hard palate of rats without alteration of the dental architecture. The novel design of this denture system (removable

portion) allows for longitudinal studies to evaluate the progression of the disease. Biofilm formation was analyzed on the denture and palate via scanning electron and confocal microscopy (Johnson et al., 2010). Biofilm formation on the denture occurred by week 4 postinoculation, characterized by the presence of hyphae coated with ECM. However, on the palate tissue, only blastospore colonization was observed with no clinical evidence of disease. By week 6 postinoculation, biofilm formation was observed on both the denture and the palate tissue and palatal erythema was evident. This suggests that during DS, *C. albicans* biofilm formation occurs initially on the denture plate, which in turn seeds the palatal tissue, resulting in mucosal biofilm formation and signs of disease. In a recent study, the biofilm deficient mutants, *efg1*/*efg1* and *bcr1*/*bcr1* were used to evaluate disease states in the rat model of DS. In a complete reversal of what was observed in VVC, clinical disease was only seen in animals inoculated with the wild-type strain. Hence, the presence of biofilm appears critical to pathogenesis of DS (Noverr, manuscript in preparation). This is consistent with the chronicity of the disease and the fact that experimental infections/disease occur after biofilm formation.

A model we envision to explain the pathogenesis of DS is illustrated in Figure A9-3. The denture acquires *Candida* first by any number of means, and a biofilm is subsequently formed. The established abiotic biofilm then proceeds to seed the mucosal tissue. Once the organism is associated with the tissue an immune response will be induced. However, due the continuous feeding and biofilm formation on the mucosa itself, the immune response can be amplified resulting in a chronic inflammatory response. Current studies are exploring the type of immune response involved and the mechanisms surrounding the response.

Conclusions

Several major points should be emphasized regarding host defense and immunomodulation against mucosal candidiasis: (1) Host defense against *Candida* is extremely different at different mucosal sites, including adaptive immunity by T cells playing a significant role at the oral mucosa, and innate immunity via PMNs and epithelial cells playing critical roles in the vagina mucosa. (2) The immune factors associated with protection and susceptibility are unique to each site and often involve immunomodulatory factors. Examples include alarmins in the vagina associated with susceptibility to VVC, epithelial cell-annexin-A1 in oral and vaginal tissues associated with protection, and E-cadherin and CD8 T cells in the oral cavity associated with protection. (3) The role of mucosal biofilm in pathogenesis of infection is likewise very different for each mucosa site, evidenced by current data showing no role for biofilm in susceptibility to vaginal candidiasis versus a critical role in susceptibility to denture stomatitis. Future research will undoubtedly further elucidate the mechanisms associated with protection and susceptibility to each infection, which will be critical to exploiting the immunomodulatory factors in therapeutics or targets thereof.

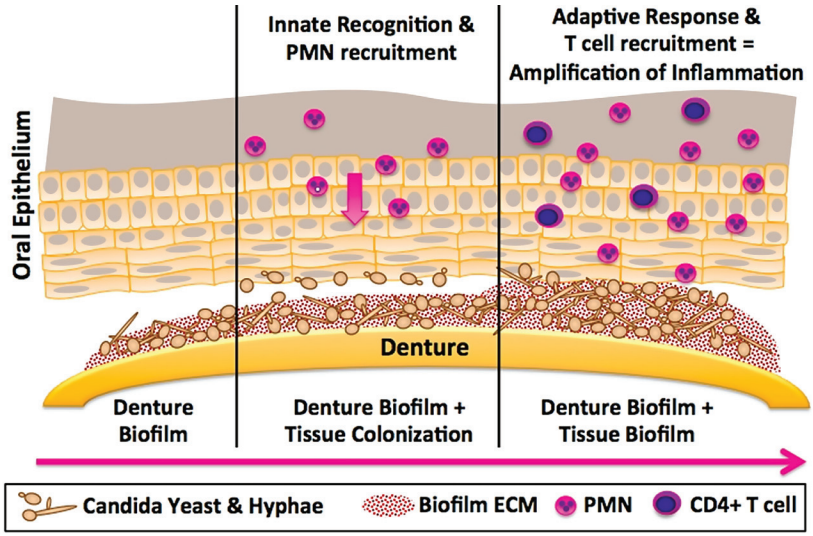


FIGURE A9-3 Proposed model of immunopathogenesis of *Candida*-associated denture stomatitis. The figure illustrates the central hypothesis that *Candida* biofilm formation on the denture (left frame) plays a significant role in denture stomatitis through the continuous inoculation of the oral mucosa from the denture (center frame) leading to tissue-associated biofilm formation and chronic erythematous inflammation due to uncontrolled innate and adaptive responses (right frame).
NOTE: PMN, polymorphonuclear neutrophil.
SOURCE: Figure courtesy of M.C. Noverr (03/2012).

References

Babula, O., G. Lazdane, J. Kroica, I. M. Linhares, W. J. Ledger, and S. S. Witkin. 2005. Frequency of interleukin-4 (il-4) -589 gene polymorphism and vaginal concentrations of il-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. *Clinical Infectious Diseases* 40(9):1258-1262.

Baillie, G. S., and L. J. Douglas. 2000. Matrix polymers of candida biofilms and their possible role in biofilm resistance to antifungal agents. *Journal of Antimicrobial Chemotherapy* 46(3):397-403.

Barousse, M. M., C. Steele, K. Dunlap, T. Espinosa, D. Boikov, J. D. Sobel, and P. L. Fidel, Jr. 2001. *Journal of Infectious Diseases* 184:1489-1493.

Barousse, M. M., T. Espinosa, K. Dunlap, and P. L. Fidel, Jr. 2005. Vaginal epithelial cell anti-candida albicans activity is associated with protection against symptomatic vaginal candidiasis. *Infection and Immunity* 73:7765-7767.

Bilhan, H., T. Sulun, G. Erkose, H. Kurt, Z. Erturan, O. Kutay, and T. Bilgin. 2009. The role of *Candida albicans* hyphae and lactobacillus in denture-related stomatitis. *Clinical Oral Investigations* 13(4):363-368.

Black, C. A., F. M. Eyers, A. Russell, M. L. Dunkley, R. L. Clancy, and K. W. Beagley. 1998. Acute neutropenia decreases inflammation associated with murine vaginal candidiasis but has no effect on the course of infection. *Infection and Immunity* 66:1273-1275.

Black, C. A., F. M. Eyers, M. L. Dunkley, R. L. Clancy, and K. W. Beagley. 1999. Major histocompatibility haplotype does not impact the course of experimentally induced murine vaginal candidiasis. *Laboratory Animal Science* 49:668-672.

Blankenship, J. R., and A. P. Mitchell. 2006. How to build a biofilm: A fungal perspective. *Current Opinion in Microbiology* 9(6):588-594.

Bodasing, N., R. A. Seaton, G. S. Shankland, and A. Pithie. 2002. Gamma-interferon treatment for resistant oropharyngeal candidiasis in an HIV-positive patient. *Journal of Antimicrobial Chemotherapy* 50:765-766.

Budtz-Jorgensen, E., A. Stenderup, and M. Grabowski. 1975. An epidemiologic study of yeasts in elderly denture wearers. *Community Dentistry and Oral Epidemiology* 3(3):115-119.

Burns, D. N., R. Tuomala, B. H. Chang, R. Hershow, H. Minkoff, E. Rodriquez, C. Zorrilla, H. Hammill, and J. Regan. 1997. Vaginal colonization or infection with *Candida albicans* in human immunodeficiency virus-infected women during pregnancy and during postpartum period. *Clinical Infectious Diseases* 24:201-210.

Calderon, L., R. William, M. Martinez, K. V. Clemons, and D. A. Stevens. 2003. Genetic susceptibility to vaginal candidiasis. *Medical Mycology* 41:143-147.

Cantorna, M. T., and E. Balish. 1990. Mucosal and systemic candidiasis in congenitally immunodeficient mice. *Infection and Immunity* 58(4):1093-1100.

Cassone, A., E. Tacconelli, F. De Bernardis, M. Tumbarello, A. Torosantucci, P. Chiani, and R. Cauda. 2002. Antiretroviral therapy with protease inhibitors has an early, immune reconstitution-independent beneficial effect on candida virulence and oral candidiasis in human immunodeficiency virus-infected subjects. *Journal of Infectious Diseases* 185(2):188-195.

Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. *Journal of Bacteriology* 183:5385-5394.

Clark, R. A., S. A. Blakley, J. Rice, and W. Brandon. 1995. Predictors of HIV progression in women. *Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology* 9:43-50.

Clemons, K. V., J. L. Spearow, R. Parmar, M. Espiritu, and D. A. Stevens. 2004. Genetic susceptibility of mice to *Candida albicans* vaginitis correlates with host estrogen sensitivity. *Infection and Immunity* 72:4878-4880.

Conti, H. R., F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. H. Hai, J. J. Yu, J. W. Jung, S. G. Filler, P. Masso-Welch, M. Edgerton, and S. L. Gaffen. 2009. Th17 cells and il-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *Journal of Experimental Medicine* 206(2):299-311.

Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. *Science* 284(5418):1318-1322.

Croxtall, J. D., D. W. Gilroy, E. Solito, Q. Choudhury, B. J. Ward, J. C. Buckingham, and R. J. Flower. 2003. Attenuation of glucocorticoid functions in an anx-a1 -/- cell line. *Biochemical Journal* 371:927-935.

Crump, J. A., and P. J. Collignon. 2000. Intravascular catheter-associated infections. *European Journal of Clinical Microbiology & Infectious Diseases* 19(1):1-8.

Daniluk, T., G. Tokajuk, W. Stokowska, K. Fiedoruk, M. Sciepuk, M. L. Zaremba, D. Rozkiewicz, D. Cylwik-Rokicka, B. A. Kedra, I. Anielska, M. Gorska, and B. R. Kedra. 2006. Occurrence rate of oral *Candida albicans* in denture wearer patients. *Advances in Medical Sciences* 51(Suppl 1):77-80.

Deslauriers, N., L. Cote, S. Montplaisir, and L. De Repentigny. 1997. Oral carriage of *Candida albicans* in murine AIDS. *Infection and Immunity* 65:661-667.

Dominic, R. M., S. Shenoy, and S. Baliga. 2007. Candida biofilms in medical devices: Evolving trends. *Kathmandu University Medical Journal (KUMJ)* 5(3):431-436.

Dongari-Bagtzoglou, A., and H. Kashleva. 2003a. *Candida albicans* triggers interleukin-8 by oral epithelial cells. *Microbial Pathogenesis* 34:169-177.

Dongari-Bagtzoglou, A., and H. Kashleva. 2003b. Granulocyte-macrophage colony-stimulating factor responses of oral epithelial cells to *Candida albicans. Oral Microbiology and Immunology* 18:165-170.

Dongari-Bagtzoglou, A., H. Kashleva, P. Dwivedi, P. Diaz, and J. Vasilakos. 2009. Characterization of mucosal *Candida albicans* biofilms. *PloS ONE* 4(11):e7967.

Douglas, L. J. 2003. Candida biofilms and their role in infection. *Trends in Microbiology* 11(1):30-36.

Duerr, A., M. F. Sierra, J. Feldman, L. M. Clarke, I. Ehlich, and J. Dehovitz. 1997. Immune compromise and prevalence of candida vulvovaginitis in human immunodeficiency virus-infected women. *Obstetrics and Gynecology* 90:252-256.

Ehrchen, J. M., C. Sunderkötter, D. Foell, T. Vogl, and J. Roth. 2009. The endogenous toll-like receptor 4 agonist s100a8/s100a9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *Journal of Leukocyte Biology* 86(3):557-566.

Eyerich, K., S. Foerster, S. Rombold, H. P. Seidl, H. Behrendt, H. Hofmann, J. Ring, and C. Traidl-Hoffmann. 2008. Patients with chronic mucocutaneous candidiasis exhibit reduced production of th17-associated cytokines il-17 and il-22. *Journal of Investigative Dermatology* 128(11):2640-2645.

Ferwerda, B., G. Ferwerda, T. S. Plantinga, J. A. Willment, A. B. van Spriel, H. Venselaar, C. C. Elbers, M. D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S. A. Morre, G. Vriend, D. L. Williams, J. R. Perfect, L. A. Joosten, C. Wijmenga, J. W. van der Meer, G. J. Adema, B. J. Kullberg, G. D. Brown, and M. G. Netea. 2009. Human dectin-1 deficiency and mucocutaneous fungal infections. *New England Journal of Medicine* 361(18):1798-1801.

Fidel, P. L., Jr. 2002. Distinct protective host defenses against oral and vaginal candidiasis. *Medical Mycology* 40:359-375.

Fidel, P. L. 2006. Candida-host interactions in HIV disease: Relationships in oropharyngeal candidiasis. *Advances in Dental Research* 19(1):80-84.

Fidel, P. L., Jr. 2007. History and update on host defense against vaginal candidiasis. *American Journal of Reproductive Immunology* 57:2-12.

Fidel, P. L., Jr., and M. C. Noverr. 2012. Mucosal immunity to *Candida albicans* In *Candida and candidiasis*. Vol. 2, edited by R. A. Calderone and C. J. Clancy. Washington, DC: ASM Press. Pp. 137-154.

Fidel, P. L., Jr., and J. D. Sobel. 1996. Immunopathogenesis of recurrent vulvovaginal candidiasis. *Clinical Microbiology Reviews* 9:335-348.

Fidel, P. L., Jr., and J. D. Sobel. 1999. Murine models of candida vaginal infections. In *Experimental models in antimicrobial chemotherapy*. Vol. 2, edited by O. Zak and M. Sande. London, UK: Academic Press Ltd. Pp. 741-748.

Fidel, P. L., Jr., J. L. Cutright, L. Tait, and J. D. Sobel. 1996a. A murine model of candida glabrata vaginitis. *Journal of Infectious Diseases* 173:425-431.

Fidel, P. L., Jr., N. A. Wolf, and M. A. KuKuruga. 1996b. T lymphocytes in the murine vaginal mucosa are phenotypically distinct from those in the periphery. *Infection and Immunity* 64:3793-3799.

Fidel, P. L., Jr., W. Luo, C. Steele, J. Chabain, M. Baker, and F. L. Wormley. 1999. Analysis of vaginal cell populations during experimental vaginal candidiasis. *Infection and Immunity* 67:3135-3140.

Fidel, P. L., Jr., J. L. Cutright, and C. Steele. 2000. Effects of reproductive hormones on experimental vaginal candidiasis. *Infection and Immunity* 68:651-657.

Fidel, P. L., Jr., M. Barousse, T. Espinosa, C. Camaratti, M. Ficarra, D. H. Martin, A. J. Quayle, and K. Dunlap. 2004. An intravaginal live candida challenge in humans leads to new hypotheses for the immunopathogenesis of vulvovaginal candidiasis. *Infection and Immunity* 72:2939-2946.

Foell, D., H. Wittkowski, Z. Ren, J. Turton, G. Pang, J. Daebritz, J. Ehrchen, J. Heidemann, T. Borody, J. Roth, and R. Clancy. 2008. Phagocyte-specific s100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. *Journal of Pathology* 216:183-192.

Frank, C. F., and M. K. Hostetter. 2007. Cleavage of e-cadherin: A mechanism for disruption of the intestinal epithelial barrier by *Candida albicans. Translational Research: The Journal of Laboratory and Clinical Medicine* 149(4):211-222.

Gebhardt, C., J. Németh, P. Angel, and J. Hess. 2006. S100a8 and s100a9 in inflammation and cancer. *Biochemical Pharmacology* 72:1622-1631.

Greenspan, D., E. Komaroff, M. Redford, J. A. Phelan, M. Navazesh, M. E. Alves, H. Kamrath, R. Mulligan, C. E. Barr, and J. S. Greenspan. 2000. Oral mucosal lesions and HIV viral load in the women's interagency HIV study (WIHS). *Journal of Acquired Immune Deficiency Syndrome* 25:44-50.

Harriott, M. M., E. A. Lilly, T. E. Rodriguez, P. L. Fidel Jr., and M. C. Noverr. 2010. *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology.* In press.

Hladik, F., G. Lentz, E. Delpit, A. McElroy, and M. J. McElrath. 1999. Coexpression of ccr5 and il-2 in human genital but not blood cells: Implications for the ontogeny of the ccr5+ th1 phenotype. *Journal of Immunology* 163:2306-2313.

Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger. 2004. Requirement of interleukin-17a for systemic anti-candida albicans host defense in mice. *Journal of Infectious Diseases* 190(3):624-631.

Ibraghimov, A. R., R. E. Sacco, M. Sandor, Z. Iakoubov, and R. G. Lynch. 1995. Resident cd4 + αβ t cells of the murine female genital tract: A phenotypically distinct t cell lineage that rapidly proliferates in response to systemic t cell activation stimuli. *International Immunology* 7:1763-1769.

Imam, N., C. C. J. Carpenter, K. H. Mayer, A. Fisher, M. Stein, and S. B. Danforth. 1990. Hierarchical pattern of mucosal candida infections in HIV-seropositive women. *American Journal of Medicine* 89:142-146.

Inghirami, G., B. Y. Zhu, L. Chess, and D. M. Knowles. 1990. Flow cytometric and immunohistochemical characterization of the gamma/delta t-lymphocyte population in normal human lymphoid tissue and peripheral blood. *American Journal of Pathology* 136:357-367.

Itohara, S., A. G. Farr, J. J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa. 1990. Homing of a gamma/delta thymocyte subset with homogenous t-cell receptors to mucosal epithelia. *Nature* 343:754-757.

Johansson, E. L., A. Rudin, L. Wassen, and J. Holmgren. 2000. Distribution of lymphocytes and adhesion molecules in human cervix and vagina. *Immunology* 96:272-277.

Johnson, C. J., H. Lee, A. Yu, P. L. Fidel Jr., and M. C. Noverr. 2010. Characterization of a contemporary animal model of candida associated denture stomatitis. *Oral Diseases.* In submission.

Kent, H. L. 1991. Epidemiology of vaginitis. *American Journal of Obstetrics and Gynecology* 165:1168-1175.

Kirkpatrick, C. H. 1984. Host factors in defense against fungal infections. *American Journal of Medicine* 77:1-12.

Kirkpatrick, C. H., and P. G. Sohnle. 1981. Chronic mucocutaneous candidiasis. In *Immunodermatology*, edited by B. Safai and R. A. Good. New York: Plenum Press. Pp. 495-514.

Kirkpatrick, C. H., J. W. Chandler, and R. N. Schimke. 1970. Chronic mucocutaneous moniliasis with impaired delayed hypersensitivity. *Clinical and Experimental Immunology* 6:375-385.

Kirkpatrick, C. H., R. R. Rich, and J. E. Bennett. 1971. Chronic mucocutaneous candidiasis: Model building in cellular immunity. *Annals of Internal Medicine* 74:955-978.

Klein, R. S., C. A. Harris, C. B. Small, B. Moll, M. Lesser, and G. H. Friedland. 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *New England Journal of Medicine* 311:354-357.

Kojic, E. M., and R. O. Darouiche. 2004. Candida infections of medical devices. *Clinical Microbiology Reviews* 17(2):255-267.

Kumar, R. K., Z. Yang, S. Bilson, S. Thliveris, B. E. Cooke, and C. L. Geczy. 2001. Dimeric s100a8 in human neutrophils is diminished after phagocytosis. *Journal of Leukocyte Biology* 70:59-64.

Kunkl, A., L. Mortara, M. T. Valle, D. Fenoglio, M. Paola, A. M. Megiovanni, A. Alessandrini, G. Li Pira, G. Mazzarello, V. Del Bono, A. Canessa, D. Bassetti, and F. Manca. 1998. Recognition of antigenic clusters of *Candida albicans* by t lymphocytes from human immunodeficiency virus-infected persons. *Journal of Infectious Diseases* 178:488-496.

LaFleur, M. D., C. A. Kumamoto, and K. Lewis. 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrobial Agents and Chemotherapy* 50(11):3839-3846.

LeBlanc, D. M., M. M. Barousse, and P. L. Fidel, Jr. 2006. A role for dendritic cells in immunoregulation during experimental vaginal candidiasis. *Infection and Immunity* 74:3213-3221.

Lee, H., A. Yu, C. J. Johnson, M. C. Noverr, and P. L. Fidel Jr. 2010. Fabrication of a multi-applicable removable intraoral denture system for rodent research. *JDR Discovery.* In submission.

Leigh, J. E., C. Steele, F. L. Wormley, Jr., W. Luo, R. A. Clark, W. R. Gallaher, and P. L. Fidel, Jr. 1998. Th1/th2 cytokine expression in saliva of HIV-positive and HIV-negative individuals: A pilot study in HIV-positive individuals with oropharyngeal candidiasis. *Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology* 19(3):373-380.

Leigh, J. E., M. Barousse, R. K. Swoboda, T. Myers, S. Hager, N. A. Wolf, J. L. Cutright, J. Thompson, J. D. Sobel, and P. L. Fidel, Jr. 2001. Candida-specific systemic cell-mediated immune reactivities in HIV-infected persons with and without mucosal candidiaisis. *Journal of Infectious Diseases* 183:277-285.

Leigh, J. E., K. M. McNulty, and P. L. Fidel, Jr. 2006. Characterization of the immune status of cd8 + t cells in oral lesions of human immunodeficiency virus-infected persons with oropharyngeal candidiasis. *Clinical and Vaccine Immunology* 13:678-683.

Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (il)-22 and il-17 are coexpressed by th17 and cooperatively enhance expression of antimicrobial peptides. *Journal of Experimental Medicine* 203(10):2271-2279.

Lilly, E., D. J. Hart, J. E. Leigh, S. Hager, K. M. McNulty, D. E. Mercante, and P. L. Fidel, Jr. 2004. Tissue-associated cytokine expression in HIV-positive persons with oropharyngeal candidiasis. *Journal of Infectious Diseases* 190:605-612.

Lilly, E. A., J. E. Leigh, K. M. McNulty, S. H. Joseph, D. E. Mercante, and P. L. Fidel, Jr. 2006. Chemokine receptor expression in HIV-positive persons with oropharyngeal candidiasis. *Oral Diseases* 12(5):493-499.

Lilly, E. A., J. Yano, and P. L. Fidel, Jr. 2010. Annexin-a1 identified as the oral epithelial cell anti-candida effector moiety. *Molecular Oral Microbiology* 25:293-304.

Liu, N., Y. P. Zhang, S. Han, J. Pei, L. Y. Xu, P. Lu, C. B. Shields, and X. Xu. 2007. Annexin a1 reduces inflammatory reaction and tissue damage through inhibition of phospholipase a 2 activation in adult rats following spinal cord injury. *Journal of Neuropathology & Experimental Neurology* 66:932-943.

Macher, A. M. 1988. The pathology of AIDS. *Public Health Report* 103:246-254.

Mathur, S., G. Virella, J. Koistinen, E. O. Horger, T. A. Mahvi, and H. H. Fudenberg. 1977. Humoral immunity in vaginal candidiasis. *Infection and Immunity* 15:287-294.

McNulty, K. M., J. Plianrungsi, J. E. Leigh, D. E. Mercante, and P. L. Fidel. 2005. Characterization of the cd8+ t-cells and microenvironment in oral lesions of HIV-infected persons with oropharyngeal candidiasis. *Infection and Immunity* 73:3659-3667.

Millon, L., C. Drobacheff, R. Piarroux, M. Monod, G. Reboux, R. Laurent, and D. Meillet. 2001. Longitudinal study of anti-*Candida albicans* mucosal immunity against aspartic proteinases in HIV-infected patients. *Journal of Acquired Immune Deficiency Syndrome* 26(2):137-144.

Myers, T. A., J. E. Leigh, A. Arribas, S. Hager, R. A. Clark, E. Lilly, and P. L. Fidel, Jr. 2003. Immunohistochemical evaluation of t cells in oral lesions from human immunodeficiency virus-positive persons with oropharyngeal candidiasis. *Infection and Immunity* 71(2):956-963.

Nandi, D., and J. P. Allison. 1991. Phenotypic analysis and gamma/delta-t cell receptor repertoire of murine t cells associated with the vaginal epithelium. *Journal of Immunology* 147:1773-1778.

Nett, J., L. Lincoln, K. Marchillo, R. Massey, K. Holoyda, B. Hoff, M. VanHandel, and D. Andes. 2007. Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrobial Agents and Chemotherapy* 51(2):510-520.

Nielsen, H., K. D. Bentsen, L. Hojtved, E. H. Willemoes, F. Scheutz, M. Schiodt, K. Stoltze, and J. J. Pindborg. 1994. Oral candidiasis and immune status of HIV-infected patients. *Journal of Oral Pathology and Medicine* 23:140-143.

Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell. 2006. Critical role of BCR1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathogens* 2:e63.

Nomanbhoy, F., C. Steele, J. Yano, and P. L. Fidel, Jr. 2002. Vaginal and oral epithelial cell anti-candida activity. *Infection and Immunity* 70:7081-7088.

Odds, F. C. 1988. Chronic mucocutaneous candidiosis. In *Candida and candidosis*. Baltimore, MD: University Park Press. Pp. 104-110.

Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards Jr., and S. G. Filler. 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biology* 5(3):e64.

Phelan, J. A., B. R. Salzman, G. H. Friendland, and R. S. Klein. 1987. Oral findings in patients with acquired immune deficiency syndrome. *Oral Surgery* 64:50-56.

Quimby, K., E. A. Lilly, M. Zacharek, K. McNulty, J. E. Leigh, J. E. Vazquez, and P. L. Fidel, Jr. 2011. Cd8 t-cells and e-cadherin in host responses against oropharyngeal candidiasis. *Oral Diseases* Epub ahead of print.

Rabeneck, L., M. M. Crane, J. M. Risser, C. E. Lacke, and N. P. Wray. 1993. A simple clinical staging system that predicts progression to AIDS using cd4 count, oral thrush, and night sweats. *Journal of General Internal Medicine* 8:5-9.

Ramage, G., K. VandeWalle, J. L. Lopez-Ribot, and B. L. Wickes. 2002. The filamentation pathway controlled by the efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans. FEMS Microbiology Letters* 214:95-100.

Ramage, G., J. P. Martinez, and J. L. Lopez-Ribot. 2006. Candida biofilms on implanted biomaterials: A clinically significant problem. *FEMS Yeast Research* 6(7):979-986.

Rhoads, J. L., D. C. Wright, R. R. Redfield, and D. S. Burke. 1987. Chronic vaginal candidiasis in women with human immunodeficiency virus infection. *JAMA* 257:3105-3107.

Romagnoli, P., N. Pimpinelli, M. Mori, P. A. Reichart, L. R. Eversole, and G. Ficarra. 1997. Immunocompetent cells in oral candidiasis of HIV-infected patients: An immunohistochemical and electron microscopical study. *Oral Diseases* 3:99-105.

Ross, K. F., and M. C. Herzberg. 2001. Calprotectin expression by gingival epithelial cells. *Infection and Immunity* 69(5):3248-3254.

Rouabhia, M., G. Ross, N. Page, and J. Chakir. 2002. Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation. *Infection and Immunity* 70:7073-7080.

Rouabhia, M., A. Semlali, J. Audoy, and W. Chmielewski. 2012. Antagonistic effect of *Candida albicans* and IFN-γ on e-cadherin expression and production by human primary gingival epithelial cells. *Cellular Immunology* 280(1):61-67.

Ryckman, C., K. Vandal, P. Rouleau, M. Talbot, and P. A. Tessier. 2003. Proinflammatory activities of s100: Proteins s100a8, s100a9, and s100a8/a9 induce neutrophil chemotaxis and adhesion. *Journal of Immunlogy* 170:3233-3242.

Schaller, M., R. Mailhammer, G. Grassl, C. A. Sander, B. Hube, and H. C. Korting. 2002. Infection of human oral epithelia with candida species induces cytokine expression correlated to the degree of virulence. *Journal of Investigative Dermatology* 118:652-657.

Schuman, P., S. Ohmit, J. D. Sobel, K. H. Mayer, V. Greene, A. Rompalo, and R. S. Klein. 1998a. Oral lesions among women living with or at risk for HIV infection. *American Journal of Medicine* 104:559-563.

Schuman, P., J. D. Sobel, S. Ohmit, K. H. Mayer, A. Rompalo, A. Duerr, D. K. Smith, D. Warren, and R. S. Klein. 1998b. Mucosal candidal colonization and candidiasis in women with or at risk for human immunodeficiency virus infection. *Clinical Infectious Diseases* 27:1161-1167.

Sobel, J. D. 1988. Pathogenesis and epidemiology of vulvovaginal candidiasis. *Annals of the New York Academy of Sciences* 544:547-557.

Sobel, J. D. 1992. Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clinical Infectious Diseases* 14(Suppl 1):S148-S153.

Sobel, J. D., G. Muller, and H. R. Buckley. 1984. Critical role of germ tube formation in the pathogenesis of candidal vaginitis. *Infection and Immunity* 44:576-580.

Sohnle, P. G., B. L. Hahn, and V. Santhanagopalan. 1996. Inhibition of *Candida albicans* growth by calprotectin in the absence of direct contact with the organisms. *Journal of Infectious Diseases* 174:1369-1372.

Spinillo, A., G. Michelone, C. Cavanna, L. Colonna, E. Capuzzo, and S. Nicola. 1994. Clinical and microbiological characteristics of symptomatic vulvovaginal candidiasis in HIV-seropositive women. *Genitrouinary Medicine* 70:268-272.

Steele, C., and P. L. Fidel, Jr. 2002. Cytokine and chemokine production by human oral and vaginal epithelial cells in response to *Candida albicans. Infection and Immunity* 70:577-583.

Steele, C., H. Ozenci, W. Luo, M. Scott, and P. L. Fidel, Jr. 1999. Growth inhibition of *Candida albicans* by vaginal cells from naive mice. *Medical Mycology* 37:251-260.

Steele, C., J. E. Leigh, R. K. Swoboda, and P. L. Fidel, Jr. 2000. Growth inhibition of candida by human oral epithelial cells. *Journal of Infectious Diseases* 182:1479-1485.

Steele, C., J. E. Leigh, R. K. Swoboda, H. Ozenci, and P. L. Fidel, Jr. 2001. Potential role for a carbohydrate moiety in anti-candida activity of human oral epithelial cells. *Infection and Immunity* 69:7091-7099.

Taylor, B. N., M. Saavedra, and P. L. Fidel, Jr. 2000. Local th1/th2 cytokine production during experimental vaginal candidiasis. *Medical Mycology* 38:419-431.

Urban, C. F., D. S. M. Ermert, U. Abu-Abed, C. Goosman, W. Nacken, V. Brinkmann, P. R. Jungblut, and A. Zychlinsky. 2009. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathogens* 5(10):e1000639.

Villar, C. C., H. Kashleva, C. J. Nobile, A. P. Mitchell, and A. Dongari-Bagtzoglou. 2007. Mucosal tissue invasion by *Candida albicans* is associated with e-cadherin degradation, mediated by transcription factor rim101p and protease sap5p. *Infection and Immunity* 75(5):2126-2135.

Webb, B. C., C. J. Thomas, M. D. Willcox, D. W. Harty, and K. W. Knox. 1998. Candida-associated denture stomatitis. Aetiology and management: A review. Part 2. Oral diseases caused by candida species. *Australian Dental Journal* 43(3):160-166.

White, M. H. 1996. Is vulvovaginal candidiasis an AIDS-related illness? *Clinical Infectious Diseases* 22(Suppl 2):S124-S127.

Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases* 39(3):309-317.

Wormley, F. L., Jr., J. Chaiban, and P. L. Fidel, Jr. 2001. Cell adhesion molecule and lymphocyte activation marker expression during experimental vaginal candidiasis. *Infection and Immunity* 69:5072-5079.

Wormley, F. L., Jr., C. Steele, K. Wozniak, K. Fujihashi, J. R. McGhee, and P. L. Fidel, Jr. 2001. Resistance of tcr δ-chain knock-out mice to experimental candida vaginitis. *Infection and Immunity* 69:7162-7164.

Wozniak, K. L., J. E. Leigh, S. Hager, R. K. Swoboda, and P. L. Fidel, Jr. 2002. A comprehensive study of candida-specific antibodies in saliva of HIV-infected persons with oropharyngeal candidiasis. *Journal of Infectious Diseases* 185:1269-1276.

Wray, D., D. H. Felix, and C. G. Cumming. 1990. Alteration of humoral responses to candida in HIV infection. *British Dental Journal* 168(8):326-329.

Yano, J., E. Lilly, C. Steele, D. Fortenberry, and P. L. Fidel, Jr. 2005. Oral and vaginal epithelial cell anti-candida activity is acid-labile and does not require live epithelial cells. *Oral Microbiology and Immunology* 20:199-205.

Yano, J., E. Lilly, M. Barousse, and P. L. Fidel, Jr. 2010. Epithelial cell-derived s100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during candida vaginitis. *Infection and Immunity* 78(12):5126-5137.

Yano, J., J. K. Kolls, K. I. Happel, F. L. Wormley, Jr., K. L. Wozniak, and P. L. Fidel, Jr. 2012. The acute neutrophil response mediated by s100 alarmins during vaginal candida infections is independent of the th17-pathway. *PloS One* 7(9):e46311. doi: 46310.41371/journal.pone.0046311.

Yeater, K. M., J. Chandra, G. Cheng, P. K. Mukherjee, X. Zhao, S. L. Rodriguez-Zas, K. E. Kwast, M. A. Ghannoum, and L. L. Hoyer. 2007. Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* 153(Pt 8):2373-2385.

Zimmer, D. B., E. H. Cornwall, A. Landar, and W. Song. 1995. The s100 protein family: History, function, and expression. *Brain Research Bulletin* 37:417-429.

A10

MICROBIOTA-TARGETED THERAPIES: AN ECOLOGICAL PERSPECTIVE[34]

Katherine P. Lemon,[35,36,*] *Gary C. Armitage,*[37] *David A. Relman,*[38,39,40] *and Michael* ***Fischbach***[41,42,*]

Abstract

The connection between disease and the disruption of homeostatic interactions between the host and its microbiota is now well established. Drug developers and clinicians are starting to rely more heavily on therapies that directly target the microbiota and on the ecology of the microbiota to

[34] From K. P. Lemon, G. C. Armitage, D. A. Relman, M. A. Fischbach, Microbiota-Targeted Therapies: An Ecological Perspective. *Sci. Transl. Med.* 4, 137rv5 (2012). Reprinted with permission from AAAS.

[35] Department of Molecular Genetics, The Forsyth Institute, Cambridge, MA 02142, USA.

[36] Division of Infectious Diseases, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA.

[37] Division of Periodontology, School of Dentistry, University of California, San Francisco, San Francisco, CA 94143, USA.

[38] Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA.

[39] Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA.

[40] Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA.

[41] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, CA 94158, USA.

[42] California Institute of Quantitative Biosciences, University of California, San Francisco, San Francisco, CA 94158, USA.

* To whom correspondence should be addressed. Email: klemon@forsyth.org (K.P.L.); fischbach@fischbachgroup.org (M.A.F.)

understand the outcomes of these treatments. The effects of those microbiota-targeted therapies that alter community composition range in scale from eliminating individual strains of a single species (for example, with antibacterial conjugate vaccines) to replacing the entire community with a new intact microbiota (for example, by fecal transplantation). Secondary infections linked to antibiotic use provide a cautionary tale of the unintended consequences of perturbing a microbial species network and highlight the need for new narrow-spectrum antibiotics with rapid companion diagnostics. Insights into microbial ecology will also benefit the development of probiotics, whose therapeutic prospects will depend on rigorous clinical testing. Future probiotics may take the form of a consortium of long-term community residents: "a fecal transplant in a capsule." The efficacy of microbiota-targeted therapies will need to be assessed using new diagnostic tools that measure community function rather than composition, including the temporal response of a microbial community to a defined perturbation such as an antibiotic or probiotic.

The human microbiota consists of hundreds of microbial species that inhabit distinct body sites, which can be thought of as ecosystems (see review by Costello et al., 2012). The healthy human state is a homeostasis between the microbiota and the host. Maladies such as Crohn's disease, chronic periodontitis, and bacterial vaginosis are characterized by a disruption of this homeostasis, a state known as dysbiosis (Tamboli et al., 2004). We envision a future in which new therapeutics and diagnostics enable the management of our microbiota to treat and prevent disease (Kolter, 2009).

Two major challenges face the development of microbiota-targeted therapies. First, changes in the composition or function of the microbiota must be proven to cause a disease or contribute to its pathology. Correlative studies are a good start, but without a causative link there is little reason to expect that a microbiota-targeted therapy will cure a disease or alleviate its symptoms. Second, we may need to be satisfied with a less detailed understanding of how microbiota-targeted therapies work than we currently expect for drug development. To date, a threshold requirement for developing a new drug is an understanding of its molecular mechanism at the level of a protein-ligand interaction. Should this requirement be applied to microbiota-targeted therapies? A central lesson of ecology is that perturbations often ripple through an ecosystem, leading to unexpected outcomes (Costello et al., 2012). Likewise, the desired effect might not occur at the level of inhibiting a specific enzyme but by restructuring a microbial community in a specific way. To advance microbiota-directed therapeutics, we will need to define new endpoints (for example, microbial community composition or function), develop therapies to achieve them, and design diagnostics to evaluate our efforts.

What Is the Goal of Therapeutically Perturbing a Microbial Community?

A clear therapeutic goal is a prerequisite for designing a drug; in a practical sense, the goal will determine the diagnostic used to judge the therapy's success in preclinical studies and clinical trials. The goal of a therapy often takes the form of a clinical endpoint or a surrogate endpoint, and the distinction between them is especially important for microbiota-targeted therapies. A clinical endpoint is the long-term goal of a therapy, such as preventing death from heart disease or eliminating the signs and symptoms of Crohn's disease (Lesko and Atkinson, 2001). If the goal of a microbiota-targeted therapy is a clinical endpoint (for example, clearing *Clostridium difficile*–associated diarrhea), then achieving it is equivalent to clinical success.

However, clinical endpoints are often impossible to measure in preclinical studies because of the use of animal models rather than human subjects. In addition, certain clinical endpoints can be impractical to measure in a clinical trial (for example, the prevention of death from heart disease). In these cases, a surrogate endpoint—a marker whose level predicts the therapy's effect on the clinical endpoint—is used (Lesko and Atkinson, 2001). For example, a reduction in serum cholesterol has been used as a surrogate endpoint for preventing death from heart disease. The goal of many microbiota-targeted therapies will be a surrogate endpoint, such as shifting the gut community from a Crohn's disease–associated state to a "healthy" state. If these therapies are to succeed, their surrogate endpoints must be predictive of the clinical endpoint; a correlation between the two is not enough.

What Is a Healthy Microbiome?

The goal of many microbiota-targeted therapeutics will be to restore a healthy host–microbiota homeostasis. Metrics for assessing the state of the microbiota can be sorted into two categories: global measurements and specific markers. However, as discussed later, the health of a community may have more to do with its ability to resist pathogen invasion (a functional property) than its static composition (a structural property).

Two commonly used global metrics derive from ecology: alpha diversity (within-community diversity) and beta diversity (between-community diversity). The relationship between alpha diversity and health differs among body sites; for example, increased alpha diversity in the gut is associated with a decreased risk of necrotizing enterocolitis in premature infants (Mshvildadze et al., 2010; Neu and Walker, 2011; Wang et al., 2009), whereas decreased alpha diversity in the vaginal community is associated with a decreased risk of bacterial vaginosis (Ravel et al., 2011). Although the relationship between beta diversity and health is not as well explored, the example of cystic fibrosis is instructive: Increased beta diversity is observed in later-stage disease after the oropharyngeal microbiota is invaded by *Pseudomonas aeruginosa* and subjected to increased antibiotic

exposure (Klepac-Ceraj et al., 2010). It is not yet known whether the increased beta diversity in cystic fibrosis is a cause or a consequence of antibiotic exposure and pathogen invasion.

In addition to diversity measures associated with health and disease, specific taxa are emerging as indicators of disease states. In many microbiota-related ailments, the disease state is marked by a change in the level of normal community members rather than the presence of a foreign pathogen. For example, a decrease in the common gut resident *Faecalibacterium prausnitzii* is associated with ileal Crohn's disease (Sokol et al., 2008; Willing et al., 2009), and the suspected causative agents of gingivitis and periodontitis are normal members of healthy periodontal communities (Hayashi et al., 2006; Li et al., 2004; Tanner et al., 2002; Wilson et al., 1993). Two key challenges for microbiota markers are to define the relationship between the marker species/genes and disease pathophysiology, especially if it is direct, and to determine how broadly these apply within the human population.

What Scale of Perturbation Is Appropriate?

Viewing a human-associated microbial community as a network of interacting species, the scale of perturbation could range from "surgically" excising a single node to eliminating the entire network and replacing it with a new one (Figure A10-1A). There is precedent for interventions at both ends of this spectrum. Antibacterial conjugate vaccines represent the simplest perturbations; these agents stimulate the immune system to remove specific strains of a single species from the community. Fecal transplantation, which lies at the opposite end of the spectrum, consists of a colonic lavage to dislodge the resident community followed by the infusion of a donor community (20 to 30 g of donor feces suspended in 100 ml of water) during endoscopy or colonoscopy. Fecal transplantation appears to be effective in clearing recurrent *C. difficile* infections that are refractory to antibiotic treatment (Gough et al., 2011), but it is not yet clear whether a perturbation this extreme is required to displace a tenacious strain of *C. difficile* from the gut community.

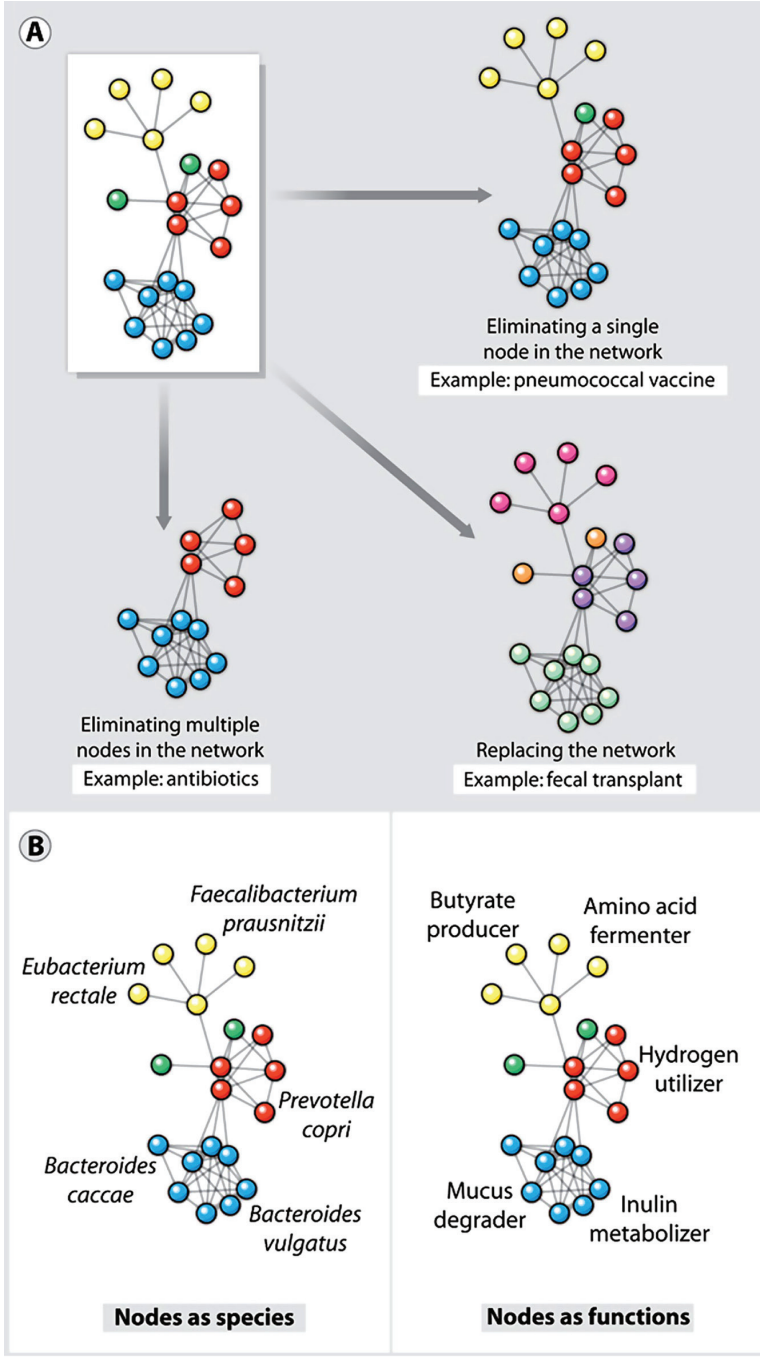
As we highlight in the examples below, the tendency of ecological perturbations to ripple through a community can make the effects of even a simple perturbation difficult to predict. A major challenge for the future will be to create predictive models of microbial community ecology that can anticipate—and ultimately circumvent—these ripple effects (Costello et al., 2012; Robinson et al., 2010).

Targeted Removal of Specific Strains from the Upper Respiratory Tract Microbiota

Several widely used vaccines target bacterial mutualists or commensals that are occasionally pathogenic (termed "*pathobionts*"). For pathobionts, colonization is the first step in infection; these strains colonize many people and cause deadly infections in a subset. Two examples of such vaccines are conjugate vaccines against *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* that protect against infection and clear or prevent colonization (Figure A10-1A). Once the leading cause of bacterial meningitis in children < 5 years old, Hib infections have been nearly eliminated by the use of a conjugate vaccine (Adams et al., 1993; CDC, 1996). Before the vaccine's introduction in the early 1990s, Hib colonized 3 to 5% of healthy children; in immunized populations, the vaccine has virtually eradicated Hib from the pharyngeal microbiota. It has apparently been replaced by less virulent *H. influenzae* strains (Mohle-Boetani et al., 1993; Takala et al., 1991).

Similarly, the introduction in 2000 of the seven-valent pneumococcal conjugate vaccine against *S. pneumoniae* resulted in a 77% decline in severe invasive disease (CDC, 2008; Whitney et al., 2003). After vaccination, nonvaccine serotypes of *S. pneumoniae* (most of which were less virulent than the vaccine serotypes) became more prevalent in the pharyngeal community, and overall *S. pneumoniae* colonization rates did not decline significantly. Thus, immunizations against Hib and *S. pneumoniae* have led to their replacement by less virulent members of the same species and appear to have caused few other effects in the ecosystem. This phenomenon may prove to be a common outcome of the targeted removal of a pathobiont, because many pathobionts have closely related, nonpathogenic relatives that can occupy the same niche. (Dethlefsen et al., 2007). An important caveat is that initial concerns about a possible increase in *Staphylococcus aureus* carriage after the use of the *S. pneumoniae* vaccine may yet prove to be valid (Regev-Yochay et al., 2008).

The success of vaccines against pathobionts underscores the need for a better understanding of the immunological aspects of host–microbiota interactions and the potential for success in developing new microbiota-targeted vaccines (see reviews by Blumberg and Powrie, 2012, and Hooper et al., 2012). It also illustrates that strain-level changes in community composition can have a clinically important impact. Because these changes are not usually detectable by 16S rRNA (ribosomal RNA) sequence analysis, there is a growing need for culture-independent diagnostics that enable species- and strain-level identification of the microbiota. Finally, our ability to surgically remove a single taxon from a community has come far in advance of our ability to understand the ways in which communities respond to such perturbations, including the role of microbe-microbe interactions in determining which species fill a niche opened by a perturbation (Figure A10-2A).



Antibiotics Cause Collateral Damage to Microbiota

Antibiotics save countless lives and are essential to clinical practice, but they profoundly perturb human-associated microbial communities (Blaser, 2011; Costello et al., 2012; Dethlefsen and Relman, 2011). Antibiotic treatment often achieves the goal of eradicating infection with help from the immune system. However, it can have the ecologically undesirable side effect of killing mutualistic (that is, helpful) bacterial symbionts, creating a rare opportunity for a hardy pathogen—whose growth usually is suppressed by these mutualists—to become a dominant species in the community (for example, alternative state 2 in Figure A10-3) (Costello et al., 2012). Two examples of such "secondary infections" are *C. difficile*–associated diarrhea and yeast infections (candidiasis), both of which are associated with antibiotic use (Kelly and LaMont, 2008; Spinillo et al., 1999). In such situations, the goal of a microbiota-targeted therapy would be to perturb the new "unhealthy" alternative stable state of the community sufficiently to shift it to a "healthier" alternative stable state (Figure A10-3).

The clinicians who prescribe antibiotics and the researchers who develop them face four challenges, which we focus on here and in the following section: (i) Can clinically useful adjunctive therapies be developed to prevent secondary infections? (ii) Can physicians be encouraged to use antibiotics with narrower activity spectra to minimize the collateral damage to bacterial mutualists? (iii) Should diagnostics be designed to identify the etiologic agent of infection and then actively monitor the microbiota for signs of a secondary infection? Clinicians routinely monitor patients for adverse effects of antibiotics on the kidneys and liver, but apart from counseling patients to be alert for symptoms of *C. difficile*–associated diarrhea and candidiasis, they have no good way to monitor the state of the microbiota. (iv) Can targeted antibiotics that cause minimal perturbation to the healthy microbiota be developed?

FIGURE A10-1 Microbial communities as networks. (A) Shown are three types of perturbation to a network of microbial species such as that found in the microbiota at various body sites. The microbiota can be perturbed by excision of a single species (node) by a vaccine or a species-specific antibiotic, by elimination of multiple nodes or a subnetwork by an antibiotic, or by replacement of a whole network using microbiota transplantation. (B) Two ways of modeling a microbial community as a network. (Left) Nodes as species, and edges as interactions among species. Species networks can be constructed directly from metagenomic sequence data, but they lack functional information. (Right) Nodes as functions, and edges as interactions among functions. Function networks can generate hypotheses about the mechanism of microbiota–host interactions, but they require mapping genes to functions or a panel of direct functional measurements.

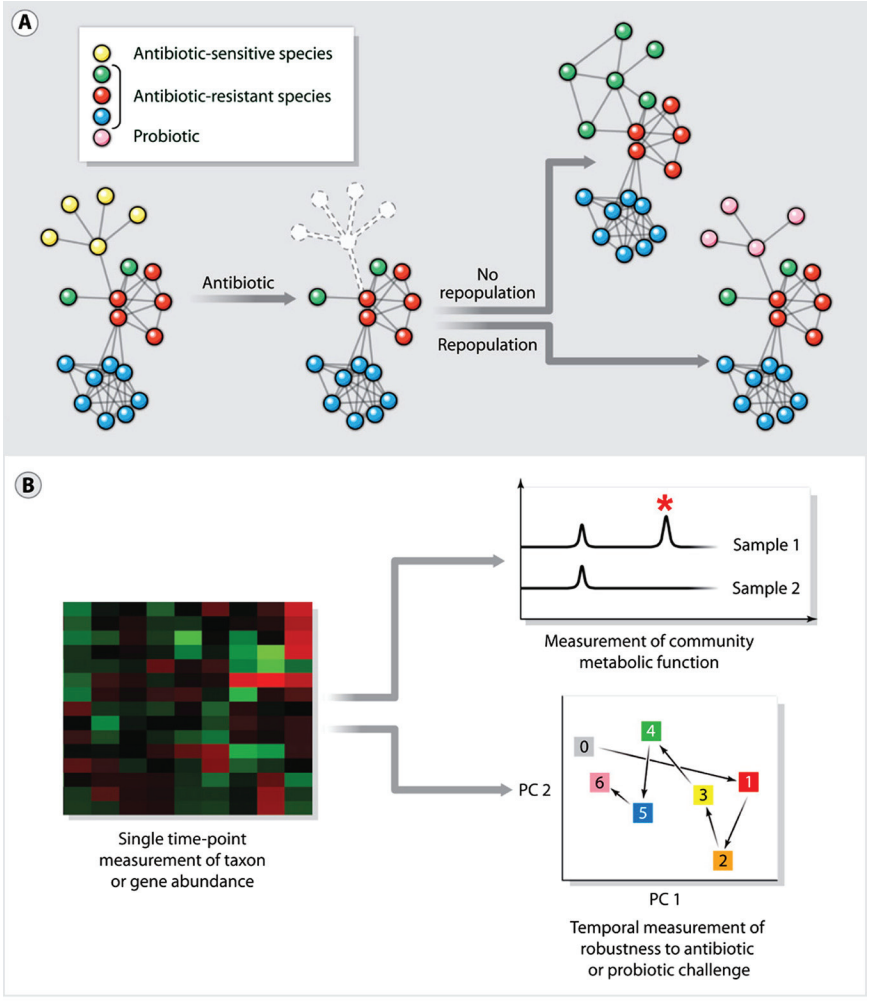


FIGURE A10-2 New opportunities in treatment and diagnostics. (A) Antibiotics save countless lives, but when they kill mutualistic (that is, helpful) microbiota that normally check the growth of pathogens, a secondary infection can ensue. Repopulating antibiotic-treated patients with probiotics is a promising strategy to prevent secondary infections. (B) Given that the human microbiota has many normal taxonomic compositions, it might be easier to develop markers of a normal or healthy community in terms of functional attributes like resistance and resilience. A single time-point measurement of taxon and gene abundance (left) is limited in its ability to provide functional information. Diagnostics based on direct measurements of metabolites (right, top) and temporal measurements of robustness to antibiotic or probiotic challenge (right, bottom) would enable community function to be assessed directly.

Passive Recovery Versus Active Repopulation

In a real sense, the current approach to antibiotic treatment is the equivalent of treating a weed-infested garden with a herbicide, and then leaving it fallow in the hope that the desired crops take root. This is an example of the ecological phenomenon of secondary succession, which describes changes in a community's species after a disturbance. One way to influence the course of secondary succession is to seed the newly cleared ecosystem with one or more pioneer species (Figure A10-2A) (Costello et al., 2012).

An intriguing example of success with ecosystem repopulation in the context of the microbiome is the use of fecal transplantation to treat refractory recurrent *C. difficile*–associated diarrhea. The incidence of *C. difficile* infections is rising rapidly; between 1996 and 2005, the number of annual reported cases in the United States tripled and the number of deaths in England rose nearly sevenfold to 3,393 (Kelly and LaMont, 2008). Nearly all *C. difficile* infections are healthcare associated, occurring in patients who are either hospitalized or were recently discharged (CDC, 2012). A recent study reported that, in patients with multiple

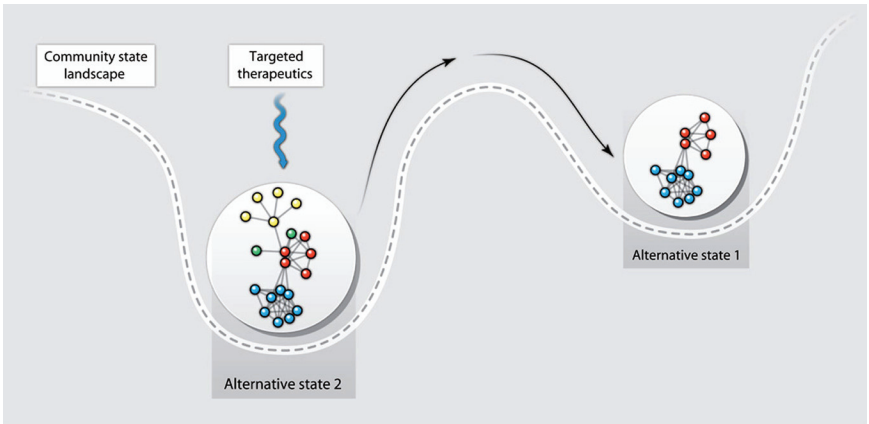


FIGURE A10-3 Microbiota-targeted therapy can shift a community to a healthier stable state. A microbial community's response to a microbiota-targeted therapy can be illustrated with a stability landscape diagram (1). The ball containing the network represents the microbial community, and the shift in its horizontal position within the landscape represents movement between alternative stable states. The depth of a basin indicates the probability that the community will stay in that specific state in response to perturbation and, therefore, reflects the degree of perturbation needed to shift the community to an alternative stable state, for example, from state 1 to state 2. In this illustration, a therapeutic perturbation that removes some nodes (yellow and green) from the community network is sufficient to shift the community to an alternative, and in this case healthier, stable state (state 1).

recurrences, fecal transplantation resolved the symptoms of 34 of 34 patients who were infected with conventional strains of *C. difficile* and 32 of 36 patients who were infected with a *C. difficile* strain from a particularly virulent group known as ribotype 027 (Mattila et al., 2012). At present, fecal transplantation is only used to treat patients for whom secondary antibiotic therapy has repeatedly failed; however, its potential use as an adjunctive or follow-up therapy to the initial course of anti–*C. difficile* antibiotics could markedly reduce the rate of recurrence, which is currently 20 to 30%. Further in the future, one might imagine autologous fecal transplantation with the patient's stored preantibiotic fecal microbiota as a way to prevent or treat *C. difficile*–associated diarrhea (Figure A10-2A).

Apart from this example, the literature suggests that a shift in clinical practice from passive recovery to active repopulation of microbiota during and after antibiotic treatment will require proof of efficacy and pharmaceutical-grade products (Thomas and Greer, 2010). A recent meta-analysis of randomized controlled trials of adjunctive probiotic therapy for the prevention of antibiotic-associated diarrhea in children suggests a possible benefit of high-dose probiotics (Johnston et al., 2011). In contrast, there are not yet sufficient data to support concurrent probiotic therapy during antibiotic treatment in adults to prevent *C. difficile*–associated diarrhea (Cohen et al., 2010). In both cases, there is a strong need for large randomized controlled trials with a consensus definition of the disease state and clinical endpoints, and standardized probiotic constituents and dosing.

Similarly, will future antibiotic treatment of pathobiont infections be followed by therapeutic repopulation with a mutualistic microbiota? For example, after treatment of *S. aureus* infections, the microbiota recovers passively but *S. aureus* is not necessarily eradicated, potentially leaving the patient at higher risk for a new *S. aureus* infection than someone who is not colonized. In certain high-risk populations, efforts have been made to eradicate *S. aureus* carriage, especially for methicillin-resistant *S. aureus*, through the use of a topical antibiotic to the anterior nares (mupirocin) and chlorohexidine body washes (Chow and Yu, 1989; Fritz et al., 2012). However, the current approach to decolonization makes no attempt to influence secondary succession. A future challenge will be to determine what role pathobionts such as *S. aureus* play in a healthy microbiota and whether a pathobiont-free consortium can be used to replace the original community.

Narrow-Spectrum Antibiotics: Policy, Diagnostics, and New Agents

Numerous evidence-based clinical practice guidelines and antibiotic stewardship programs promote the use of narrow-spectrum antibiotics. For example, a children's hospital in Kansas introduced a clinical practice guideline for the empiric treatment (that is, before the etiologic agent of infection is identified) of uncomplicated community-acquired pneumonia that favored the use of ampicillin, a relatively narrow-spectrum antibiotic, over ceftriaxone, which has a much

broader activity spectrum. The result was a shift from empiric use of ceftriaxone in 72% of cases to empiric use of ampicillin in 63% of patients, with no significant change in the failure rate of the initial antibiotic (Newman et al., 2012). Similarly, a 2011 national clinical practice guideline recommends ampicillin for hospitalized children with uncomplicated community-acquired pneumonia (Bradley et al., 2012).

For patients with severe acute infections, the empiric use of broad-spectrum antibiotics remains a life-saving measure. In these situations, the switch to equally effective narrow-spectrum agents is limited by the fact that current diagnostics require 24 to 72 hours to cultivate, identify, and determine the antibiotic susceptibilities of the bacterium causing the infection. The increased use of narrow-spectrum antibiotics will require companion diagnostics that are not only more rapid and sensitive but also just as specific as current cultivation-based methods. Nucleic acid–based tests for pathogen-specific genes seem to meet these criteria, but their exquisite sensitivity raises a question that is both ecological and practical in nature: If such a test comes back positive, how can a clinician be certain that the pathogen is still alive, and if so, that it is the etiologic agent of infection and not a contaminant (for samples from sterile sites/compartments) or a bystander (for samples from nonsterile sites)?

In addition to providing equally effective treatment of susceptible bacterial pathogens, narrow-spectrum antibiotics minimize the collateral damage to microbiota community structure, decrease the selective pressure for the spread of antibiotic resistance genes within the microbiota, and lower the probability of acquiring resistant strains. Much of this is predicted by ecological studies showing that perturbations render a community more susceptible to invasion (Costello et al., 2012). Further research on the impact of antibiotics on the microbiota is needed to determine how the loss of antibiotic-susceptible species leads to unintended changes in the species network (Little et al., 2008); even a narrow-spectrum antibiotic may remove a well-connected node from a network, which could have an unexpectedly large impact on community structure.

How Should New Antibiotics Be Designed

Two key insights from ecology are likely to change the way future antibiotics are designed and developed. First, a long-standing paradigm has been that for activity spectrum, "broader is better." The unintended consequences of broad-spectrum antibiotics provide an impetus for supplementing their position in the physician's toolbox with targeted agents. Two recent efforts have proven that antibiotics can be designed with the narrowest of spectra: Both inhibit *S. aureus* but not closely related bacteria. One molecule, a dehydrosqualene synthase inhibitor, blocks the production of the glycolipid virulence factor staphyloxanthin, the yellow pigment from which the species name of *S. aureus* is derived. This antibiotic—which was originally developed as a candidate cholesterol

biosynthesis inhibitor for use in humans—does not kill *S. aureus* but makes the bacterium more sensitive to innate immune clearance in an animal model of infection (Liu et al., 2008). The other molecule, an FtsZ inhibitor, has been proposed to bind FtsZ in a region that is polymorphic among Firmicutes; the result is an antibiotic with activity against various species of *Staphylococcus* and *Bacillus subtilis*, but not against *S. pneumoniae* and *Enterococcus faecalis* (Haydon et al., 2008). To be used as single-agent therapies very early in the course of treating an infection, these drugs would require rapid companion diagnostics as described above.

Second, although antibiotics are often designed and tested against bacteria in liquid culture, pathogens often live in structured, surface-associated communities known as biofilms (López et al., 2010). Numerous infections are caused by bacteria living in biofilms: These include dental caries, periodontal infections, otitis media, endocarditis, osteomyelitis, artificial joint infections, pulmonary infections in cystic fibrosis, and device-associated infections (Darouiche, 2004; Furukawa et al., 2006). Given that existing antibiotics are rarely able to clear the biofilm from the affected site, patients often require weeks to months of therapy and surgical/mechanical intervention. To more effectively treat biofilm-associated infections, agents that promote the dispersion of biofilms or block their formation altogether are needed. A recent promising discovery comes from the observation that the commonly studied soil bacterium *B. subtilis* produces a diffusible factor that promotes the disassembly of its own biofilms, which is a mixture of D-amino acids (Kolodkin-Gal et al., 2010). The generality of this result was demonstrated by showing that D-amino acids can prevent biofilm formation by the gram-positive pathogen *S. aureus* and the gram-negative pathogen *P. aeruginosa*. Studies into the mechanism by which D-amino acids act could open the door to a new class of agents that reverse or prevent biofilm formation. This would make their constituent pathogens easier to treat with conventional antibiotics, many of which are more active against free-living bacteria than bacteria in a biofilm (Stewart and Costerton, 2001).

What Is the Path Forward for Probiotic Therapies

Probiotics are another class of microbiota-targeted therapies whose properties are intimately connected to ecological principles. One key question for the design of future probiotics is whether they should be short-term or long-term residents of a community. Most current probiotics (for example, species of *Lactobacillus* and *Bifidobacterium*) are transient members of the gut community, present for only a few days (Denou et al., 2008). Among the advantages of short-term colonization are a lower risk of unintended consequences and the ability to determine empirically which probiotic is most effective through rounds of trial and observation. However, long-term residents (for example, species of *Bacteroides* and *Clostridium*) would be capable of modulating community structure and

function in ways that short-term residents could not, including signaling to the host via receptors expressed in gut epithelial cells that may have evolved to monitor chemical cues from common long-term residents (see review by Holmes et al., 2012, and review by Nicholson et al., 2012). If future probiotic therapies supplying long-term residents become widely used, an important challenge—made famous by ecological studies of agriculture—will be to ensure that no single strain is used in too broad a swath of the human population; a limited choice of probiotic strains could inadvertently create a population-level vulnerability to disease (Hughes et al., 2008).

In addition to residence time, another key property of a probiotic therapy is the number of bacterial species it comprises. Most existing probiotics consist of one or a few strains, and the constituents of those that are simple consortia appear to have been chosen on the basis of the dictum of "more is better" rather than for scientific reasons. At the other end of the complexity spectrum, fecal transplants consist of an intact, highly complex community of hundreds of species. Will new probiotics occupy the middle of this spectrum? Two possibilities are synthetic communities containing ~20 species, chosen to represent the major taxa of common gut bacteria (Faith et al., 2011), and, more ambitiously, a defined mixture of 50+ species chosen to mimic the healthy state of one of the common "enterotypes" (Arumugam et al., 2011)—a simplified fecal transplant in a capsule.

Currently, most probiotics target the gut, but the principles of designing and testing gut probiotics may also apply to the skin, oral, vaginal, and upper respiratory tract communities. For example, for atopic dermatitis, a topical ointment with a probiotic *Corynebacterium* species that blocks colonization by *S. aureus* may be just as effective as an antibiotic or an immunosuppressant (Kong et al., 2012). Likewise, probiotic toothpastes and mouthwashes could be highly effective in reversing or preventing caries and periodontal disease (Zahradnik et al., 2009).

The widespread clinical use of probiotics will require proof of efficacy and safety from large randomized controlled trials with a consensus definition of the disease state, pharmaceutical-grade products, and standardized dosing (Cohen et al., 2010; Johnston et al., 2011; Thomas and Greer, 2010). A few notable studies have led the way in showing a positive effect of probiotics on disease outcome (Shanahan, 2010). Clear successes have been seen with the use of probiotics for preventing necrotizing enterocolitis in preterm neonates (Dshpande et al., 2010) and acute diarrhea in children (Sur et al., 2011), and promising data exist for preventing antibiotic-associated diarrhea and *C. difficile*–associated diarrhea in adults (Gao et al., 2010; Hempel et al., 2012). Probiotics are beneficial for ulcerative colitis and pouchitis, and although the data look less promising for Crohn's disease, differences in study design (the use of different bacterial strains and underpowered studies) make the results difficult to interpret and compare (Isaacs and Herfarth, 2008). A hint of benefit has been seen for irritable bowel syndrome, but a recent meta-analysis highlighted that most studies to date have been poorly

designed (Brenner et al., 2009), emphasizing the need for better study design in the future. Notably, low success rates in individual trials need not be a reason to abandon the effort; as long as a subset of patients are clear responders, much could be learned from pursuing the biological basis of their response, just as a subset of responders have revealed the molecular basis for a response to targeted kinase inhibitors in oncology trials (Jänne et al., 2009).

For probiotics with proven safety and efficacy, elucidating the molecular mechanisms by which they exert their effects will also be important for convincing the academic and clinical communities of their importance. Notably, a probiotic does not need to cause a change in the composition of the community to exert an effect, because community function (or that of the host) may be altered without changing community membership. Indeed, a recent report shows that a mixture of probiotics does not alter the composition of the gut microbiota, but it changes their metabolic function (McNulty et al., 2011).

Will Microbiota Diagnostics Lead or Follow Therapeutics?

The microbiota has two characteristics that make it an excellent source of diagnostics (Figure A10-2B). First, information richness: Bacterial cells are exquisitely sensitive to their environment, often changing in number or activating transcriptional circuits in response to extracellular cues. The community roster (metagenome) and state (metatranscriptome, metaproteome, and metametabolome) will therefore be sensitive readouts of, inter alia, inflammation, and metabolism (Blumberg and Powrie, 2012; Holmes et al., 2012; Hooper and Littman, 2012; Nicholson et al., 2012). Second, ease of collection and analysis: Fecal samples, saliva, and nasal swabs are easier to provide than blood. For a patient at risk for developing Crohn's disease, mailing a monthly fecal sample from home would be simpler and more cost effective than an annual colonoscopy. Tools being developed for cancer genotyping could be adapted for microbiota analysis, so the big challenge will be less technical in nature and more scientific: What question should be asked of the sample?

A simple combination of perturbation and measurement, such as used in the glucose tolerance test, should be the goal of some diagnostics for microbiota (Figure A10-2B). For example, administering a probiotic or antibiotic and then measuring community composition at a few longitudinal time points would determine the community's resistance to perturbation and its resilience (ability to return to its original state). Although single time-point measurements of community composition are informative, perturbation-based diagnostics will be especially important for the microbiota, which has many "normal" taxonomic compositions. It might be easier to define normal or healthy in terms of functional attributes like resistance and resilience rather than by a single measurement of composition.

An emerging theme that links ecology to the latest studies of the microbiota is the need to map a community's composition to its function (Figure A10-1B).

The mapping is rarely obvious, because closely related taxa can have distinct functions and unrelated species can have surprisingly similar functions. Nevertheless, a mechanistic understanding of how composition determines function is critical to developing useful diagnostics and therapeutics. The host may be indifferent to which species are present, but the community's ability to liberate nutrients from the diet and resist pathogen invasion are vital.

Two questions thus arise: First, at what level of granularity should a function be interrogated? Is it enough to know that a certain gut species produces short-chain fatty acids, or is the distinction between propionate and butyrate important? Second, what will function-based diagnostics look like? One possibility, which takes advantage of the wealth of expertise in analyzing metagenomic samples from the microbiome, would be to develop ways of predicting functions from the taxa and genes present in a community. An alternative would be to develop simple tests, akin to a serum creatinine test for renal function, which could quickly assess an important metabolic index of community function from an easily obtained sample (Holmes et al., 2012; Nicholson et al., 2012). Whether diagnostics lead or follow therapeutics, an emphasis on measuring functions rather than taxa will help build what is becoming an increasingly critical bridge between ecology and therapeutics.

Efforts to make microbial ecology easier to model and more predictable will pay dividends, boosting our ability to understand and manipulate the human microbiota. Not surprisingly, industrial and academic drug discovery groups rarely have an ecologist on staff. That may need to change; rigorous ecological analysis will be an essential component of designing microbiota-targeted therapies, measuring their efficacy, and assessing their unintended effects on the community.

Acknowledgments

We thank L. Costello, B. Bohannon, J. Gordon, V. Klepac-Ceraj, the members of the Forsyth Host-Microbiota Focus Group, and the three reviewers for helpful comments. Funding: Research in the authors' laboratories is supported by a Medical Research award from the W. M. Keck Foundation (M.A.F.), a Fellowship for Science and Engineering from the David and Lucile Packard Foundation (M.A.F.), NIH DP2 OD007290 (M.A.F.), NIH DE020751 (K.P.L.), a Children's Hospital Boston, Office of Faculty Development Career Development Fellowship (K.P.L.), a Distinguished Clinical Scientist Award from the Doris Duke Charitable Trust (D.A.R.), an NIH Pioneer Award DP1OD000964 (D.A.R.), and the Thomas C. and Joan M. Merigan Endowment at Stanford University (D.A.R.).

References

E. Costello, K. Stagaman, L. Dethlefsen, B. J. M. Bohannon, D. A. Relman, The application of ecological theory towards an understanding of the human microbiome. *Science*, 336, 1255–1262 (2012).

C. P. Tamboli, C. Neut, P. Desreumaux, J. F. Colombel, Dysbiosis in inflammatory bowel disease. *Gut* 53, 1–4 (2004).

R. Kolter, A visit to the paediatrician in the not-so-distant future. *Environ. Microbiol. Rep*. 1, 12–16 (2009) (10.1111/j.1758-2229.2008.00010.x).

L. J. Lesko, A. J. Atkinson Jr., Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: Criteria, validation, strategies. *Annu. Rev. Pharmacol. Toxicol*. 41, 347–366 (2001).

M. Mshvildadze, J. Neu, J. Shuster, D. Theriaque, N. Li, V. Mai, Intestinal microbial ecology in premature infants assessed with non–culture-based techniques. *J. Pediatr*. 156, 20–25 (2010).

J. Neu, W. A. Walker, Necrotizing enterocolitis. *N. Engl. J. Med*. 364, 255–264 (2011).

Y. Wang, J. D. Hoenig, K. J. Malin, S. Qamar, E. O. Petrof, J. Sun, D. A. Antonopoulos, E. B. Chang, E. C. Claud, 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J*. 3, 944–954 (2009).

J. Ravel, P. Gajer, Z. Abdo, G. M. Schneider, S. S. Koenig, S. L. McCulle, S. Karlebach, R. Gorle, J. Russell, C. O. Tacket, R. M. Brotman, C. C. Davis, K. Ault, L. Peralta, L. J. Forney, Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A*. 108 (Suppl. 1), 4680–4687 (2011).

V. Klepac-Ceraj, K. P. Lemon, T. R. Martin, M. Allgaier, S. W. Kembel, A. A. Knapp, S. Lory, E. L. Brodie, S. V. Lynch, B. J. Bohannan, J. L. Green, B. A. Maurer, R. Kolter, Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. *Environ. Microbiol*. 12, 1293–1303 (2010).

H. Sokol, B. Pigneur, L. Watterlot, O. Lakhdari, L. G. Bermúdez-Humarán, J. J. Gratadoux, S. Blugeon, C. Bridonneau, J. P. Furet, G. Corthier, C. Grangette, N. Vasquez, P. Pochart, G. Trugnan, G. Thomas, H. M. Blottière, J. Doré, P. Marteau, P. Seksik, P. Langella, *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. U.S.A*. 105, 16731–16736 (2008).

B. Willing, J. Halfvarson, J. Dicksved, M. Rosenquist, G. Järnerot, L. Engstrand, C. Tysk, J. K. Jansson, Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm. Bowel Dis*. 15, 653–660 (2009).

F. Hayashi, M. Okada, Y. Soda, K. Miura, K. Kozai, Subgingival distribution of Campylobacter rectus and *Tannerella forsythensis* in healthy children with primary dentition. *Arch. Oral Biol*. 51, 10–14 (2006).

J. Li, E. J. Helmerhorst, C. W. Leone, R. F. Troxler, T. Yaskell, A. D. Haffajee, S. S. Socransky, F. G. Oppenheim, Identification of early microbial colonizers in human dental biofilm. *J. Appl. Microbiol*. 97, 1311–1318 (2004).

A. C. Tanner, P. M. Milgrom, R. Kent Jr., S. A. Mokeem, R. C. Page, C. A. Riedy, P. Weinstein, J. Bruss, The microbiota of young children from tooth and tongue samples. *J. Dent. Res*. 81, 53–57 (2002).

M. Wilson, D. Lopatin, G. Osborne, J. B. Kieser, Prevalence of *Treponema denticola* and *Porphyromonas gingivalis* in plaque from periodontally-healthy and periodontally-diseased sites. *J. Med. Microbiol*. 38, 406–410 (1993).

E. Gough, H. Shaikh, A. R. Manges, Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clin. Infect. Dis*. 53, 994–1002 (2011).

C. J. Robinson, B. J. Bohannan, V. B. Young, From structure to function: The ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev*. 74, 453–476 (2010).

Centers for Disease Control and Prevention (CDC), Progress toward elimination of *Haemophilus influenzae* type b disease among infants and children—United States, 1987–1995. *MMWR Morb. Mortal. Wkly. Rep*. 45, 901–906 (1996).

W. G. Adams, K. A. Deaver, S. L. Cochi, B. D. Plikaytis, E. R. Zell, C. V. Broome, J. D. Wenger, Decline of childhood *Haemophilus influenzae* type b (Hib) disease in the Hib vaccine era. *JAMA 269, 221–226 (1993).*

J. C. Mohle-Boetani, G. Ajello, E. Breneman, K. A. Deaver, C. Harvey, B. D. Plikaytis, M. M. Farley, D. S. Stephens, J. D. Wenger, Carriage of *Haemophilus influenzae* type b in children after widespread vaccination with conjugate *Haemophilus influenzae* type b vaccines. *Pediatr. Infect. Dis. J*. 12, 589–593 (1993).

A. K. Takala, J. Eskola, M. Leinonen, H. Käyhty, A. Nissinen, E. Pekkanen, P. H. Mäkelä, Reduction of oropharyngeal carriage of *Haemophilus influenzae* type b (Hib) in children immunized with an Hib conjugate vaccine. *J. Infect. Dis*. 164, 982–986 (1991).

Centers for Disease Control and Prevention (CDC), Invasive pneumococcal disease in children 5 years after conjugate vaccine introduction—Eight states, 1998–2005. *MMWR Morb. Mortal. Wkly. Rep*. 57, 144–148 (2008).

C. G. Whitney, M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, R. Lynfield, A. Reingold, P. R. Cieslak, T. Pilishvili, D. Jackson, R. R. Facklam, J. H. Jorgensen, A. Schuchat; Active Bacterial Core Surveillance of the Emerging Infections Program Network, Decline in invasive pneumococcal disease after the introduction of protein–polysaccharide conjugate vaccine. *N. Engl. J. Med*. 348, 1737–1746 (2003).

L. Dethlefsen, M. McFall-Ngai, D. A. Relman, An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* 449, 811–818 (2007).

G. Regev-Yochay, D. Bogaert, R. Malley, P. W. M. Hermans, R. H. Veenhoven, E. A. M. Sanders, M. Lipsitch, E. Rubinstein, Does pneumococcal conjugate vaccine influence Staphylococcus aureus carriage in children? *Clin. Infect. Dis*. 47, 289–291 (2008).

R. Blumberg, F. Powrie, Microbiota, disease, and back again to health: A metastable journey. *Sci. Transl. Med*. 4, 137rv7 (2012).

L. V. Hooper, D. R. Littman, A. J. Macpherson, Interactions between the microbiota and the immune system. *Science* 336, 1268–1273 (2012).

M. Blaser, Antibiotic overuse: Stop the killing of beneficial bacteria. *Nature* 476, 393–394 (2011).

L. Dethlefsen, D. A. Relman, Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci. U.S.A*. 108, (Suppl. 1), 4554–4561 (2011).

A. Spinillo, E. Capuzzo, S. Acciano, A. De Santolo, F. Zara, Effect of antibiotic use on the prevalence of symptomatic vulvovaginal candidiasis. *Am. J. Obstet. Gynecol*. 180, 14–17 (1999).

C. P. Kelly, J. T. LaMont, *Clostridium difficile*—More difficult than ever. *N. Engl. J. Med*. 359, 1932–1940 (2008).

Centers for Disease Prevention (CDC), Vital signs: Preventing *Clostridium difficile* infections. *MMWR. Morb. Mortal. Wkly. Rep*. 61, 157–162 (2012).

E. Mattila, R. Uusitalo-Seppälä, M. Wuorela, L. Lehtola, H. Nurmi, M. Ristikankare, V. Moilanen, K. Salminen, M. Seppälä, P. S. Mattila, V. J. Anttila, P. Arkkila, Fecal transplantation, through colonoscopy, is effective therapy for recurrent *Clostridium difficile* infection. *Gastroenterology* 142, 490–496 (2012).

D. W. Thomas, F. R. Greer; American Academy of Pediatrics Committee on Nutrition; American Academy of Pediatrics Section on Gastroenterology, Hepatology, and Nutrition, Probiotics and prebiotics in pediatrics. *Pediatrics* 126, 1217–1231 (2010).

B. C. Johnston, J. Z. Goldenberg, P. O. Vandvik, X. Sun, G. H. Guyatt, Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *Cochrane Database Syst. Rev*. 11, CD004827 (2011).

S. H. Cohen, D. N. Gerding, S. Johnson, C. P. Kelly, V. G. Loo, L. C. McDonald, J. Pepin, M. H. Wilcox; Society for Healthcare Epidemiology of America; Infectious Diseases Society of America, Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect. Control Hosp. Epidemiol*. 31, 431–455 (2010).

J. W. Chow, V. L. Yu, *Staphylococcus aureus* nasal carriage in hemodialysis patients. Its role in infection and approaches to prophylaxis. *Arch. Intern. Med*. 149, 1258–1262 (1989).

S. A. Fritz, P. G. Hogan, G. Hayek, K. A. Eisenstein, M. Rodriguez, E. K. Epplin, J. Garbutt, V. J. Fraser, Household versus individual approaches to eradication of community-associated *Staphylococcus aureus* in children: A randomized trial. *Clin. Infect. Dis*. 54, 743–751 (2012).

R. E. Newman, E. B. Hedican, J. C. Herigon, D. D. Williams, A. R. Williams, J. G. Newland, Impact of a guideline on management of children hospitalized with community-acquired pneumonia. *Pediatrics* 129, e597–e604 (2012).

J. S. Bradley, C. L. Byington, S. S. Shah, B. Alverson, E. R. Carter, C. Harrison, S. L. Kaplan, S. E. Mace, G. H. McCracken Jr., M. R. Moore, S. D. St Peter, J. A. Stockwell, J. T. Swanson; Pediatric Infectious Diseases Society and the Infectious Diseases Society of America, Executive summary: The management of community-acquired pneumonia in infants and children older than 3 months of age: Clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America. *Clin. Infect. Dis*. 53, 617–630 (2011).

A. E. Little, C. J. Robinson, S. B. Peterson, K. F. Raffa, J. Handelsman, Rules of engagement: Interspecies interactions that regulate microbial communities. *Annu. Rev. Microbiol*. 62, 375–401 (2008).

C. I. Liu, G. Y. Liu, Y. Song, F. Yin, M. E. Hensler, W. Y. Jeng, V. Nizet, A. H. Wang, E. Oldfield, A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. *Science* 319, 1391–1394 (2008).

D. J. Haydon, N. R. Stokes, R. Ure, G. Galbraith, J. M. Bennett, D. R. Brown, P. J. Baker, V. V. Barynin, D. W. Rice, S. E. Sedelnikova, J. R. Heal, J. M. Sheridan, S. T. Aiwale, P. K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, L. G. Czaplewski, An inhibitor of FtsZ with potent and selective anti-staphylococcal activity. *Science* 321, 1673–1675 (2008).

D. López, H. Vlamakis, R. Kolter, Biofilms. *Cold Spring Harb. Perspect. Biol*. 2, a000398 (2010).

R. O. Darouiche, Treatment of infections associated with surgical implants. *N. Engl. J. Med*. 350, 1422–1429 (2004).

S. Furukawa, S. L. Kuchma, G. A. O'Toole, Keeping their options open: Acute versus persistent infections. *J. Bacteriol*. 188, 1211–1217 (2006).

I. Kolodkin-Gal, D. Romero, S. Cao, J. Clardy, R. Kolter, R. Losick, D -Amino acids trigger biofilm disassembly. *Science* 328, 627–629 (2010).

P. S. Stewart, J. W. Costerton, Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138 (2001).

E. Denou, R. D. Pridmore, B. Berger, J. M. Panoff, F. Arigoni, H. Brüssow, Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. *J. Bacteriol*. 190, 3161–3168 (2008).

E. Holmes, J. Kinross, G. R. Gibson, R. Burcelin, W. Jia, S. Pettersson, J. K. Nicholson, Therapeutic modulation of microbiota-host metabolic interactions. *Sci. Transl. Med*. 4, 137rv6 (2012).

J. Nicholson, E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, S. Pettersson, Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267 (2012).

A. R. Hughes, B. D. Inouye, M. T. Johnson, N. Underwood, M. Vellend, Ecological consequences of genetic diversity. *Ecol. Lett*. 11, 609–623 (2008).

J. J. Faith, N. P. McNulty, F. E. Rey, J. I. Gordon, Predicting a human gut microbiota's response to diet in gnotobiotic mice. *Science* 333, 101–104 (2011).

M. Arumugam, J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, D. R. Mende, G. R. Fernandes, J. Tap, T. Bruls, J. M. Batto, M. Bertalan, N. Borruel, F. Casellas, L. Fernandez, L. Gautier, T. Hansen, M. Hattori, T. Hayashi, M. Kleerebezem, K. Kurokawa, M. Leclerc, F. Levenez, C. Manichanh, H. B. Nielsen, T. Nielsen, N. Pons, J. Poulain, J. Qin, T. Sicheritz-Ponten, S. Tims, D. Torrents, E. Ugarte, E. G. Zoetendal, J. Wang, F. Guarner, O. Pedersen, W. M. de Vos, S. Brunak, J. Doré; MetaHIT Consortium, M. Antolín, F. Artiguenave, H. M. Blottiere, M. Almeida, C. Brechot, C. Cara, C. Chervaux, A. Cultrone, C. Delorme, G. Denariaz, R. Dervyn, K. U. Foerstner, C. Friss, M. van de Guchte, E. Guedon, F. Haimet, W. Huber, J. van Hylckama-Vlieg, A. Jamet, C. Juste, G. Kaci, J. Knol, O. Lakhdari, S. Layec, K. Le Roux, E. Maguin, A. Mérieux, R. Melo Minardi, C. M'Rini, J. Muller, R. Oozeer, J. Parkhill, P. Renault, M. Rescigno, N. Sanchez, S. Sunagawa, A. Torrejon, K. Turner, G. Vandemeulebrouck, E. Varela, Y. Winogradsky, G. Zeller, J. Weissenbach, S. D. Ehrlich, P. Bork, Enterotypes of the human gut microbiome. *Nature* 473, 174–180 (2011).

H. H. Kong, J. Oh, C. Deming, S. Conlan, E. A. Grice, M. A. Beatson, E. Nomicos, E. C. Polley, H. D. Komarow; NISC Comparative Sequence Program, P. R. Murray, M. L. Turner, J. A. Segre, Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res*. 22, 850–859 (2012).

R. T. Zahradnik, I. Magnusson, C. Walker, E. McDonell, C. H. Hillman, J. D. Hillman, Preliminary assessment of safety and effectiveness in humans of ProBiora3, a probiotic mouthwash. *J. Appl. Microbiol*. 107, 682–690 (2009).

F. Shanahan, Probiotics in perspective. *Gastroenterology* 139, 1808–1812 (2010).

G. Deshpande, S. Rao, S. Patole, M. Bulsara, Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics* 125, 921–930 (2010).

D. Sur, B. Manna, S. K. Niyogi, T. Ramamurthy, A. Palit, K. Nomoto, T. Takahashi, T. Shima, H. Tsuji, T. Kurakawa, Y. Takeda, G. B. Nair, S. K. Bhattacharya, Role of probiotic in preventing acute diarrhoea in children: A community-based, randomized, double-blind placebo-controlled field trial in an urban slum. *Epidemiol. Infect*. 139, 919–926 (2011).

X. W. Gao, M. Mubasher, C. Y. Fang, C. Reifer, L. E. Miller, Dose–response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. *Am. J. Gastroenterol*. 105, 1636–1641 (2010).

S. Hempel, S. J. Newberry, A. R. Maher, Z. Wang, J. N. V. Miles, R. Shanman, B. Johnsen, P. G. Shekelle, Probiotics for the prevention and treatment of antibiotic-associated diarrhea: A systematic review and meta-analysis. *JAMA* 307, 1959–1969 (2012).

K. Isaacs, H. Herfarth, Role of probiotic therapy in IBD. *Inflamm. Bowel. Dis*. 14, 1597–1605 (2008).

D. M. Brenner, M. J. Moeller, W. D. Chey, P. S. Schoenfeld, The utility of probiotics in the treatment of irritable bowel syndrome: A systematic review. *Am. J. Gastroenterol*. 104, 1033–1049 (2009).

P. A. Jänne, N. Gray, J. Settleman, Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. *Nat. Rev. Drug Discov*. 8, 709–723 (2009).

N. P. McNulty, T. Yatsunenko, A. Hsiao, J. J. Faith, B. D. Muegge, A. L. Goodman, B. Henrissat, R. Oozeer, S. Cools-Portier, G. Gobert, C. Chervaux, D. Knights, C. A. Lozupone, R. Knight, A. E. Duncan, J. R. Bain, M. J. Muehlbauer, C. B. Newgard, A. C. Heath, J. I. Gordon, The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci. Transl. Med*. 3, 106ra106 (2011).

A11

COMMUNITY ECOLOGY AND THE VAGINAL MICROBIOME

*Larry J. **Forney***[43,44] *and Jacques Ravel*[45,46]

Abstract

Human vaginas are home to complex communities of microorganisms that are partners in mutualistic associations with their hosts that are critical to maintaining health and protecting the host from infectious urogenital diseases. Recent studies have begun to shed light on how vaginal microbial communities vary among and within individuals at various stages of a woman's life span. However, relatively little is known about the function of these communities or how their constituent members interact with each other and the host to form a dynamic ecosystem that responds to environmental disturbances. To better define variation in the species composition of these communities we conducted a cross-sectional study in which the vaginal bacterial communities of 396 asymptomatic North American women that represented four ethnic groups (white, black, Hispanic, and Asian) were sampled and the species composition was characterized by pyrosequencing of barcoded 16S rRNA genes. The communities clustered into five groups; four were dominated by *Lactobacillus iners*, *L. crispatus*, *L. gasseri*, or *L. jensenii*, while the fifth had lower proportions of lactic acid bacteria and higher proportions of anaerobic organisms. Although it appears that a key ecological function—the production of lactic acid—is conserved, there are statistically significant differences in the proportions of each community group in the four ethnic groups. Moreover, the vaginal pH of women in different ethnic groups also differed and was higher in Hispanic (pH 5.0±0.59) and black (pH 4.7±1.04) women as compared to Asian (pH 4.4±0.59) and white (pH 4.2±0.3) women. In a longitudinal study we investigated the temporal dynamics of vaginal bacterial communities in 32 reproductive age women over a 16-week period. The dynamics were highly personalized and generally varied both within and between women over time, but five major classes of community dynamics could be discerned. These vaginal communities sometimes changed markedly over short periods of time, while others were relatively stable. It is not yet known whether community-level functions varied as the species

[43] Institute for Bioinformatics and Evolutionary Studies.
[44] Department of Biological Sciences, University of Idaho, Moscow, Idaho.
[45] Institute for Genome Sciences.
[46] Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland.

composition of communities changed. Modeling community stability indicated that deviation from stability was correlated with time in the menstrual cycle, community composition, and sexual activity. The women studied were healthy; thus, it appears that neither variation in community composition per se, nor higher levels of observed diversity (codominance) are necessarily indicative of dysbiosis. It is envisioned that better knowledge of vaginal community dynamics and the mutualism between the host and indigenous bacterial communities will lead to the development of strategies to manage the vaginal ecosystem in a way that promotes health and minimizes the use of antibiotics. These efforts should take into account the temporal dynamics of vaginal communities and the potential problems that might arise from diagnostic and therapeutic strategies based on cross-sectional studies.

Introduction

The female lower reproductive tract is home to diverse microbial communities that are thought to play a critical role in maintaining health and protecting individuals from infectious disease. These communities and the host are an example of a finely balanced mutualistic association in which the microbes confer a protective advantage upon their hosts in exchange for a nutrient rich, oxygen-depleted habitat (Danielsson et al., 2011). In reproductive age women, this protective effect is assumptively due to the production of lactic acid and other antimicrobial substances with the former reducing the pH of the vaginal environment making it inhospitable to other invading organisms and precluding unwanted microbial growth (Linhares et al., 2010; O'Hanlon et al., 2011). Lactic acid–producing bacteria (LAB) are thought to be primarily responsible for creating and maintaining this characteristic low-pH environment through the fermentation of glucose liberated from the vaginal epithelium during glycogenolysis (Danielsson et al., 2011). Consequently, high proportions of LAB, particularly *Lactobacillus* species have been paradigmatically considered the hallmark of vaginal health, while the lack thereof is typically associated with a "disturbance" or a "disease" (O'Hanlon et al., 2011).

Coevolutionary processes between the human host and specific microbial partners have shaped the vaginal ecosystem, although the selective forces driving this mutualistic association are not known. The specificity of this host–microbe association is exemplified by the vaginal lactobacilli. Although these organisms are ubiquitous in the environment, they inhabit only three regions of the human body: the oral cavity, the vagina, and the intestines (Pavlova et al., 2002). Of the more than 100 of species of lactobacilli known to exist, only four species are commonly found as dominant members of vaginal microbial communities, namely *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii* (Ravel et al., 2011; Zhou et al., 2007). This suggests these species possess certain characteristics that allow them to outcompete others in the vaginal environment for resources or to actively

exclude nonindigenous species by producing antimicrobial compounds and creating an inhospitable environment.

Lactobacilli have been considered the keystone species[47] of the normal postpubertal vaginal community since 1892, when Professor Albert Döderlein, a renowned German gynecologist, first cultured the organism from vaginal secretions obtained from healthy, pregnant women (Döderlein, 1892; Martin, 2011). These long, slender rods—originally called "Döderlein's bacillus"—were renamed *Lactobacillus acidophilus* in 1928 after their ability to produce lactic acid and reduce the vaginal pH (Danielsson et al., 2011). In the 1980s, it was later discovered that *L. acidophilus* was not a single species, but a group of closely related, obligately homofermentative species collectively known as the *Lactobacillus acidophilus* complex (Lauer et al., 1980). Since species in this complex are difficult to differentiate phenotypically or biochemically (Johnson et al., 1980), they have been classified on the basis of DNA homology (Du Plessis and Dicks, 1995; Schleifer and Ludwig, 1995). All species of *Lactobacillus* recovered from the vagina to date belong to this complex, including *L. iners*, *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. cellobiosus*, *L. brevis*, *L. minutus* (now classified as *Atopobium minutum*) (Collins and Wellbanks, 1992), and *L. salivarius* (Antonio et al., 1999; Levison et al., 1977; Reid et al., 1996; Rogosa and Sharpe, 1960).

Lactic acid bacteria that predominate in the vaginas of reproductive-age women also metabolize extracellular glycogen into lactic acid by anaerobic glycolysis, and also release the acid into the vagina. Epithelial cells produce only the L-lactate isomer, while many bacteria are capable of producing both D- and L-lactate. Moreover, many organisms produce organic acids such as formic acid, acetic acid, propionic acid, succinic acid and others through fermentative metabolism and thereby contribute to acidification of the vaginal environment. The environmental pH of the vagina, traditionally considered to be around 4.0–4.5 in healthy women, is presumably determined by the organic acid contributions from both epithelial cells, resident LAB, and other anaerobic and strictly anaerobic bacteria (Linhares et al., 2010). In addition to lactic acid, vaginal lactobacilli are also capable of producing other antimicrobial compounds, such as antibiotics, target-specific bacteriocins—proteinaceous substances capable of permeabilizing target cell membranes (Aroutcheva, 2001; Oscariz and Pisabarro, 2001)—and the broad-spectrum antimicrobial, hydrogen peroxide (Hawes et al., 1996; Klebanoff et al., 1991). The vagina is, however, a virtually anoxic environment, and as a result it is unlikely that any significant amounts of hydrogen peroxide would be produced and allowed to accumulate to a toxic antimicrobial level *in vitro* (O'Hanlon et al., 2011).

[47] A keystone species is one that plays a unique and crucial role in the way an ecosystem functions.

Common Wisdom of the Vaginal Microbiota

Vaginal microbial communities undergo significant structural changes at various stages in a woman's life span that are directly linked to the level of estrogen in the body (Farage and Maibach, 2006). While numerous studies have been done to understand the vaginal microbiology of reproductive age women, far fewer studies have been done on the vaginal microbiota[48] of premenarcheal, perimenopausal, and postmenopausal females. Despite the paucity of data a plausible sequence of successional events can be described that occur in the vaginal microbiota over a woman's life span.

Initial colonization of the vagina occurs at birth when the infant is first exposed to the mother's vaginal microbiota via passage through the birth canal, or by the skin bacteria of persons handling infants delivered via Caesarian section (Domingez-Bello, 2010). This initial colonization event is believed to establish the gut, skin, and vaginal microbiota, allowing them to differentiate into habitat-specific communities in the weeks and months following birth (Koenig et al., 2011). During the first 2–4 weeks of life, maternal estrogen mediates maturation of the vaginal epithelium and the accumulation of glycogen that is fermented by indigenous bacteria resulting in a lowering of the vaginal pH (~5.0). This effect is transitory, however, as waning maternal estrogen, subsequent thinning of the vaginal mucosa, and a concomitant increase in vaginal pH occur. During childhood the vagina is colonized by diverse assemblages of aerobic, strictly anaerobic, and enteric species of bacteria and the pH is nearly neutral (Farage and Maibach, 2006). At the onset of puberty, adrenal and gonadal maturation causes an increase in estrogen production, which once again causes thickening of the vaginal epithelium and accumulation of glycogen. These environmental conditions selectively favor the proliferation of glycogen-fermenting LAB and the concomitant acidification of the vaginal environment (pH~4.5), which is sustained throughout the reproductive years. The vaginal microbiota of adolescent girls (13–18 years) are comparable to those found in adults, but less is known whether this is also the case for premenarcheal or perimenarcheal girls (Farage and Maibach, 2006). During menopause, a decrease in estrogen levels and the cessation of menstruation are accompanied by atrophy of the vaginal epithelium and reduced cervicovaginal secretions (Farage and Maibach, 2006). In many women this is accompanied by a shift in the vaginal microbiota from populations of lactic acid-producing bacteria to an assortment of species that include anaerobic bacteria comparable to those found during childhood or bacterial vaginosis. Likewise, vaginal pH typically rises to near-neutral levels (6.5–7.0) in

[48] The term *microbiota* refers collectively to all the microorganisms present in a given environment without regard to whether they interact with one another. This is in contrast to the ecological concept of a "microbial community," which refers to a group of actually or potentially *interacting* species that inhabit a place. These terms are distinct from the broader term "microbiome" that is more holistic and includes the microorganisms as well as the environment in which they are found.

women who do not use hormone-replacement therapy (HRT), whereas women who do use HRT typically maintain a vaginal pH comparable to that of reproductive age women (4.5–5.0) and a *Lactobacillus* dominated microbiota (Danielsson et al., 2011). The dynamic nature of this ecosystem underscores the importance of resolving its microbial constituents at different stages of human development and the important role of estrogen on the vaginal environment.

Cultivation-Dependent and Cultivation-Independent Studies

More recently cultivation-independent methods reliant on DNA sequencing of 16S rRNA genes have provided a means to study fine-scale variation in host-associated microbial communities within and among individuals and exploration of ecological relationships between bacterial species and the host. Typically, partial 16S rRNA gene sequences are amplified from total genomic DNA from a sample, and the resulting amplicons are sequenced. Phylogenetic analyses of the sequences allows for classification of phylotypes and determination of the numerically dominant taxa in a community. Major advances in DNA sequencing technologies over the last decade have fundamentally changed the way we assess microbial community structure and composition, and this has facilitated more expansive and intensive studies of vaginal community structure and dynamics. Below we summarize the results of studies done by our group using this approach and the insights to the structure, function, and dynamics of vaginal microbial communities that have been obtained.

Results

Vaginal Bacterial Community Composition and Structure

We characterized the vaginal microbiota and vaginal pH of 396 asymptomatic, sexually active women that fairly equally represented four self-reported ethnic groups: Caucasian (N = 98), black (N = 104), Asian (N = 97), and Hispanic (N = 97) (Ravel et al., 2011). Self-collected midvaginal samples were obtained and used to determine the species composition and structure of the resident bacterial communities by phylogenetic analysis of 16S rRNA gene sequences (Hamady and Knight, 2009). Whole genomic DNA was extracted from each swab and variable regions 1 and 2 (V1–V2) of 16S rRNA genes were PCR amplified using the barcoded universal primers. The resulting amplicons were pyrosequenced, and the 897,345 high-quality sequences (~2,200 reads per sample) were classified using the RDP Naïve Bayesian Classifier (Wang et al., 2007). Species-level taxonomic assignments of *Lactobacillus* sp. was done using a bioinformatics algorithm based on a combination of species-level Hidden Markov Models (HMM) and clustering as described by Ravel et al. (2011). Overall, a total of 282 taxa were observed in the vaginal communities of these women. The depth of coverage

for each community was sufficient to detect taxa that constitute ~0.1% of the community. While taxa present at less than this level are often referred to as low abundance or "rare" taxa, they are only rare in the context of sampling depth. If a vaginal bacterial community has ~10^8 cells per ml of vaginal secretion, then high numbers (10^5 cells per ml) of "rare" members are present in the community, while phylotypes present at densities less than 10^5 cells per ml would remain undetected. These rare taxa could play major roles in the ecology of a community while undetected members may constitute a seed bank of species whose numbers increase under conditions that favor their growth.

The vaginal bacterial communities were grouped based on community composition (Figure A11-1, panel A), and the phylotypes were clustered based on their correlation profiles (Figure A11-1, panel C). The heat map in Figure A11-1 shows the results obtained using $\log_{10}$ transformed percent abundance of each taxon. It highlights the diversity found in all vaginal bacterial communities, even those where the phylotypes abundance is highly skewed and dominated by a single phylotype, and identifies taxa with similar correlation profiles. The analysis revealed five major groups of microbial communities, which we named community state types (CST) and is reminiscent of previously published studies on microbial diversity in the human vagina (Zhou et al., 2007, 2010). The five CSTs, designated CST I, CST II, CST III, CST IV, and CST V, contained 104, 25, 135, 108, and 21 taxa, respectively. The most diverse communities were those of CST IV, and this was reflected in the Shannon diversity indices of the communities (Figure A11-1).

Unlike any other anatomical site on the human body, most vaginal communities (73%) were dominated by one or more species of *Lactobacillus* that constitute more than 50% of all sequences obtained. Communities in CST I, which occurred in 26.2% of the women sampled were dominated by *L. crispatus*, while CST II (6.3%), CST III (34.1%), and CST V (5.3%) were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively. The skewed rank abundances of species in these communities leaves the impression that these communities are species poor, but this may not be the case since there are an unknown number of rare species. The remaining communities found in 27% of the women formed a large heterogeneous group (CST IV), and were typified by higher proportions of anaerobic and strictly anaerobic bacteria, including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*. This finding is consistent with those of previous studies wherein the species composition of vaginal communities was investigated by cloning and sequencing of 16S rRNA genes (Ferris et al., 2004, 2007; Fredricks et al., 2005; Srinivasan and Fredricks, 2008; Verhelst et al., 2004; Verstraelen et al., 2004; Zhou et al., 2007, 2010). It should be pointed out that while communities in CST IV appear to be diverse relative to the other CST, it could simply reflect greater species evenness. Although communities in CST IV were not dominated by *Lactobacillus* sp., *L. iners*, and *L. crispatus* were detected

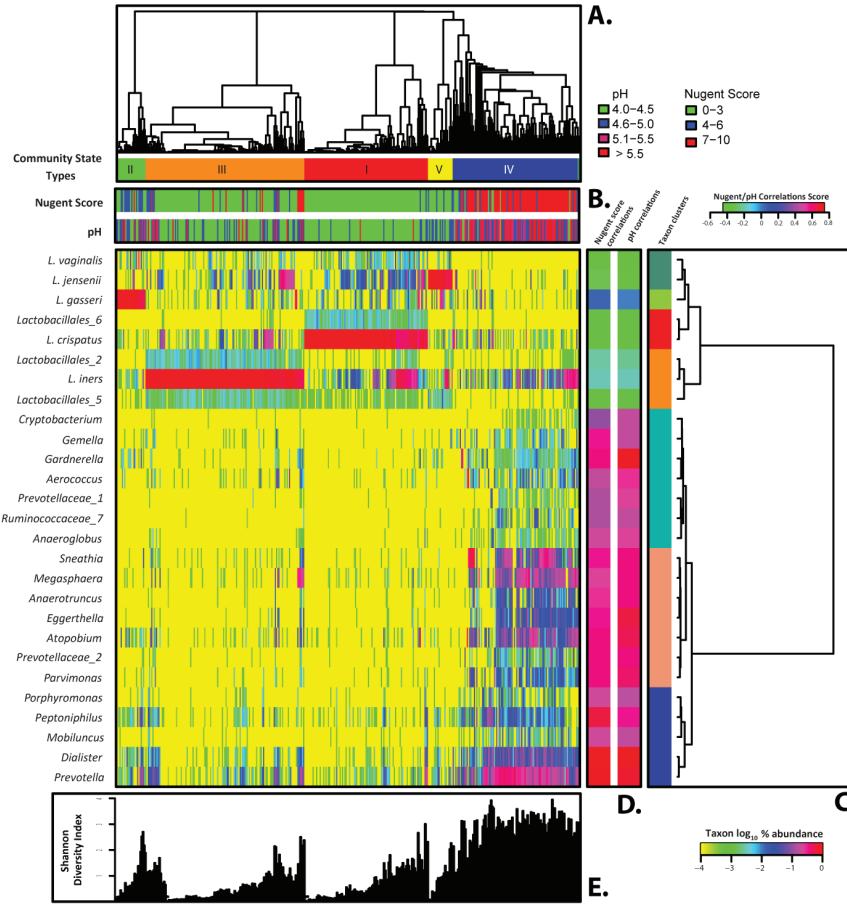


FIGURE A11-1 Heat map of $\log_{10}$ transformed proportions of microbial taxa found in the vaginal bacterial communities of 394 women of reproductive age (color key is indicated in the lower-right corner). (A) Complete linkage clustering of samples based the species composition and abundance of vaginal bacterial communities that defines community groups I to V. (B) Nugent scores and pH measurements for each of the 394 community samples (color key is indicated above panel C). (C) Complete linkage clustering of taxa based on Spearman's correlation coefficient profiles, which were defined as the set of Spearman's correlation coefficients calculated between one taxon and all the other taxa. (D) Spearman's correlation coefficients between the presence of a taxon and the Nugent score or pH of a sample. (E) Shannon diversity indices calculated for 394 vaginal communities. SOURCE: Modified from Ravel et al. (2011).

in 78.7% and 51.9% of CST IV communities, respectively. Only four subjects in CST IV lacked detectable *Lactobacillus* sp. in their vaginas, and those communities were dominated by *Prevotella*, *Sneathia*, *Megasphaera*, or *Streptococcus*. Interestingly, all communities contained members that have been assigned to genera known to produce lactic acid including *Lactobacillus*, *Megasphaera*, *Streptococcus*, and *Atopobium*. This suggests that an important catabolic function, namely the production of lactic acid, might be conserved among each community's state types despite differences in the species composition.

Core Microbiomes of the Human Vagina

One objective of studies on the human microbiome is to determine if there is a core set of microbial species associated with the bodies of all humans. It is postulated that changes to this "core microbiome" may be correlated with changes in human health or risk to disease. The results from this study suggest that for the human vagina there is no single core microbiome. Instead, it appears there are multiple core microbiomes that can be defined by CST I–V depicted in Figure A11-1. As noted above, these groups can be readily distinguished on the basis of two criteria: (a) whether the constituent communities are dominated by *Lactobacillus* or not, and (b) the particular species of *Lactobacillus* present. The vast majority of communities in CST I, CST II, CST III, and CST V had more than one phylotype of lactic acid bacteria suggesting a degree of functional redundancy, but they differed widely in abundance. Although no core microbiome can be identified based on the taxa found in these communities, we posit that core functions are conserved among communities despite differences in their species composition, and that functional redundancy would be associated with increased community reliability in the face of environmental changes (Konopka, 2009).

Differences in the Vaginal Microbiomes of Ethnic Groups

The study cohort consisted of roughly equal numbers of four self-described ethnicities (Caucasian, Asian, black, and Hispanic), and this offered the opportunity to assess the relationship of ethnic background on vaginal bacterial community composition. The proportions of each CST varied among the four ethnic groups (Figures A11-2 and A11-3), and these differences were statistically significant ($\chi^2 = 36.8$ on 10 df, $P < .0001$). No statistically significant associations were observed between age and community types within or across ethnic groups.

Vaginal bacterial communities dominated by species of *Lactobacillus* (CSTs I, II, III, and V) were found in 80.2% and 89.7% of Asian and white women, respectively, but in only 59.6% and 61.9% of Hispanic and black women, respectively. The higher median pH values in Hispanic (pH 5.0±0.59) and black (pH 4.7±1.04) women reflects the higher prevalence of communities not dominated by *Lactobacillus* sp. (CST IV) in these two ethnic groups when compared

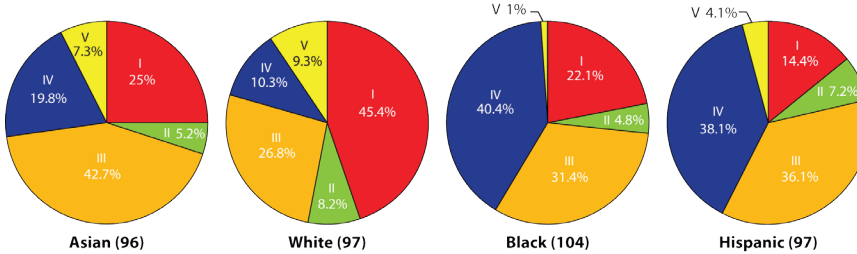


FIGURE A11-2 Representation of vaginal bacterial community groups within each ethnic group of women.
NOTE: The number of women from each ethnic group is in parentheses.
SOURCE: Modified from Ravel et al. (2011).

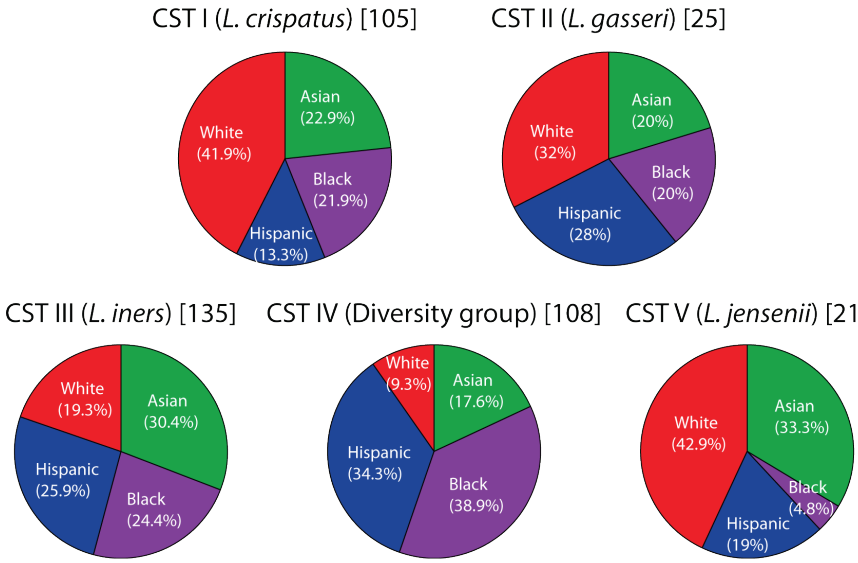


FIGURE A11-3 Contribution of ethnicity to each of the five vaginal community groups expressed as percentage.
NOTE: Sectors are colored according to ethnicity and labeled accordingly. The percentage represents the proportion of subjects of each ethnicity divided by the total number of subjects assigned to a community group (indicated in square brackets). The dominant species for each community group is indicated in parentheses.
SOURCE: Modified from Ravel et al. (2011).

to Asian (pH 4.4±0.59) and white (pH 4.2±0.3) women (Table A11-1). This is significant because the occurrence of high numbers of lactobacilli and pH < 4.5 have become synonymous with :healthy." If accepted at face value, this common wisdom suggests that while most Asian and white women are healthy, a significant proportion of asymptomatic Hispanic and black women are "unhealthy"; a notion that seems implausible. It also begs the question of what kinds of bacterial communities should be considered normal in Hispanic and black women. We found that CST IV was overrepresented in Hispanic (34.3%) and black (38.9%) women as compared to Asian (17.6%) and white (9.3%) women (Figure A11-3). From these data we conclude that vaginal bacterial communities not dominated by species of *Lactobacillus* are common and normal in black and Hispanic women. The data from this study are in accordance with the results of Zhou et al. (2007, 2010) who studied the vaginal bacterial communities of white, black, and Japanese women. The reasons for these differences among ethnic groups are unknown, but it is tempting to speculate that the species composition of vaginal communities could be governed by genetically determined differences between hosts. These might include differences in innate and adaptive immune systems, the composition and quantity of vaginal secretions, and ligands on epithelial cell surfaces, among others. While these may be key to shaping vaginal communities, previous studies have also shown that human habits and practices including personal hygiene, methods of birth control, and sexual behaviors also exert strong influences (Schwebke, 2009).

The small number of different kinds of vaginal communities is somewhat surprising given that these communities are probably assembled independently after birth. The repeatability of community assembly suggests that a host exerts strong selection for a rather limited number of different kinds of bacteria. This is especially evident in the limited number of *Lactobacillus* phylotypes and other lactic acid producing bacteria that are abundant in these communities. The prominence of these populations and their important role in modulating vaginal pH suggests they might be drivers in these communities and thought of in terms of Walker's driver-passenger model (Peterson et al., 1998, Walker, 1992). This model posits that ecological function resides in "driver" species or in functional groups of such species that have key ecological functions that significantly structure ecosystems, while passenger species are those that have minor ecological impact. Studies done to tease out the influence of these various factors on vaginal community ecology will be important to understanding community stability, resistance, and resilience so that strategies can be developed to maintain human vaginal health and prevent disease.

Vaginal Community Space

The relationships among communities were visualized by principal component analysis and displayed in three-dimensional (3-D) space. The three principle

TABLE A11-1 Median Vaginal pH by Community State Types Within Ethnic Background

	Community State Types[a]					
	CST I (*L. crispatus*)		CST II (*L. gasseri*)		CST III (*L. iners*)	
Ethnic groups	Subjects[b]	Median pH ±MAD[c]	Subjects[b]	Median pH ±MAD[c]	Subjects[b]	Median pH ±MAD[c]
Asian	24	4.4 ± 0.52	5	4.4 ± 0.44	41	4.0 ± 0.0
White	44	4.0 ± 0.0	8	4.7 ± 0.44	26	4.3 ± 0.30
Black	23	4.0 ± 0.0	5	5.0 ± 0.0	33	4.0 ± 0.0
Hispanic	14	4.0 ± 0.0	7	4.7 ± 0.22	35	4.4 ± 0.59

[a] Community groups are defined as in Figure A11-1.
[b] Total number of subjects within a CST.
[c] Median absolute deviation.

components explained 82% of the variance. Each point in Figure A11-4 represents the vaginal community of an individual. Communities dominated by species of *Lactobacillus* and representing CSTs I, II, III, and V are shown at each of the four outer vertices of the tetrahedron, with CST IV at the inner vertex. Communities found on the edges joining two vertices are mixtures of the two *Lactobacillus* species that dominate the communities found at the corresponding vertices, with an equal proportion of each species at the midpoint of the edge. We refer to each location in this 3-D space as a community state, and one can consider the entire space to represent the plausible alternative community states, or vaginal bacterial community space.

The cross-sectional design of this study with only one sample from each subject precludes knowing whether the locations of these communities in vaginal community space vary over time. Nonetheless, at this stage we can propose four distinct conceptual models for the variation of community composition over time. The first is the "dynamic equilibrium hypothesis" in which the composition of a community is comparatively invariant over time and exists in a single dynamic equilibrium. A second "community space hypothesis" is the opposite of the first, and each community can and does occupy any position in community space over time and throughout a woman's lifetime. These changes are postulated to occur in response to hormonal cycles, an individual's habits and practices, changes in diet, or some other ecological force. A third model is an "alternative equilibrium states hypothesis" wherein a woman's community can change over time, but the number of alternative states are limited in number and governed by unknown factors. A fourth possibility is a "community resilience hypothesis" in which a

CST IV (Diversity group)		CST V (*L. jensenii*)		All CSTs	
Subjects[b]	Median pH ±MAD[c]	Subjects[b]	Median pH ±MAD[c]	Subjects[b]	Median pH ±MAD[c]
19	5.5 ± 0.44	7	5.0 ± 0.89	96	4.4 ± 0.59
10	5.5 ±.74	9	4.85 ± 0.22	97	4.2 ± 0.30
42	5.3 ± 0.44	1	4.7 ± 0.44	104	4.7 ± 1.04
37	5.3 ± 0.44	4	5.0 ± 0.59	97	5.0 ± 074

community normally resides in a single region of space. Under this scenario the composition and structure of a vaginal community can change to a transitional state in response to disturbance, but the resistance and resilience of a community determine the extent and duration of a change, while homeostatic mechanisms drive communities back to their "ground state." We expect that no single hypothesis will explain the dynamics of all communities.

The pH and Nugent scores of each community are depicted in the 3-D community space on Figures A11-4B and A11-4C. The figures show a strong correlation between high pH and high Nugent scores.[49] The lowest pH values were associated with community states dominated by *L. iners* and *L. crispatus*, and the highest pH values were associated with community states not dominated by species of *Lactobacillus*. Both Nugent scores and pH values increased as the proportion of non-*Lactobacillus* sp. increased. This was most readily seen in communities that contained a decreasing proportion of *L. iners*. Interestingly, elevated pH and high Nugent scores were observed in some communities that have high proportions of *Lactobacillus* species (also shown in Figure A11-1) suggesting that these metrics cannot be predicted with absolute certainty based solely on the proportion of *Lactobacillus* in a community. Clearly additional research is needed to understand the various factors that govern vaginal pH.

[49] Nugent scores are used to diagnose bacterial vaginosis. The scores range from 0–10 and are based on the weighted counts of different bacterial cell morphologies observed in a Gram-stained smear of a vaginal sample. For further information see Nugent, P. et al. (1991) *J Clin Microbiol 29*(2), 297–301.

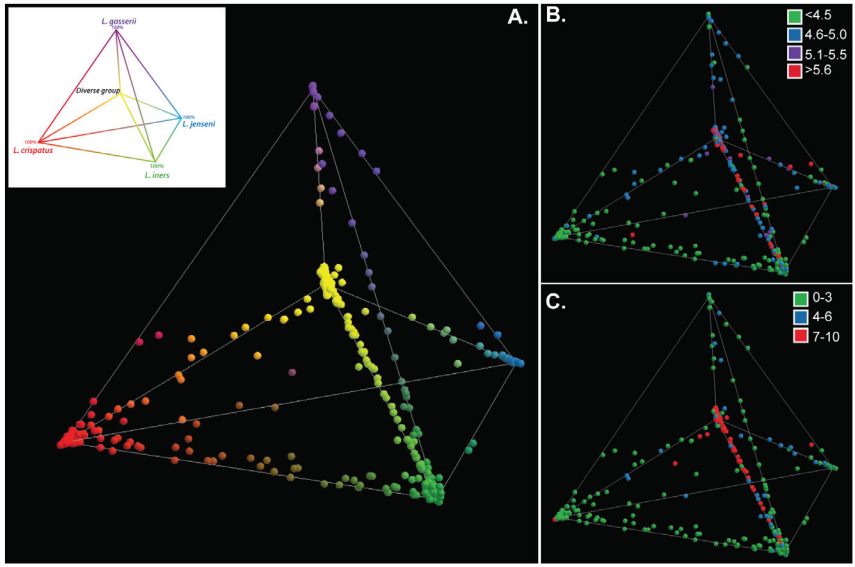


FIGURE A11-4 Relationships among vaginal bacterial communities visualized by principal component analysis in which the relative abundances are expressed as proportions of the total community and displayed in three-dimensional (3-D) space. Communities dominated by species of *Lactobacillus* and representing CST I, II, III, and V are shown at each of the four outer vertices of the tetrahedron, with CST IV at the inner vertex and shown in the inset. Panel A: Each point corresponds to a single subject and was colored according to the proportions of phylotypes in each community. Panel B: The pH of each vaginal community shown in Panel A. Panel C: The Nugent score category of each vaginal community shown in Panel A.
SOURCE: Modified from Ravel et al. (2011).

By analogy with other ebiological communities, it is reasonable to assume that vaginal microbial communities exist in a state of dynamic equilibrium, and that homeostatic mechanisms exist to provide resilience. Given the fundamental differences in the species composition of these communities, one can speculate that they will differ in terms of number and strength of interspecies interactions. This will in turn have implications for the relative resistance and resilience of each community type to disturbances. If that is the case then invasive species, including both opportunistic and overt pathogens, are more likely to become established in communities that exhibit low stability while the converse will also be true (Hobbs and Huenneke, 1992). This has direct implications for the assessment of susceptibility to infectious disease. Importantly, it also suggests that differences in vaginal bacterial community composition should be taken into account in the estimation of disease risks. This would constitute the first

step toward personalized medicine for women's reproductive health, wherein differences between the vaginal microbiomes of individuals would be taken into account in risk assessment, and for disease diagnosis and treatment.

Temporal Dynamics of the Vaginal Microbiome

To evaluate the hypotheses described above, we determined the temporal dynamics of vaginal bacterial communities in healthy reproductive-age women in a longitudinal study that was conducted in which 32 women self-collected midvaginal swabs twice-weekly for 16 weeks using a validated self-collection protocol (Gajer et al., 2012). The bacterial diversity in the vaginal communities sampled was determined by pyrosequencing variable regions 1 and 2 (V1–V2) of bacterial rRNA genes. A dataset of 2,522,080 high-quality classifiable 16S rRNA gene sequences was obtained from 937 samples with an average of 2,692±910 (SD) sequences per sample. Using the methods described above the bacterial communities sampled were classified into one of five community state types based on differences in species composition and their relative abundances (Figure A11-5). Three of these CSTs (I–III), were dominated by *L. crispatus*, *L. gasseri*, or *L. iners*, respectively. Unlike the study described above none of the communities examined in this study were dominated by *L. jensenii*, probably because the low prevalence of this CST and too few women were sampled. Communities that clustered in CST IV-A and IV-B were heterogeneous in composition, lacked significant numbers of *Lactobacillus* sp. but differed in species composition (Figure A11-5). Communities in CST IV-A were generally characterized by modest proportions of either *L. crispatus*, *L. iners*, or other *Lactobacillus* spp., along with low proportions of various species of anaerobic and strictly anaerobic bacteria such as *Anaerococcus*, *Corynebacterium*, *Finegoldia*, or *Streptococcus*. In contrast, CST IV-B had higher proportions of the genus *Atopobium*, in addition to *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella*, *Mobiluncus*, or *Peptoniphilus* and several other taxa. Some of the taxa in CST IV-B have previously been shown to be associated with bacterial vaginosis (Fredricks et al., 2005). Overall, high Nugent scores were most often associated with CST IV-B, while low Nugent scores were associated with CST IV-A (Figure A11-6).

Community State Types Transitions

Profiles of community state types were derived from time series of community samples and clustered into five classes of longitudinal dynamics, which we refer to as community classes (Figure A11-6), designated LC, LG, LI, DA, and DB. These classes reflect similarities in how community composition changed over time (Figure A11-6). Nearly all the temporal profiles were complex and somewhat individualized. Not all kinds of transitions between community states were equally likely to occur within a time series, and some were not

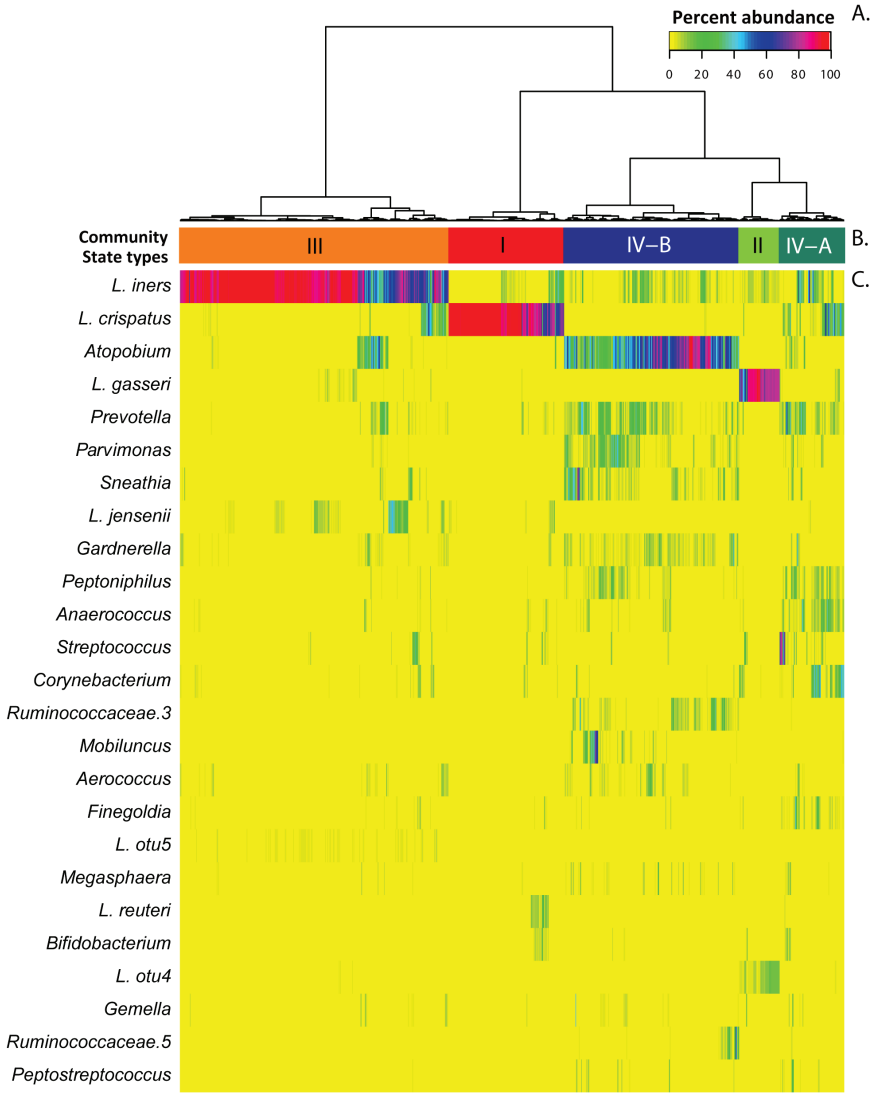


FIGURE A11-5 Assignment of vaginal community state types. (A) Dendogram of Jensen-Shannon distances between bacterial community states in all samples collected twice weekly for 16 weeks among all 32 women. (B) Community state types resulting from clustering analysis wherein each sample was assigned to one of the five community state type (CST I, CST II, CST III, CST IV-A, and CST IV-B), and these are color coded. (C) Heat map of phylotype relative abundances. The color key for abundance is in the top-right corner. NOTE: The 25 most prevalent phylotypes in all communities are listed on the left side of the heat map.
SOURCE: Modified from Gajer et al. (2012).

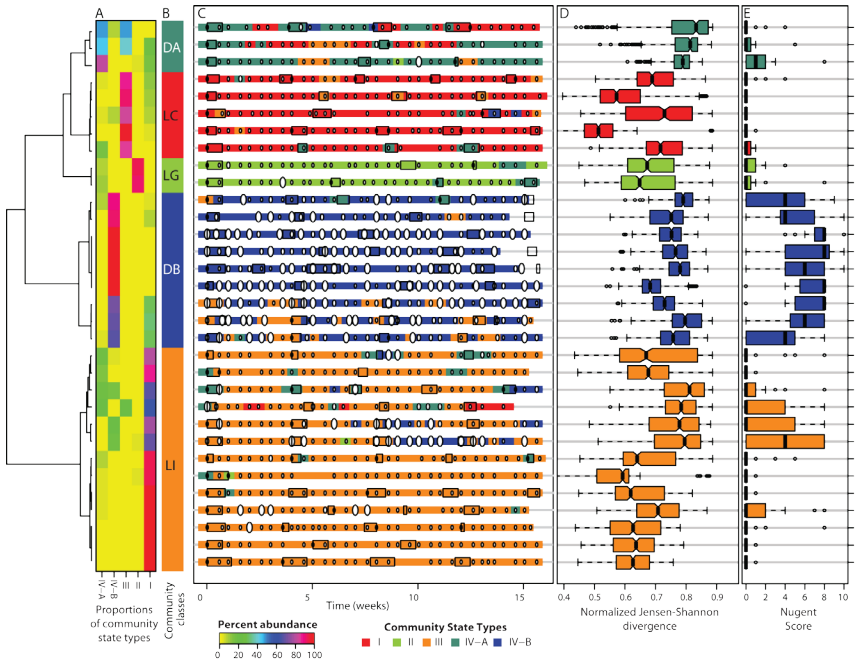


FIGURE A11-6 Dynamics of vaginal community state types in 32 women over 16 weeks. (A) Heat map showing the proportions of community state types (I, II, III, IV-A and IV-B) observed within a woman over time (color key is indicated below panel C) that were used to generate the dendogram that depicts distances between proportions of the five community state types identified. (B) Color bar indicating community class (DA, LC, LG, DB, and LI) as defined by clusters of proportions of community state types within a woman over time. (C) Profiles of community state types, Nugent scores, and menses for 32 women over a 16-week period. Each dot (white or black) represents one sample in the time series. The absence of a dot indicates missing samples. The community state types in the time series for each woman are color-coded according to the schema shown below panel C. Nugent scores is indicated: high Nugent score (7–10, large ovals; intermediate Nugent score (4–6; medium ovals); and low Nugent score (1–3; small ovals). Menses for each woman are indicated by boxes. (D) Box plot of normalized Jensen-Shannon distances between all pairs of community states within each subject. Community deviation from constancy is represented by the Jensen-Shannon Index (black bar, see Supplementary Online Material), with higher values reflecting decreased constancy. (E) Box plot of average Nugent scores for each woman over 16 weeks. Panel D and E, the whiskers represent the lowest and highest datum still within 1.5 interquartile range (IQR) of the lower and upper quartile. The middle 50% of the data is represented by the height of the box.
SOURCE: Modified from Gajer et al. (2012).

observed (Figure A11-7). For example, vaginal communities of CST IV-B often transitioned to CST III, but only rarely to CST I. Likewise, the results show that vaginal communities of CST I, which are dominated by *L. crispatus*, most often transition to become community CST III, which are dominated by *L. iners*, or CST IV-A. Of note, CST III were two times more likely to switch to CST IV-B than to CST IV-A. The community states of CST II are dominated by *L. gasseri* and rarely underwent transitions to other CST. Conversely, we observed no transitions from CST I to CST II, and there were only two transitions from CST III to CST II.

Index of vaginal bacterial community stability By definition, communities that persist in the same state type over time display a high level of stability, while those that often transition to different state types have low levels of stability. We introduce the normalized Jensen-Shannon divergence index,[50] defined as the median of Jensen-Shannon distances between all pairs of community states, which provides a quantitative measure of community stability (Figure A11-6E). As the index becomes smaller less change is observed between different community states, and the community is more constant over time. The vaginal bacterial communities of subjects 3, 4, 5, and 6 were of class DB, and displayed higher levels of overall constancy and persistently high Nugent score even though the subjects did not report any BV symptoms. This calls into question current widely held views on what is considered to be a "normal" vaginal bacterial community that are founded on the premise that the communities of healthy women must contain high proportions of *Lactobacillus* species (Srinivasan and Fredricks, 2008). This premise is also undermined by the observation that vaginal bacterial communities of subjects 32, 26, and 25 of class DA and subjects 30, 24, and 31 of class LC, which had high Jensen-Shannon indices yet persistently low Nugent scores. Thus, highly variable vaginal bacterial communities do not always have persistently high Nugent scores, so variation per se does not always equate with disease. These results suggest that neither variation in community composition, nor constantly high levels of apparent diversity (codominance) are necessarily indicative of dysbiosis. The meaning and implications of these observations will remain unknown until molecular characterization of vaginal microbiota is applied to studies of adverse outcomes.

Lactobacillus *sp. and Vaginal Bacterial Community Stability*

The stability of vaginal communities was associated with certain species of lactic acid bacteria that dominated a community. For example, the vaginal communities of women in community classes LC (five women) and LG (two women)

[50] The Jensen-Shannon divergence index reflects the similarity between two probability distributions. In this case it was used to quantitatively compare the kinds and abundances of bacterial phylotypes in consecutive pairs of samples.

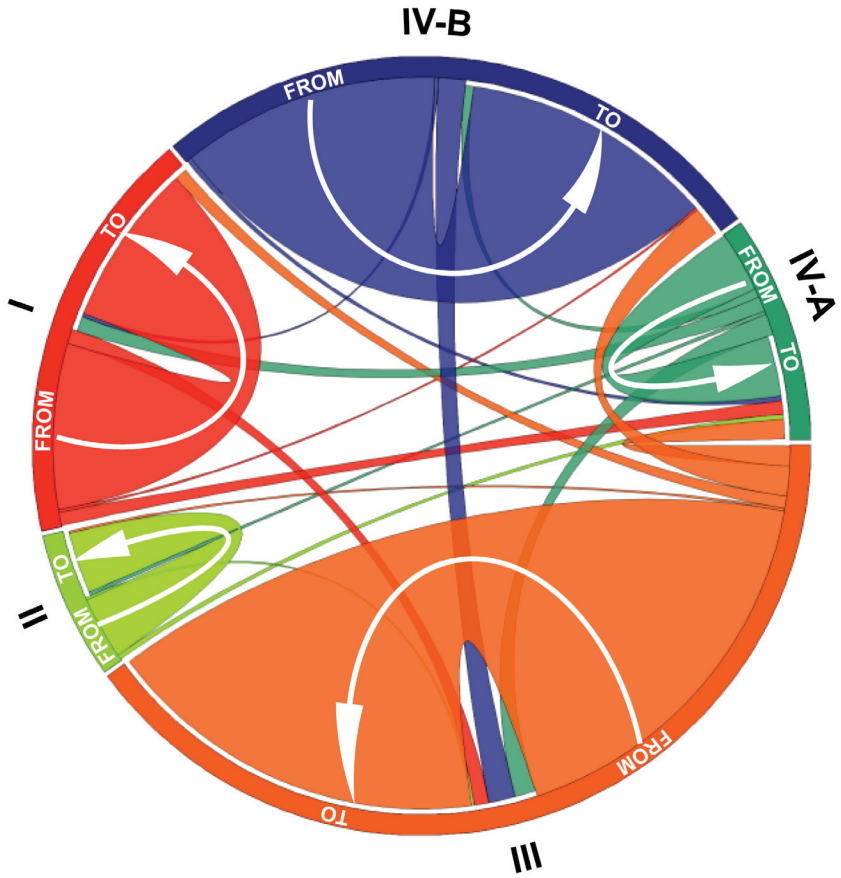


FIGURE A11-7 Graphical representation of community state type transitions observed between all consecutive pairs of time points (905 transitions) and their frequencies among all women. For each community state type, the length of the outer arcs represents the proportion of each community state type among all women combined. The width of the inner arcs (at its base) represents the frequency of transition to the same community state type or to another community state type. The directionality of the transition is indicated by the labels FROM and TO as well by an arrow for transition to the same community state type. SOURCE: Modified from Gajer et al. (2012).

were most often dominated by *L. crispatus* and *L. gasseri*, respectively. These communities were rather stable, exhibited fewer transitions between community states, and typically had low Nugent scores. In these women most transitions between community states were associated with menses (Figure A11-6). For example, in subject 28 (Figure A11-8A), a community dominated by *L. crispatus* was resilient, being replaced by a community dominated by *L. iners* during

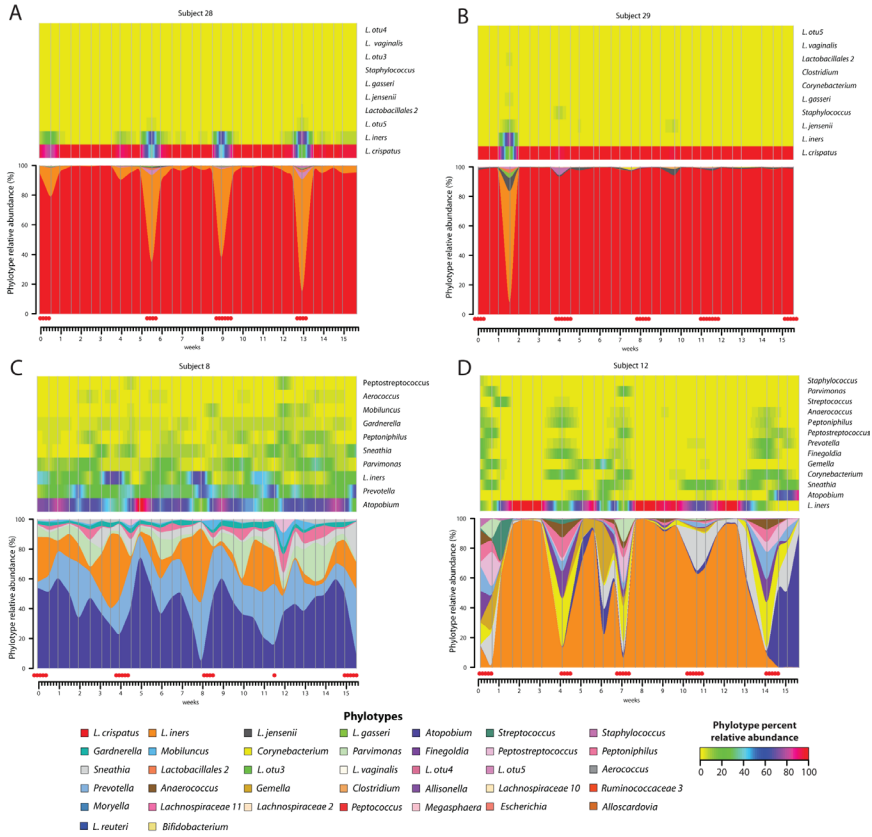


FIGURE A11-8 (A–D) Heat maps (top) and interpolated bar plots (bottom) of phylotype relative abundance observed in four selected subjects over 16 weeks (heat map color key is indicated in the lower-right corner).
NOTE: Color-codes for each phylotype represented in the interpolated bar plots are shown below the figure. Red dots below the interpolated bar graphs represent menstruation days.
SOURCE: Modified from Gajer et al. (2012).

menses, which then reverted to a community dominated by *L. crispatus* at the end of menses. Communities of class LI (14 women) were typically dominated by *L. iners*, but varied widely in terms of species composition and stability. This is illustrated by the communities of subjects 14, 15, 16, 18, and 19 that appeared rather stable over time, while others such as 11 and 27 commonly shifted to different community types that were more often associated with higher Nugent scores. These differences might reflect genomic heterogeneity in the dominating *Lactobacillus* sp.

The rapid and sometimes extensive turnover of human vaginal communities was visualized by mapping temporal changes in community composition onto the 3-D community space defined previously in a cross-sectional study described above of vaginal communities in 396 women (Ravel et al., 2011) (Figure A11-9). These illustrations show that while the vaginal community composition of many

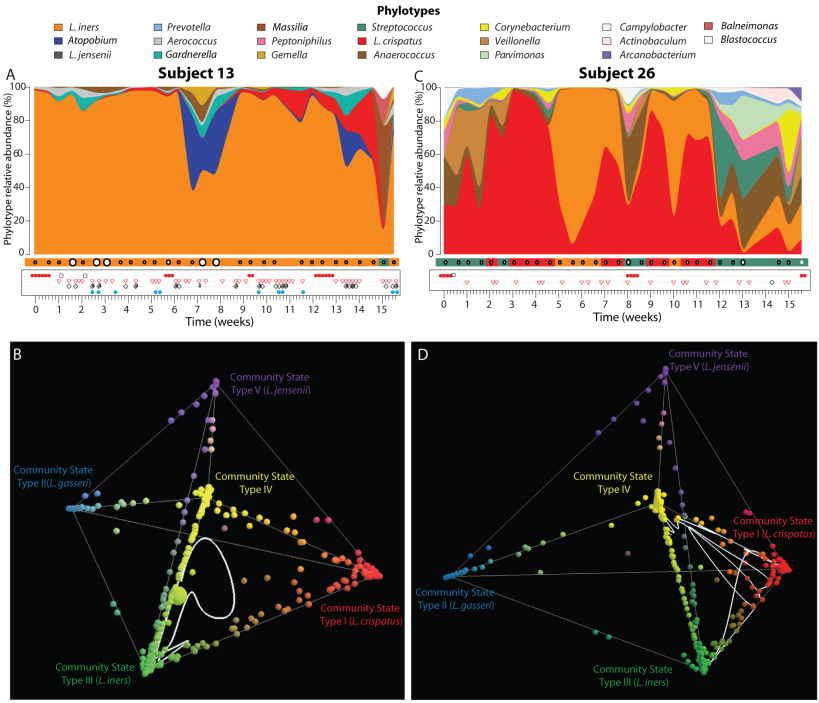


FIGURE A11-9 Temporal dynamics of vaginal bacterial communities in two women over 16 weeks. (A, C) Interpolated bar graph of phylotype relative abundance for subjects 13 (A) and 26 (C). Profiles of community state types in which Nugent scores have been superimposed (high Nugent score, 7–10, large open circles; intermediate Nugent score, 4–6, medium open circles; and low Nugent score, 1–3, small open circles) are shown below the interpolated bar graphs. Daily metadata is represented by the following: red balls, menstruation; black open square, douching; open red triangle, vaginal intercourse; open black diamond, oral sex; vertical black bar, digital penetration (insertion of finger(s) in the vagina); light blue closed circle, lubricant use. (B, D) Representation of vaginal community dynamics in 3-D community space (3) for subjects 13 (B) and 26 (D). Communities dominated by species of *Lactobacillus* and representing CST I (red), II (light blue), III (green), and V (purple) are shown at each of the four outer vertices of the tetrahedron, with CST IV (yellow) at the inner vertex. The white line represents the succession of community states for each subject over time.
SOURCE: Modified from Gajer et al. (2012).

individuals changed over time the alternatives were often restricted to specific community states. This is consistent with the notion that vaginal communities can exist in alternative equilibrium states. In contrast, communities of most other women evinced dynamics consistent with the "community resilience hypothesis," in which a community, though dynamic, normally resides in a single region of space. In these cases the composition and structure of a community occasionally changed to a transition state, but they were resilient, and homeostatic mechanisms returned them to their "ground state."

In many women the bacterial species composition and rank abundances of bacterial species changed markedly over short periods of time (Figure A11-8D for example). Consistent with this, the communities of some women seemed resilient and showed simple and predictable changes between community states that occurred only during menses (e.g., subjects 28, 12, and 19). In contrast the communities of other women were almost invariant during menses (e.g., subject 29), while still others changed states continuously over time regardless of whether the subjects were menstruating or not (e.g., subjects 1, 2, 7, and 27). The latter might be examples of communities transitioning to alternative equilibrium states. Most of the transitions to other state types were, however, transient in nature with 35% of all state types persisting for not more than a week. This is consistent with previous studies that showed significant yet short-lived changes in Nugent scores (Brotman et al., 2010; Schwebke et al., 1999) and with daily fluctuations in the composition of the vaginal microbiota that have been previously documented by microscopy (Brotman et al., 2010; Hay et al., 1997; Keane et al., 1997; Schwebke et al., 1999).

Identifying Factors of Stability Disturbance

In an effort to evaluate the dependence of stability of vaginal bacterial communities on the time in the menstrual cycle and other time-varying factors, we modeled the log Jensen-Shannon divergence rate of change over normalized menstrual cycles using a linear mixed effects model with a Fourier polynomial of the normalized menstrual time component adjusted for hormonal contraception, community type, sexual activities, lubricant use and douching (with the last three evaluated 1 day prior to sampling), and with subject-based degree two polynomial random effects to account for correlation in repeated measurements on each subject (Figure A11-10). The Fourier polynomial captured population-level dependence of the log Jensen-Shannon divergence rate of change (i.e., community deviation from constancy) on the time in the menstrual cycle. The lowest constancy was associated with menses. Interestingly, the two minima of the population-level Jensen-Shannon divergence rate of change (indicating highest community constancy) coincided with the two maxima of estradiol concentration as reported by Minassian et al. (1993), while the maximum of progesterone concentration coincided with the second minimum of the population-level constancy

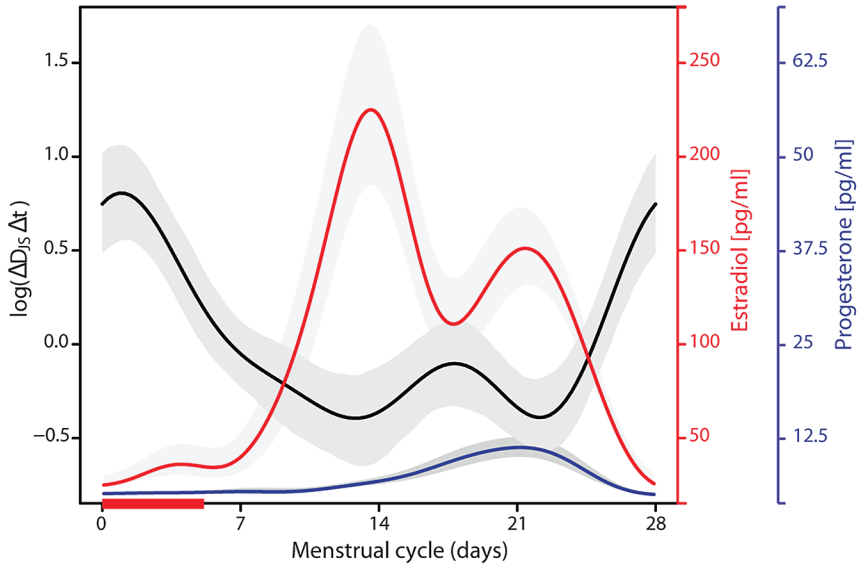


FIGURE A11-10 Modeling the dependence of the log of Jensen-Shannon divergence rate of change over the menstrual cycle. The length of menstrual cycles within and between women were normalized to 28 days with day 1 to 5 corresponding to menses (red bar). The red line shows concentrations of estradiol as a function of menstrual time (data from Brotman et al., 2008) and the blue line shows concentrations of progesterone (data from Brotman et al., 2008). The shaded areas around each curve show 95% point-wise confidence bands.
SOURCE: From Gajer et al. (2012).

function (Figure A11-10). Of all the metadata evaluated in the model, sexual activity was the only one that had a significant (negative) effect on constancy independent of time in the menstrual cycle, but its effect was rather weak in comparison to that of community class.

Discussion

A Need for Personalized Medicine?

The species that make up vaginal microbial communities vary between individuals as do the dynamics of these communities. The differences between individuals can be demonstrated by what has been learned about the vaginal microbiome wherein at least five bacterial community state types are common in healthy reproductive age women, and the frequencies of these types vary among women of different ethnic groups. These communities remain dynamic within an individual,

and dramatic changes, which seem highly individualized in the species composition, can occur over short time scales. Because differences exist among individuals, different individuals can be affected differently by common risks. Hence, the rate of disease occurrence and the efficacy of various treatments may not be pertinent to some individuals in a population since the conclusions are often drawn from studies based on a population as a whole. The reasons for these differences in composition and variance in stability are unknown. Among the possible causes are differences that exist among host's medical history, diet, genotype, ecological interactions of bacteria within hosts, and a plethora of other factors just now being discovered, as well as purely stochastic factors. Further research is needed to characterize heterogeneity in the microbiomes of the vagina, especially at a functional level, which will lead to a better understanding of disease risks for specific individuals and avoid the potential pitfalls of population-based studies. By doing so it will be possible to more reliably identify individuals at risk to bacterial vaginosis, various sexually transmitted diseases, and ascending urogenital infections.

In the data described here and reported previously (Ravel et al., 2011; Gajer et al., 2012), considerable variability exists within the definition of a "normal" vaginal microbiome. When surveyed cross-sectionally, it appears that several distinct "community state types" exist in normal, otherwise healthy women, each with a markedly different bacterial species composition and pH profile, and that the prevalence of each varies with ethnicity (Ravel et al., 2011). Nonetheless, the vaginal communities of women in the four different ethnic groups studied (Asian, Hispanic, white, and black) could be clustered into five different CSTs based on the numerically dominant species in the vaginal communities. Four of the CSTs were dominated by one of four *Lactobacillus* species (*L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii*), while communities in the fifth CST included a diverse array of strict and facultative anaerobes and lower proportions of lactobacilli (Ravel et al., 2011). This group accounted for slightly over 25% of the women studied, a notable finding given the paradigmatic view that high proportions of *Lactobacillus* are prerequisite for vaginal health and "normality." Indeed, the inherent differences within and between women in different ethnic groups strongly argues for a more refined definition of the kinds of bacterial communities normally found in healthy women, and the need to appreciate differences between individuals so they can be taken into account in risk assessment and disease diagnosis. Indeed the variability seen in the composition of the vaginal microbiome between healthy women and over a woman's life span beg for us to reassess and broaden our thinking of what constitutes "normal" or at the very least to accept that "normal" is a continuum and not a categorical variable.

If the maintenance of a low pH is indeed a key function of the vaginal microbial community, then perhaps it may be more appropriate to consider all lactic acid bacteria found in vaginal communities as members of the same ecological "guild" (Jaksic, 1981) since they all rely on the same resource pool to fulfill the same ecological niche. If this is the case, then the prevailing view that

species of *Lactobacillus* are both necessary and sufficient for maintaining health may be overly simplistic because functionally equivalent species may "substitute" for one another. With this in mind these types of vaginal bacterial communities might be considered normal and healthy if signs and symptoms of disease are absent even though the composition of these communities closely resembles those associated with symptomatic bacterial vaginosis.

Ecological Dynamics of the Vaginal Microbiota

The dynamics of vaginal communities were found to vary widely among women even among those that clustered in the same community class, and the communities of some women were shown to have low constancy and high levels of species turnover. These findings suggest that point estimates of community composition that arise from cross-sectional studies could be misleading for most women because they experience a distribution of community states over time I. This calls into question whether data from cross-sectional studies can be reliably used to link vaginal bacterial community composition to health outcomes. Intervals of increased susceptibility to disease may occur because vaginal microbiota fluctuate.

Fluctuation in community composition and constancy appear to be mainly affected by time in the menstrual cycle, community class, and to a certain extent by sexual activity. Other unknown or less well-studied factors such as host diet and the state of the innate immune system are almost certainly at play as well. It should be noted that in many cases community function is probably maintained despite changes in community composition, because shifts in community structure often only involved changes in the relative dominance of a limited number of different lactic acid bacterial species. Such fluctuations could occur while maintaining community performance (e.g., lactic acid production) when there is functional redundancy among community members and shifts in the relative abundances of guild members occur due to changes in environmental conditions that favor one population over another. This coupled with the observation that a limited number of community types are found in women of different ethnicities suggest that deterministic processes driven by interspecies interaction, host–microbe interaction, and niche partitioning account for vaginal community composition. In some cases changes in community performance occur as a result of changes in community composition. These are reflected in the shift in composition of the metabolome,[51] which in this strictly anaerobic environment is dominated by fermentation products such as lactic, succinic, and acetic acids that accumulate in vaginal secretions (Gajer et al., 2012).

[51] A metabolome is comprised of all the intermediates and products of metabolism that are present in a biological sample (cell, tissue, organ, or organism).

The causes of shifts in community structure and function are unknown. However the common occurrence of highly abundant lactic acid bacteria and the pronounced effect they have on the vaginal environmental in terms of pH is consistent with Walker's driver-passenger model (Walker, 1995). If the role of lactic acid bacteria is to create and maintain the low pH environment to protect from unwanted microbial colonization, then these lactic acid bacteria could be considered the "drivers" of the vaginal environment because they set the stage for other microbial populations who must be able to withstand a pH of around 4.0 to 4.5. In this way the lactic acid bacteria act to stabilize communities. Anaerobic non-lactic acid bacteria would be considered the "passengers," as their presence would depend on their ability to conform to the environment created by the "drivers." These members, typically present at much lower abundance, would have much less influence on the ecosystem, and may even be lost over time without markedly affecting the community's function. At least from a numerical perspective vaginal communities seem to conform to this model since the rank abundance of species is highly skewed, and lactic acid bacteria often outnumber others by at least an order of magnitude. However, changes in environmental conditions that favor the outgrowth of "passengers" might place them in the driver's seat, and they might cause the demise of lactic acid bacteria via their metabolic activity or the production of antimicrobial compounds. Such marked changes in community structure might be precipitated by changes in the kinds and amounts of resources available to the bacteria by the host, disturbances of the community induced by various habits and practices (e.g., douching), or changes in host immune status. Changes in the microbiome of this sort might account for some of the symptoms classically associated with bacterial vaginosis, which include an elevated pH and the malodor of vaginal secretions that result from metabolic by-products such as amines and short-chain fatty acids (Amsel et al., 1983). The change in environmental pH and accumulation of metabolic by-products may make the environment less hospitable for lactic acid bacteria allowing anaerobic bacteria to displace them as dominant community members.

Community Stability: Resistance Versus Resilience

Vaginal bacterial communities that differ in species composition are expected to differ in their resilience and resistance to environmental change, and this is manifest in the degree of stability that each exhibits over time. The resistance of a community is a measure of its ability to resist changes in response to a disturbance event, and it is measured by the amount of change the community can withstand without having an impact on community function (McCann, 2000). Resilience, on the other hand, refers to a community's ability to recover and return to a quasi-stable state following a disturbance. It is a measure of how often or how strong a disturbance must be to actually alter community function (Gunderson, 2000; McCann, 2000). Both aspects contribute to overall community stability,

and are heavily dependent on various characteristics of the underlying microbial network, such as their ability to tolerate various types of stresses, the strength and types of interactions present, and the degree of functional redundancy existing within the community (Hobbs and Huenneke, 1998). Those systems with greater degrees of functional redundancy in roles known to stabilize or "drive" the community, tend to have greater buffering to disturbances. This insurance hypothesis (McCann, 2000) suggests that more diverse communities are more likely to exhibit functional redundancy and be more stable in terms of their response to environmental perturbations.

Ecologists have long known that the biological communities of disturbed ecosystems are more susceptible to invasion by nonindigenous species, and the bacterial communities of the vaginal microbiota are probably no exception. Hence if the resistance or resilience of a vaginal community are low, then transitory changes to the structure of these communities may occur more readily in response to disturbances making these disturbed communities more susceptible to invasion by nonindigenous species that might include transient species of fecal origin and opportunistic pathogens (Hobbs and Huenneke, 1992). It should be kept in mind that disturbance events that cause shifts in population densities with concomitant changes in community function can vary in intensity, frequency, and duration (White and Jentsch, 2001), and whether an event will actually alter community composition or structure depends on characteristics of the community itself. For instance, communities with low levels of resistance and resilience may be disturbed by a single but intense event of relatively short duration that results in a transient shift in community structure that temporarily increases the community's susceptibility to invasion. More robust communities, however, may be able to retain community structure and function, despite more frequent events of low to moderate intensity. Given this, we postulate that stability and resilience of vaginal bacterial communities is likely to vary widely since the species composition and structure of these communities differs among women and this variance in turn may account for differences in the susceptibility of individuals to urogenital infectious diseases. This is important since vaginal communities are continually subjected to a wide range of potential acute and chronic disturbances related to human activities, such as the use of various birth control methods, antibiotics, and sexual intercourse, as well as natural "disturbances" such as hormonal fluctuations (Eschenbach et al., 2000), aging (Larsen et al., 1982), and stress (Culhane et al., 2002; Nansel et al., 2006) of which may in turn affect their ability to maintain human health.

Community Dynamics and Stability

Most vaginal microbiome studies done to date have employed cross-sectional designs, wherein samples are obtained from some number of individuals at a single point in time or with multiple sampling points separated by relatively

long intervals (weeks or months). While these studies have yielded valuable information in terms of species composition, they do not allow for any assessment of community stability, therefore painting an incomplete picture of vaginal microbial ecology. This reliance on data from cross-sectional studies is surprising since daily fluctuations in the composition of the vaginal microbiota have been previously documented by microscopy and other means. The data presented here shows that nearly all the vaginal communities studied exhibit some degree of variability, with some changing markedly over a short time and others remaining relatively constant. Usually these shifts involved changes in the relative proportions of species present, but in some cases, a distinct and persistent turnover in species composition occurred, marking the presence of an apparent alternative equilibrium state. Several factors may have contributed to these differing levels of community stability. For instance, menses was identified as having the largest effect on changes in community composition, while communities were less variable during periods of the menstrual cycle marked by high levels of estrogen (late follicular phase) or estrogen and progesterone (luteal phase; Gajer et al., 2012). These findings highlight the potential of prospective longitudinal studies to identify causes of community instability that are associated with increased risk to infectious disease and the community disturbances that give rise to bacterial vaginosis.

Prospective

Host–microbe interactions are notoriously complex, yet in the case of the vaginal microbiome there has been a willingness to accept that highly abundant lactobacilli and an acidic environment (< 4.5) are both necessary and sufficient for vaginal health. However, the evidence from recent studies suggests there is a need to critically reassess this common wisdom because the vaginal communities of a large fraction of asymptomatic, healthy, reproductive-age women (~25%) are not dominated by species of *Lactobacillus*; an observation that is equally true for apparently the majority of healthy premenarcheal and postmenopausal females. Likewise, the vaginal pH of a large proportion of reproductive age women (especially Hispanics and blacks) as well as that of premenarcheal and postmenopausal females is greater than 5.0. These facts indicate that our understanding of other processes and mechanisms that contribute to avoiding infectious disease and the maintenance of health is incomplete.

Certainly there is compelling evidence that the low pH resulting from lactic acid production is important to precluding the colonization of nonindigenous organisms in the vagina. However, lactic acid may have other roles as well. For example, lactic acid itself is known to antimicrobial activity and has been shown to be more effective than acidity alone as a microbicide against HIV and pathogens like *Neisseria gonorrhea* (Graver and Wade, 2011). Furthermore, Witkin et al. and others have shown that L-lactic acid, in addition to its role in influencing

vaginal acidity, stimulates the IL-23/IL-17 T lymphocyte pathway (Shime et al., 2008; Witkin et al., 2011), and induces the production of pro-inflammatory cytokines by vaginal epithelial cells in the presence of a synthetic viral RNA (Mossop et al., 2011), induction of tumor angiogenesis (Vegran et al., 2011), lymphocyte activation (Murray et al., 2005), and inhibition of bacterial growth (Alakomi et al., 2000; O'Hanlon et al., 2011). Witkin et al. suggest that lactic acid may have stimulatory effects on the host innate defense system by enhancing cytokine release in response to lipopolysaccharide (LPS) exposure (Witkin et al., 2011). In these studies, lactic acid enhanced the release of IL-23 by peripheral monocytes and macrophages in response to LPS exposure (Witkin et al., 2011). Given that IL-23 is one of the primary cytokines involved in neutrophil recruitment, mobilization, and activation at mucosal surfaces, this is thought to translate to better immune surveillance of mucosal surfaces characterized by high lactic acid levels in vivo (Witkin et al., 2011). This is consistent with the findings of Fichorova et al. (2011) that colonization of vaginal epithelial cell monolayers with common bacteria such as *L. crispatus, Prevotella bivia,* and *Atopobium vaginae* may regulate the epithelial innate immunity in a species-specific manner. Finally, recent studies by Witkin et al. (*mBio*, in press) provided evidence that the ratio of D- and L-lactate isomers influences the expression of matrix metalloproteinase (MMP-8) production by controlling extracellular matrix metalloproteinase inducer (EMMPRIN) levels. Based on these studies it is plausible to suggest that in addition to lowering the environmental pH lactic acid, it also serves as a signaling molecule that influences host gene expression.

It seems likely that products of bacterial species other than lactobacilli will also contribute to the maintenance of host vaginal health in humans as well as in other host species by influencing influence host gene expression. These might include other low molecular weight metabolites, including various short-chain fatty acids that are produced by strict anaerobes that inhabit the vagina. This is an appealing idea because vaginal bacterial species have coevolved with their hosts over time, and it seems likely that they collectively provide benefits to the host as part of a complex mutualistic relationship. Perhaps future studies should move away from the perhaps overly simplistic notion that lactobacilli are "good" simply because they lower the vaginal pH, and all other species are inconsequential at best, or at worst opportunistic pathogens.

Acknowledgments

This work was in part supported by the National Institute of Allergies and Infectious Diseases, National Institutes of Health (grant numbers U19 AI084044, UO1 AI070921 and UH2AI083264) and a Center of Biomedical Research Excellence grant (P30 GM103324) from the National Institute for General Medical Sciences.

References

Alakomi, H-L., E. Skytta, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander. 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology* 66:2001-2005.

Amsel, R., P. A. Totten, C. A. Spiegel, K. C. Chen, D. Eschenbach, and K. K. Holmes. 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *American Journal of Medicine* 74(1):14-22.

Antonio, M. A., S. E. Hawes, and S. L. Hillier. 1999. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. *Journal of Infectious Diseases* 180(6):1950-1956.

Aroutcheva, A., D. Gariti, M. Simon, S. Shott, J. Faro, J. A. Simoes, A. Gurguis, and S. Faro. 2001. Defense factors of vaginal lactobacilli. *American Journal of Obstetrics and Gynecology* 185(2):375-379.

Brotman, R. M., K. G. Ghanem, M. A. Klebanoff, T. E. Taha, D. O. Scharfstein, and J. M. Zenilman. 2008. The effect of vaginal douching cessation on bacterial vaginosis: A pilot study. *American Journal of Obstetrics and Gynecology* 198(6):628.e1–628.e7.

Brotman, R. M., J. Ravel, R. A. Cone, and J. M. Zenilman. 2010. Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. *Sexually Transmitted Infections* 86(4):297-302.

Collins, M. D., and S. Wallbanks. 1992. Comparative sequence analyses of the 16S rRNA genes of Lactobacillus minutus, Lactobacillus rimae and Streptococcus parvulus: proposal for the creation of a new genus Atopobium. *FEMS Microbiology Letters* 74(2-3):235-240.

Culhane, J. F., V. Rauh, K. F. McCollum, I. T. Elo, and V. Hogan. 2002. Exposure to chronic stress and ethnic differences in rates of bacterial vaginosis among pregnant women. *American Journal of Obstetrics and Gynecology* 187(5):1272-1276.

Danielsson, D., P. K. Teigen, and H. Moi. 2011. The genital econiche: focus on microbiota and bacterial vaginosis. *Annals of the New York Academy of Sciences* 1230:48-58.

Döderlein A. 1892. Das scheidensekret und seine bedeutung fur puerperalfieber. *Zentralblatt fur Bakteriology* 11:699.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences of the United States of America* 107(26):11971-11975.

Du Plessis, E. M., and L. M. Dicks. 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri, and Lactobacillus johnsonii. *Current Microbiology* 31(2):114-118.

Eschenbach, D. A., S. S. Thwin, D. L. Patton, T. M. Hooton, A. E. Stapleton, K. Agnew, C. Winter, A. Meier, and W. E. Stamm. 2000. Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. *Clinical Infectious Diseases* 30(6):901-907.

Farage, M., and H. Maibach. 2006. Lifetime changes in the vulva and vagina. *Archives of Gynecology and Obstetrics* 273(4):195-202.

Ferris, M. J., A. Masztal, K. E. Aldridge, J. D. Fortenberry, P. L. Fidel Jr., and D. H. Martin. 2004. Association of Atopobium vaginae, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infectious Disease* 4:5.

Ferris, M. J., J. Norori, M. Zozaya-Hinchliffe, and D. H. Martin. 2007. Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. *Journal of Clinical Microbiology* 45:1016–1018.

Fichorova, R. N., H. S. Yamamoto, M. L. Delaney, A. B. Onderdonk, and G. F. Doncel. 2011. Novel vaginal microflora colonization model providing new insight into microbicide mechanism of action. *MBio* 2(6):e00168-00111.

Fredricks, D. N., T. L. Fiedler, and J. M. Marrazzo. 2005. Molecular identification of bacteria associated with bacterial vaginosis. *New England Journal of Medicine* 353(18):1899-1911.

Gajer, P., R. M. Brotman, G. Bai, J. Sakamoto, U. M. Schutte, X. Zhong, S. S. Koenig, L. Fu, Z. S. Ma, X. Zhou, Z. Abdo, L. J. Forney, and J. Ravel. 2012. Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine* 4(132):132ra152.

Graver, M. A., and J. J. Wade. 2011. The role of acidification in the inhibition of Neisseria gonorrhoeae by vaginal lactobacilli during anaerobic growth. *Annals of Clinical Microbiology and Antimicrobials* 10:8.

Gunderson, L. H. 2000. Ecological resilience—in theory and application. *Annual Review of Ecology and Systematics* 31(1):425-439.

Hamady, M., and R. Knight. 2009. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Research* 19(7):1141-1152.

Hawes, S. E., S. L. Hillier, J. Benedetti, C. E. Stevens, L. A. Koutsky, P. Wolner-Hanssen, and K. K. Holmes. 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. *Journal of Infectious Diseases* 174(5):1058-1063.

Hay, P. E., A. Ugwumadu, and J. Chowns. 1997. Sex, thrush and bacterial vaginosis. *International Journal of STD & AIDS* 8(10):603-608.

Hobbs, R. J., and L. F. Huenneke. 1992. Disturbance, Diversity, and Invasion: Implications for Conservation. *Conservation Biology* 6(3):324-337.

Jaksić, F. M. 1981. Abuse and Misuse of the Term "Guild" in Ecological Studies. *Oikos* 37(3):397-400.

Keane, F. E., C. A. Ison, and D. Taylor-Robinson. 1997. A longitudinal study of the vaginal flora over a menstrual cycle. *International Journal of STD & AIDS* 8(8):489-494.

Klebanoff, S. J., S. L. Hillier, D. A. Eschenbach, and A. M. Waltersdorph. 1991. Control of the microbial flora of the vagina by H2O2-generating lactobacilli. *Journal of Infectious Diseases* 164(1):94-100.

Koenig, J. E., A. Spor, N. Scalfone, A. D. Fricker, J. Stombaugh, R. Knight, L. T. Angenent, and R. E. Ley. 2011. Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America* 108 Suppl 1:4578-4585.

Konopka, A. 2009. What is microbial community ecology? *ISME J* 3(11):1223-1230.

Larsen, B., and R. P. Galask. 1982. Vaginal microbial flora: composition and influences of host physiology. *Annals of Internal Medicine* 96(6 Pt 2):926-930.

Lauer, E., C. Helming, O. Kandler. 1980. Heterogeneity of the species Lactobacillus acidophilus (Moro) Hansen and Mocquot as revealed by biochemical characteristics and DNA-DNA hybridization. *Zentbl Bacteriol Mikrobial Hyg Abt* 1:150-168.

Levison, M. E., L. C. Corman, E. R. Carrington, and D. Kaye. 1977. Quantitative microflora of the vagina. *American Journal of Obstetrics and Gynecology* 127(1):80-85.

Linhares, I. M., P. R. Summers, B. Larsen, P. C. Giraldo, and S. S. Witkin. 2011. Contemporary perspectives on vaginal pH and lactobacilli. *American Journal of Obstetrics and Gynecology* 204(2):120 e121-125.

Martin, D. H. 2012. The microbiota of the vagina and its influence on women's health and disease. *American Journal of the Medical Sciences* 343(1):2-9.

McCann, K. S. 2000. The diversity-stability debate. *Nature* 405(6783):228-233.

Minassian, S. S., and C. H. Wu. 1993. Free and protein-bound progesterone during normal and luteal phase defective cycles. *International Journal of Gynaecology and Obstetrics* 43(2):163-168.

Mossop, H., I. M. Linhares, A. M. Bongiovanni, W. J. Ledger, and S. S. Witkin. 2011. Influence of lactic acid on endogenous and viral RNA-induced immune mediator production by vaginal epithelial cells. *Obstetrics & Gynecology* 118:840-846.

Murray, C. M., R. Hutchinson, J. R. Bantick, G. P. Belfield, A. D. Benjamin, D. Brazma, R. V. Bundick, I. D. Cook, R. I. Craggs, S. Edwards, L. R. Evans, R. Harrison, E. Holness, A. P. Jackson, C. G. Jackson, L. P. Kingston, M. W. D. Perry, A. R. J. Ross, P. A. Rugman, S. S. Sdhu, M. Sullivan, D. A. Taylor-Fishwick, P. C. Walker, Y. M. Whitehead, D. J. Wilkinson, A. Wrigth, and D. K. Donald. Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nature Chemical Biology* 1:371-376.

Nansel, T. R., M. A. Riggs, K. F. Yu, W. W. Andrews, J. R. Schwebke, and M. A. Klebanoff. 2006. The association of psychosocial stress and bacterial vaginosis in a longitudinal cohort. *American Journal of Obstetrics and Gynecology* 194(2):381-386.

O'Hanlon, D. E., T. R. Moench, and R. A. Cone. 2011. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infectious Diseases* 11:200.

Oscáriz, J. C., and A. G. Pisabarro. 2001. Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *International Microbiology* 4(1):13-19.

Pavlova, S. I., A. O. Kilic, S. S. Kilic, J. S. So, M. E. Nader-Macias, J. A. Simoes, and L. Tao. 2002. Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. *Journal of Applied Microbiology* 92(3):451-459.

Peterson, G., C. R. Allen, and C. S. Holling. 1998. Ecological Resilience, Biodiversity, and Scale. *Ecosystems* 1(1):6-18.

Ravel, J., P. Gajer, Z. Abdo, G. M. Schneider, S. S. Koenig, S. L. McCulle, S. Karlebach, R. Gorle, J. Russell, C. O. Tacket, R. M. Brotman, C. C. Davis, K. Ault, L. Peralta, and L. J. Forney. 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America* 108 Suppl 1:4680-4687.

Reid, G., J. A. McGroarty, L. Tomeczek, and A. W. Bruce. 1996. Identification and plasmid profiles of Lactobacillus species from the vagina of 100 healthy women. *FEMS Immunology and Medical Microbiology* 15(1):23-26.

Rogosa, M., and M. E. Sharpe. 1960. Species differentiation of human vaginal lactobacilli. *Journal of General Microbiology* 23:197-201.

Schleifer K. H. and W. Ludwig. 1995. Phylogeny of the genus Lactobacillus and related genera. *Systematic and Applied Microbiology* 18:461-467.

Schwebke, J. R. 2009. New concepts in the etiology of bacterial vaginosis. *Current Infectious Disease Reports* 11(2):143-147.

Schwebke, J. R., C. M. Richey, and H. L. Weiss. 1999. Correlation of behaviors with microbiological changes in vaginal flora. *Journal of Infectious Diseases* 180(5):1632-1636.

Shime, H., M. Yabu, T. Akazawa, K. Kodama, M. Matsumoto, T. Seya, and N. Inoue. 2008. Tumor-secreted lactic acid promotes IL-23/Il-17 proinflammatory pathway. *Journal of Immunology* 180: 7175-7183.

Srinivasan, S., and D. N. Fredricks. 2008. The Human Vaginal Bacterial Biota and Bacterial Vaginosis. *Interdisciplinary Perspectives on Infectious Diseases* 2008.

Vegran, F. R. Boidot, C. Michiels, P. Sonveaux, and O. Feron. 2011. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-κB/IL-8 pathway that drives tumor angiogenesis. *Cancer Research* 71(7):2550-2560.

Verhelst, R., H. Verstraelen, G., Claeys, G. Verschraegen, J. Delanghe, L. Van Simaey, C. De Ganck, M. Temmerman, and M. Vaneechoutte..2004. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between Atopobium vaginae, Gardnerella vaginalis and bacterial vaginosis. *BMC Microbiology* 4:16.

Verstraelen, H., R. Verhelst, G. Claeys, M. Temmerman, and M. Vaneechoutte. 2004. Culture-independent analysis of vaginal microflora: The unrecognized association of Atopobium vaginae with bacterial vaginosis. *American Journal of Obstetrics and Gynecology* 191:1130–1132.

Walker, B. H. 1992. Biodiversity and Ecological Redundancy. *Conservation Biology* 6(1):18-23.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73(16):5261-5267.

Witkin, S. S., S. Alvi, A. M. Bongiovanni, I. M. Linhares, and W. J. Ledger. 2011. Lactic acid stimulates interleukin-23 production by peripheral blood mononuclear cells exposed to bacterial lipopolysaccharide. *FEMS Immunology & Medical Microbiology* 61:153-158.

Zhou, X., C. J. Brown, Z. Abdo, C. C. Davis, M. A. Hansmann, P. Joyce, J. A. Foster, and L. J. Forney. 2007. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME J* 1(2):121-133.

Zhou, X., M. A. Hansmann, C. C. Davis, H. Suzuki, C. J. Brown, U. Schutte, J. D. Pierson, and L. J. Forney. 2010. The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. *FEMS Immunology and Medical Microbiology* 58(2):169-181.

A12

INVESTIGATING BACTERIAL-ANIMAL SYMBIOSES WITH LIGHT SHEET MICROSCOPY[52]

Michael J. Taormina,[53,*] *Matthew Jemielita*,[53,*] *W. Zac Stephens*,[54] *Adam R. Burns*,[55] *Joshua V. Troll*,[54] *Raghuveer Parthasarathy*,[53,54,56] *and Karen* ***Guillemin***[54]

Abstract

Microbial colonization of the digestive tract is a crucial event in vertebrate development, required for maturation of host immunity and establishment of normal digestive physiology. Advances in genomic, proteomic, and metabolomic technologies are providing a more detailed picture of the constituents of the intestinal habitat, but these approaches lack the spatial and temporal resolution needed to characterize the assembly and dynamics of microbial communities in this complex environment. We report the use of light sheet microscopy to provide high-resolution imaging of bacterial colonization of the intestine of *Danio rerio*, the zebrafish. The method allows us to characterize bacterial population dynamics across the entire organ and the behaviors of individual bacterial and host cells throughout the

[52] Taormina et al., 2012. Biol. Bull. Vol. 223: Pp. 7–20. Reprinted with permission from the Marine Biological Laboratory, Woods Hole, MA.

[53] Department of Physics, University of Oregon, Eugene, Oregon 97403.

[54] Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403.

[55] Institute of Ecology and Evolution, University of Oregon, Eugene, Oregon 97403.

[56] Materials Science Institute, University of Oregon, Eugene, Oregon 97403.

* Co-first authors.

colonization process. The large four-dimensional data sets generated by these imaging approaches require new strategies for image analysis. When integrated with other "omics" data sets, information about the spatial and temporal dynamics of microbial cells within the vertebrate intestine will provide new mechanistic insights into how microbial communities assemble and function within hosts.

Background and Significance

Introduction

All plants and animals are ecosystems for microbial communities. With the advent of high-throughput "omics" technologies (including genomics, proteomics, and metabolomics) that can provide comprehensive catalogs of a microbial sample's nucleic acids, proteins, and metabolites, the stature of these underexplored and often overlooked communities is on the rise. Now that we can identify the members of these host-associated microbial communities and their functional capacities, we can ask fundamental questions about these symbiotic associations: How do particular microbial communities assemble on and within hosts? How are these communities maintained over time? How do these communities influence the development and physiology of their hosts? These questions are further motivated by human health concerns, as recent insights suggest that many diseases, including inflammatory bowel diseases, type II diabetes, colorectal cancer, autoimmune diseases, and possibly even autism are correlated with altered gut microbiota (Dethlefsen et al., 2007; Spor et al., 2011). A still deeper question in the field of symbiosis is the extent to which there are universal rules and mechanisms that govern the coexistence of plants and animals with their microbial associates and whether lessons learned from model symbioses can be applied to the associations between humans and their complex microbial consortia.

Although they provide extraordinarily powerful tools for probing multispecies systems, omics approaches deliver only homogenized inventories of community components, incapable of revealing the spatial organization and dynamics of host-associated microbial communities. To truly understand these associations, we need to be able to visualize them on the spatial and temporal scales at which microbes operate—an experimental challenge. Here we describe the application of light sheet microscopy to the field of symbiosis. In particular, we describe the utility of this imaging technique for exploring unanswered questions about the microbial colonization of the vertebrate intestine by using the model organism *Danio rerio,* the zebrafish. Hand-in-hand with omics technologies has come an explosion of data, spurring the need for analytic approaches that can make sense of information being generated about host-associated microbiota. A major thrust of these approaches is to draw correlations between the composition of a

microbial community and the physiology of the colonized host (Kuczynski et al., 2012). Analogously, large data sets documenting the three-dimensional spatial distributions of microbial and host cells over time can be analyzed to reveal spatial and temporal correlations between microbial species and between microbes and host cells to build quantitative models of microbial dynamics within a host organ.

One instructive example of insights into host colonization by bacteria comes from live imaging of the partnership between the bobtail squid *Euprymna scolopes* and the luminescent marine bacterium *Vibrio fischerii,* which colonizes the light organ of its host and provides light to erase the squid's shadow when the squid is foraging at night in shallow seawater (Nyholm and McFall-Ngai, 2004). Direct visualization of colonization using fluorescently labeled bacteria revealed host-mediated control of the microbial population, as the symbionts are selectively recruited from the seawater and collected on specialized mucus-rich external surfaces before migrating into the pores leading to the light organ (Nyholm et al., 2000, 2002). The squid maintains long-term control over its symbiont population by purging the contents of the light organ each dawn, thereby allowing a fresh population to regrow from a small residual inoculum (Boettcher et al., 1996). In this simple system, bacterial growth can be well described by analogy to bacterial population dynamics in a flask. An initial clonal inoculum grows exponentially until the population reaches a maximum density, and the diurnal venting of the squid is the equivalent of diluting the culture into a flask of fresh broth.

In contrast to the squid light organ, the vertebrate intestine is an open-ended tube with continual flux of microbial and dietary contents. Experimentally, researchers have attempted to model this system as a series of chemostats with climax populations of bacteria maintained at a fixed density by a continual influx of nutrients and efflux of contents (Macfarlane et al., 1998). In reality, we know very little about the microbial population dynamics within this organ and the extent to which the chemostat model accurately simulates the intestinal environment. For example, to what extent is the efflux balanced by regrowth of permanent residents, as in a chemostat or the squid light organ, as opposed to influx of new members from the external environment? Are there conditions that favor internal regrowth *versus* influx? Answers to these questions are crucial for developing treatments for human disease that minimize disturbances of gastrointestinal ecology or that maximize invasion by, for example, a probiotic. To investigate colonization of the vertebrate intestine, one needs an experimentally tractable model vertebrate that can be subject to live imaging. Ideally the host would be optically transparent, small enough for its entire digestive tract to be visualized, and amenable to microbiological manipulations, such as the ability to generate gnotobiotic animals with defined microbial associations. The zebrafish larva offers all of these features, as well as a rich research history that has generated many valuable protocols and reagents for experimental manipulations.

Zebrafish as a Model for Studying Colonization of the Vertebrate Gut

Zebrafish were pioneered as a model for studying vertebrate development because of their optical transparency, rapid embryonic development, fecundity, inexpensive husbandry, and ability to be manipulated genetically and embryologically (Grunwald and Eisen, 2002). These same attributes make them extremely useful for studying host-microbe associations (Cheesman and Guillemin, 2007; Kanther and Rawls, 2010). Zebrafish first encounter microbes in their environment when they hatch out of their chorions as larvae between 2 and 3 days post-fertilization (dpf). By 4 dpf, their digestive tracts are open to the environment at both ends and begin to be colonized by bacteria in their environment. At 7 dpf, the larval stage shown in Figure A12-1, they are still optically transparent and their internal organs, including the intestine, can be readily visualized. Their ex-utero development enables easy derivation of germ-free, or axenic, zebrafish larvae by surface sterilization of the chorion prior to hatching (Milligan-Myhre et al., 2011). Because of their fecundity and the fact that embryos can be fertilized in vitro, it is possible to derive thousands of germ-free individuals at a time, a scale that is unobtainable in mammalian systems. Our gnotobiotic methodologies have allowed us to infer the roles that the resident microbiota play in host development by comparing the traits of germ-free and conventionally reared zebrafish (Bates et al., 2006, 2007; Cheesman et al., 2011). The ability to rear germ-free zebrafish also provides a starting point for visualizing the process of colonization in real time.

Zebrafish, like all vertebrates, are colonized by complex microbial communities, with the most numerically abundant communities being found in their digestive tracts. As in humans, the gut microbiota of zebrafish are dominated by a small number of bacterial phyla, with a high diversity of species and strains within these phyla (Rawls et al., 2006). In teleost fish the dominant phylum is the Proteobacteria (Roeselers et al., 2011), which fortuitously contains the most experimentally tractable bacterial species. We and others have successfully genetically engineered a growing number of zebrafish-derived bacteria to express fluorescent proteins for visualizing their interactions with host tissues and cells. Direct visualization of infection by pathogenic bacteria in zebrafish has upended conventional wisdom about aspects of infectious disease; for example, demonstrating the dynamic nature of mycobacterial granulomas (Davis and Ramakrishnan, 2009). The first description of bacterial dynamics in the zebrafish intestine by Rawls and colleagues used wide-field fluorescence microscopy (Rawls et al., 2007). These authors described different types of bacterial movement in the intestinal habitat, including active swimming and passive movement of large clumps of bacteria. Their studies were, however, limited by the lack of optical sectioning and three-dimensional resolution inherent in widefield imaging. Below we describe imaging technology that overcomes these limitations.

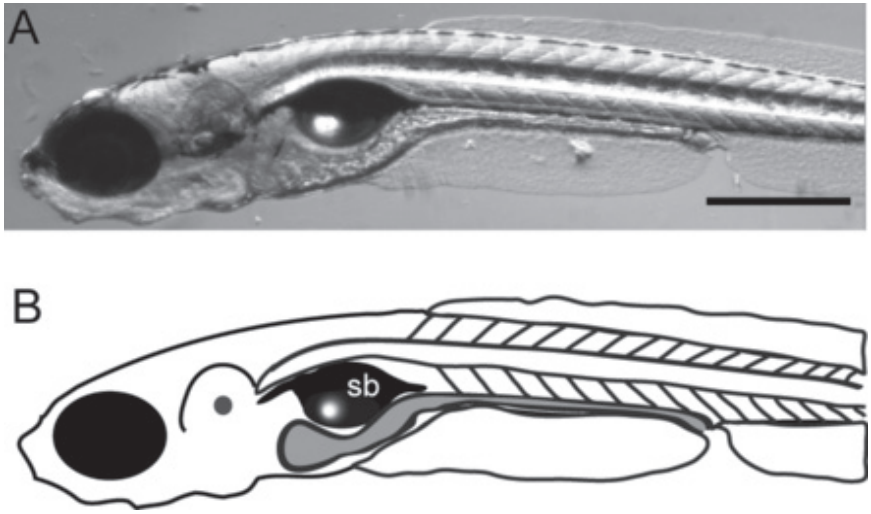


FIGURE A12-1 A zebrafish larva at 7 dpf (days post-fertilization). A brightfield image (A) and a schematic representation (B) illustrating the intestinal tract. sb indicates the swim bladder, and gray fill highlights the intestine. Scale bar: 0.5 mm.

Four-Dimensional Microscopy and Light Sheet Imaging

Three-dimensional fluorescence imaging of live specimens over time, often denoted four-dimensional microscopy, provides molecule-specific, nondestructive information about the spatial structure and temporal dynamics of biological systems. Four-dimensional imaging of host and microbial cells in a developing intestine presents particular technical challenges, as it simultaneously demands fine spatial resolution to visualize individual cells; fine temporal resolution to track individual motions and cellular shape changes; large fields of view spanning, for example, a larval zebrafish intestine that is several hundred microns long; and low levels of photodamage to enable nondestructive imaging over hours or days. There are a variety of three-dimensional imaging techniques, the most widely adopted of which are confocal and multiphoton microscopies, all of which provide access to different ranges of resolution, speed, photodamage, and tissue penetration. A rather new approach, light sheet fluorescence microscopy, offers a route to high-speed, light-efficient imaging of large-scale systems such as developing animals (Keller et al., 2008) and is particularly well suited to visualizing host-associated microbes in vivo, as we show here.

The performance characteristics that differentiate light sheet methods and other three-dimensional fluorescence microscopies are straightforward consequences of their geometries. As illustrated in Figure A12-2A, simple wide-field microscopy is incompatible with optical sectioning of a sample. Light emitted by

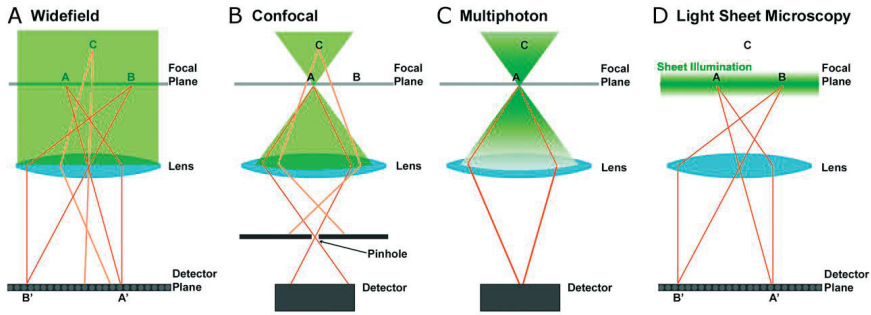


FIGURE A12-2 Schematic illustrations of fluorescence imaging techniques: (A) wide-field, (B) point scanning confocal, (C) multiphoton, and (D) light sheet microscopy. In all diagrams, points A and B lie in the focal plane of the lens while point C lies outside the focal plane. Optical sectioning in light sheet microscopy works by creating a plane of excitation light that is coincident with the focal plane of an imaging objective. Since the sectioning occurs during excitation, the emitted light from the whole field of view can be gathered with a standard camera as a two-dimensional image.

points in the focal plane of an objective lens is mapped onto points on a detector (typically a camera). Points outside this plane, however, are also excited, emitting light that reaches the detector as an out-of-focus blur, reducing contrast and destroying depth information. Point scanning confocal microscopy rectifies the problem at the cost of imaging speed and photon efficiency. As shown in Figure A12-2B, an excitation laser is focused into the specimen, exciting a point in the focal plane as well as points throughout roughly conical volumes around this point. Placement of a pinhole in a conjugate plane blocks most of the light emitted by non-focal points, allowing the in-focus signal to be collected at a detector. At any point in time, information is collected from just one point, though a considerably larger volume is illuminated and subject to photodamage. This point must be scanned through all three dimensions to construct a three-dimensional image. Clever use of multiple focal points and pinholes in spinning disk confocal microscopy speeds up this process but retains the intrinsic illumination inefficiency. Multiphoton microscopy (Figure A12-2C) involves excitation of fluorophores with light of wavelengths two (or more) times that of the normal excitation. The probability of mulitphoton excitation is a nonlinear function of intensity and hence is appreciable only near the focal point, so it is primarily light from this point that is collected at the detector (Figure A12-2C). As with confocal microscopy, the focal point of the laser must be scanned to obtain a rasterized optical section or three-dimensional image.

The distinguishing characteristic of light sheet fluorescence microscopy is that it illuminates the entire focal plane from the side, exciting all points in the plane and capturing all of their emission at once with a camera, as in wide-field

imaging (Figure A12-2D). Points outside of the focal plane are not excited and do not contribute to the image. Imaging an entire plane at once provides a dramatic advantage in speed compared to point scanning methods. Moreover, illuminating only the focal plane is intrinsically photon-efficient: there is (ideally) a one-to-one correspondence between illuminated and imaged points, minimizing photobleaching and photodamage by orders of magnitude compared to confocal and multiphoton methods (Keller et al., 2008; Truong et al., 2011). A disadvantage of light sheet microscopy compared to confocal and multiphoton imaging is resolution, a consequence of sheet illumination. Generating a uniform, thin plane of excitation light is made difficult by the diffraction of light; the thinner one makes the center of the sheet, the smaller the lateral extent of this thin region. Issues of axial resolution and field uniformity can be overcome, however, by a variety of adaptations including multiview reconstruction (Swoger et al., 2007; Keller et al., 2008), structured illumination (Keller et al., 2010; Planchon et al., 2011), non-Gaussian laser beam profiles (Planchon et al., 2011), and two-photon sheet excitation (Truong et al., 2011). In general, light sheet microscopy is amenable to many sorts of modifications and variations. This feature, together with a rather complex history (Santi, 2011), has unfortunately led to a proliferation of names and acronyms – light sheet fluorescence microscopy (LSFM), selective plane illumination microscopy (SPIM), digital scanned light sheet microscopy (DSLM), orthogonal-plane fluorescence optical sectioning (OPFOS), and more; all refer to essentially the same combination of sheet illumination and perpendicular detection. For the purposes of this discussion, we use the term *light sheet microscopy* to refer to this technology collectively.

Materials and Methods

Light Sheet Microscope Design

Light sheet microscopes are to date commercially unavailable, necessitating custom constructions. Various microscope designs and their use in developmental biology have recently been reviewed (Huisken et al., 2004), so we will briefly note the design considerations underlying our home-built light sheet microscope setup (Figure A12-3), which largely mirrors that of Keller et al. (2008). Key components are listed in Table A12-1. Multiple laser lines are used to excite different fluorescent probes such as labels of different microbial populations or host cells. Our setup uses single-photon excitation; though multiphoton light sheet excitation can enhance tissue penetration and resolution (Truong et al., 2011), its implementation requires expensive pulsed laser sources and, moreover, may be nontrivial to integrate with multicolor imaging due to the large spectral bandwidth of short laser pulses (Butko et al., 2011). Rapid switching between colors is provided by an acousto-optic tunable filter. The emerging light is incident on a mirror that rapidly scans the beam. A telecentric scan lens transforms the angular

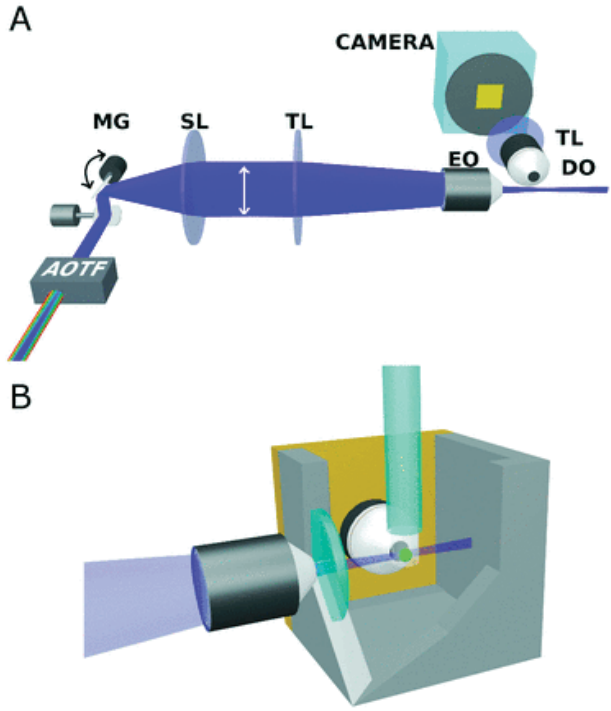


FIGURE A12-3 (A) Schematic illustration of our light sheet microscope setup. Abbreviations: AOTF, acousto-optic tunable filter; MG, mirror galvanometer; SL, scan lens; TL, tube lens; EO, excitation objective lens; DO, detection objective lens. (B) Schematic illustration of our specimen holder, in which the EO axis, DO axis, and capillary tube are all mutually perpendicular. The green sphere denotes the specimen, which is embedded in agarose gel and held by a glass capillary from above.

TABLE A12-1 List of Key Parts for the Author's Light Sheet Microscope

Description	Manufacturer	Part No.
Laser 1: 488/568/647 nm	Melles Griot	35-LTL-835-208
Laser 2: 594 nm	Research Electro-Optics	LHYP-0201
Scan Lens	Sill Optics	S4LFT0061/065
Excitation Objective: 4×0.10 NA	Olympus Corporation	PLN 4×
Detection Objective: 40×1.0 NA	Carl Zeiss, Inc.	441452-9900-000
Mirror Galvonometer	Cambridge Technology	6210H
Stage: x, y, z Translation	Applied Scientific Instrumentation	LS-50 (×3)
Filter Wheel	Applied Scientific Instrumentation	FW-1000
Camera: 5.5 Mpx sCMOS	Cooke Corporation	pco.Edge
Deconvolution Software	Scientific Volume Imaging	Huygens Professional

scan into a translating scan that when imaged through the tube lens and objective lens produces a sheet of illumination in the specimen.

Alternatively, a laser sheet can be formed by focusing a laser beam through a cylindrical lens (Huisken et al., 2004); while this avoids the need for scanning, interference between scattered light from different parts of the sheet can degrade image quality and resolution. Each optical section is imaged through an (orthogonally mounted) high numerical aperture (NA) objective lens and captured by a fast, high-resolution camera. Because of its wide-field method of image collection, the limiting factor for imaging speed in light sheet microscopy is the readout of large-resolution sensors; in order to maximize image acquisition speed, we employ a recently developed CMOS camera that has high readout speeds, resolution, sensitivity, and dynamic range. For some data sets in this paper (Figure A12-5H–M) deconvolution methods were applied to improve contrast and resolution, using commercially available software noted in Table A12-1.

Interestingly, the sample chamber necessary to house a submerged specimen and water immersion lens creates a nontrivial design hurdle. Although not discussed in the literature, we have found that many light sheet microscope builders experience difficulty obtaining satisfactory designs, and a number of different configurations have been explored. The challenge lies in the geometry of the design: one needs to control the position and orientation of the specimen as well as the position of the imaging objective. High NA objectives need an immersion medium with a high index of refraction (relative to air), necessitating that the imaging objective protrude into the sample chamber yet maintain a water-tight seal. Linear motion in one dimension is necessary to adjust the focus of this objective. The sample being imaged, however, requires linear motion in three spatial dimensions and rotation about one axis for positioning and scanning. For the specimen to have linear motion in three dimensions, it must be anchored to suitable translation stages (stages that enable linear motion). Because such devices are typically large and non-submersible, control of the specimen is best achieved from above the surface of the liquid medium, leaving access for the imaging objective restricted to one of the vertical walls or the bottom of the chamber.

The illumination objective, in contrast, is typically of low NA to create a small angle of divergence for the sheet, and has a large working distance. It can therefore be kept outside the sample chamber.

The approach to sample chamber design that we have taken is to replace one side with a sheet of silicone rubber that has been custom-formed to create a water-tight seal around the imaging objective (Figure A12-3B). This was accomplished using commercially available tin-cured silicone molded around a slightly undersized disk to match the objective.

Live fish are drawn into a small capillary containing low-concentration agarose gel. After the gel sets, it is partially extruded out of the capillary such that the specimen is suspended below the end of the glass, with the opposite end attached to the motion-control hardware above the specimen chamber.

Regardless of the details of particular designs, light sheet microscopes possess an intrinsic speed and photon efficiency that are useful for large-scale imaging of organisms, as has been demonstrated by several recent studies (e.g., Keller et al., 2008; Scherz et al., 2008; Truong et al., 2011). These characteristics make light sheet methods valuable for studying the dynamics of bacterial-host interactions. The studies illustrated below exemplify the importance of imaging speed—for example, to discern the motility of individual bacteria—and large fields of view and low levels of photodamage—for example, to image an entire organ for long times.

Zebrafish Husbandry and Bacterial Colonization

All experiments with zebrafish were performed using protocols approved by the University of Oregon Institutional Animal Care and Use Committee and following standard protocols (Westerfield, 2000). The zebrafish line *Tg[BACmpo:gfp]* (Renshawet al., 2006) (referred to as *mpo:gfp* below) was used to visualize neutrophils, and*Tg[nkx2.2a:mEGFP]* (Ng et al., 2005) (referred to as *nkx2.2a:egfp* below) was used to visualize enteroendocrine cells. Larvae were reared at a density of about 1 fish/ml of embryo medium (EM) at 28.5°C. Larvae were not fed during the experiments, instead subsisting off their egg yolks for nourishment. Embryos from natural or in vitro fertilization crosses of wild-type AB/Tu fish were derived germ-free as described (Milligan-Myhre et al., 2011). Germ-free larvae were inoculated with bacteria at 3 dpf, unless otherwise noted. Larvae were removed from flasks with bacteria, rinsed once in sterile EM, and anesthetized in MS222 (tricaine methanesulfonate), then mounted in agar prior to imaging on the light sheet microscope. Anesthetized fish could be maintained for up to 24 h of continuous imaging. Viability of the fish was assessed by the beating of the heart and the occurrence of peristalsis in the intestine. We observed, as has been previously noted (Rombough, 2007), that older larval fish were more susceptible to MS222 toxicity, so we employed doses no higher than 80 mg/l and larvae no older than 6 dpf.

Aeromonas veronii strains used to inoculate germ-free fish were grown overnight (16 h) aerobically at 30°C in Luria broth shaking at 185 rpm prior to inoculation at a target total density of 10^4 CFU/ml in EM. Antibiotics were added to overnight cultures as needed (Kanamycin 100 µg/ml, Gentamycin 10 µg/ml, Rifampicin 100 µg/ml). Strains used were derived from *A. veronii* HM21 (Graf, 1999). The dTomato-expressing strain (HMTn7RFP) is derived from a naturally isolated rifampicin resistant strain, HM21R, into which Gm:tac-dTomato (Singer et al., 2010) was inserted by Tn7-mediated transposition. The GFP-expressing strain (HMTn7GFP) is derived from HM21 into which Kan:GFP was inserted by Tn7-mediated transposition. The doubling times during logarithmic growth at 30°C in Luria broth of the RFP- and GFP-expressing strains were determined to be 38.8 min and 39.1 min respectively.

Results

Visualizing Bacterial Colonization Dynamics in the Zebrafish Intestine

To begin to investigate bacterial colonization dynamics in the zebrafish intestine using light sheet microscopy, we performed experiments in which we inoculated initially germ-free larvae with fluorescently labeled *Aeromonas veronii*, a common bacterial resident of the zebrafish intestine, and imaged them over an extended time period. We incubated the fish with the bacteria for 6 h prior to imaging, which was the shortest inoculation time that ensured consistent colonization across all of the fish. *A. veronii* colonization of the germ-free intestine could occur by many possible mechanisms. In one extreme model, a single bacterial cell could colonize the organ and clonally expand. A second model, at the opposite extreme, would involve continuous influx and efflux of bacterial cells, with no growth occurring within the intestine itself. A third model, commonly evoked in the human gastrointestinal literature, posits that microbial growth in the intestine resembles growth in a chemostat (Macfarlane et al., 1998), with bacterial cell proliferation being driven by influx of nutrients into the system and balanced by efflux of contents out of the system. To discriminate between these and other possible colonization dynamic models, we simultaneously exposed germ-free zebrafish larvae to two isogenic strains of wild-type *A. veronii* with similar in vitro growth rates, one expressing green fluorescent protein (HMTn7GFP) and the other dTomato (HMTn7RFP). The larvae were incubated with the bacterial strains for 6 h, after which three-dimensional images spanning the entire intestine were collected every 20 min for 18 h. Each three-dimensional data set, roughly 3 GB in size, was obtained in approximately 60 s per color channel.

In all fish examined, we observed both fluorescently marked *A. veronii* strains within the zebrafish intestine (Figure A12-4), ruling out the first model of colonization by a single cell. The second model of continuous influx of bacteria from the environment without population growth in the intestine was also not supported by our data. In media in which the fish were inoculated, we observed a well-mixed distribution of red and green cells in suspension and some clumps of cells that contained both red and green members (Figure A12-4A). The pattern of multicolored clumps of cells is expected because the cells are genetically identical except for the fluorescent proteins they produce and thus should have no bias for homotypic aggregation. In contrast, in the fish intestines, we observed single-colored aggregates of cells, which we interpret to occur through clonal expansion of single cells (Figure A12-4B–J and Appendix Figure 1). We speculate that some of the larger clumps consist of clonally expanded cells adhering in biofilms to flocculent material such as host mucus or dead cells shed from the intestinal epithelium (Sonnenburg et al., 2004). Interestingly, some of these clumps persist for a long time, others transit through the intestine rapidly, while still others disintegrate or become dislodged from formerly stable locations, indicating that the fluid dynamics within this organ are extremely complex. The heterogeneous

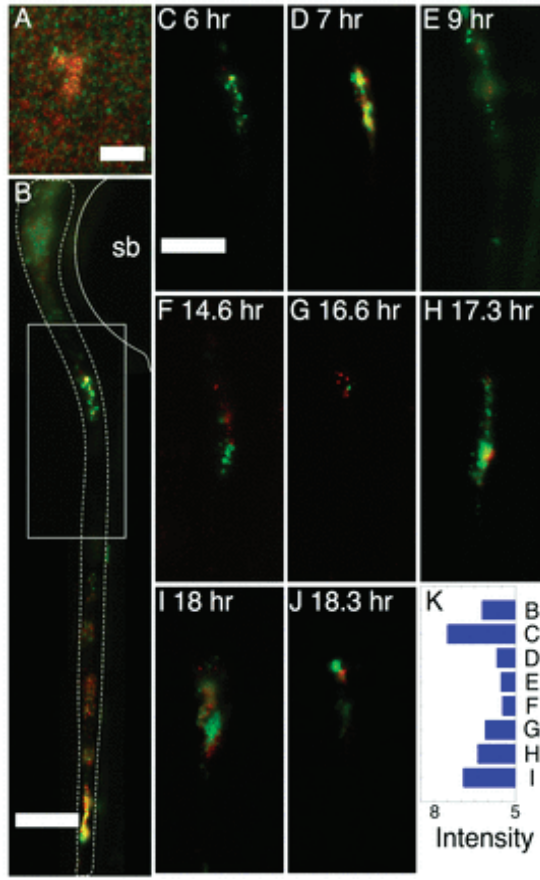


FIGURE A12-4 Colonization of a larval zebrafish gut by GFP-expressing and dTomato-expressing *Aeromonas veronii* bacteria. (A) Wild type GFP-expressing and dTomato-expressing *A. veronii* coinoculated in culture. Panels B–J show representative two-dimensional optical sections from three-dimensional data sets. (B) The entire gut 6 h after inoculation. The gut is approximately outlined with a dashed line, and the swim bladder (sb) is outlined with a solid line. (C–J) The distribution of the two microbial populations in the region corresponding to the box in (B) at various times after inoculation, with $t = 6$ h corresponding to panel B. The contrast of each panel was independently adjusted so that the bacterial populations are clearly visible despite fluctuations in overall bacterial abundance. In each panel, both red and green intensities were rescaled by the same amount. (K) The intensity rescaling factor in each of panels (B–J)—that is, the sum of the red and green intensities each normalized by the typical brightness of a typical bacterium, providing a measure of the total bacterial abundance at each time. Scale bars: (A) 10 μm; (B–J) 100 μm.

distribution of clumps of bacterial cells we observed was also inconsistent with the model of a well-mixed chemostat, although more complex models of unmixed chemostats could apply (Smith and Waltman, 1995).

The light sheet microscope allows us to monitor bacterial population abundance and dynamics in real time in the zebrafish intestine. Some of the fluorescent signal observed emanated from the fish tissue itself, as is apparent when we image germ-free animals or animals colonized with low numbers of bacteria. Certain host cells of unknown identity emit very bright autofluorescent signals, but these are easily distinguished from colonizing bacteria because of their large size, their position outside of the gut lumen, and the fact that their autofluorescence is detected in both the red and green channels. The intensities of red and green fluorescence within the intestinal lumen, above a threshold intensity applied to remove low-level fluorescent background, correlates fairly well with the relative abundance of red- and green-labeled cells, as measured by dilution plating (Appendix Figure 2), validating our use of fluorescence intensity as a surrogate for bacterial population size and location. In contrast to a chemostat in which the entire volume is occupied by microbial cells utilizing available resources to support their growth, our imaging of the zebrafish intestine revealed bacterial colonization to be spatially highly heterogeneous, with punctate colonies and nonuniform distribution of colonies along the length of the gut (Figure A12-4B–J and Appendix Figure 1). Additionally, the spatial distribution of bacteria was highly dynamic, with large changes in overall abundance as well as localization occurring during the imaging period (Figure A12-4C–J and Appendix Figure 1). Our observations support two possible models of the intestinal environment, which are not mutually exclusive: first that resources for bacterial growth are distributed nonuniformly and highly dynamically in the zebrafish intestine; and second that other determinants, such as peristaltic forces or immune cell surveillance, play significant roles in bacterial population distributions. It is clear that the simple model of a chemostat with evenly distributed bacterial cells does not accurately describe the real behavior of bacterial populations in the zebrafish intestine.

The high speed of light sheet fluorescence imaging also enables observations of the motility of individual bacterial cells. Movie S1 (http://www.biolbull.org/content/supplemental) shows a two-dimensional section of a larval zebrafish intestine with dTomato–labeled *A. veronii* bacteria (HMTn7RFP) (as in Figure A12-4). Several distinct motility patterns are evident, including bacteria in stationary clumps, bacteria passively moving with the fluid flow, and bacteria that are actively swimming. While motility is relatively straightforward to study in vitro, its properties in vivo are largely unexplored and may not reflect those of cultured bacteria due both to the complex physical environment of the intestine and to signaling among intestinal constituents. Future analyses of bacterial cell dynamics in the zebrafish intestine will investigate how individual cells explore their local chemical and spatial environments, what proportion of the community are permanent residents as opposed to transients, and whether

motility is a prerequisite for permanent residency. *Aeromonas* species are of particular interest, as they have been shown to exhibit chemotaxis toward fish mucus (van der Marel et al., 2008) and to engage in different modes of motility mediated by different types of flagella (Altarriba et al., 2003).

Visualizing Host Cell Dynamics in the Intestine

An important question in the field of symbiosis is how the process of colonization and the presence of microbial associates change the development and function of the host. We have characterized numerous zebrafish traits that are affected by the microbiota. For example, germ-free zebrafish intestines have a paucity of secretory cells, including enteroendocrine cells, which secrete hormones, and neutrophils, which are phagocytic cells of the innate immune system (Bates et al., 2006, 2007). In addition, the physiology of the intestine is altered in the absence of the microbiota, with more rapid peristaltic contractions (Bates et al., 2006). The high speed of light sheet microscopy provides an opportunity to explore host–microbe interactions at much higher resolution and to ask how the microbiota affect the dynamics of individual zebrafish cells, and reciprocally, how host cells affect the microbiota.

To demonstrate the feasibility of this approach, we used the transgenic zebrafish line *nkx2.2a:egfp*, which drives expression of a membrane-bound GFP in enteroendocrine cells in the intestinal epithelium (Ng et al., 2005). Six dpf larvae were imaged for several hours (Figure A12-5A–G and Movie S2 [http://www.biolbull.org/content/supplemental]), demonstrating the ability of future in-depth studies to follow the genesis of these cells in the presence and absence of microbiota. Imaging also shows the dynamics of these cells and emphasizes the extent to which they experience mechanical forces during the propulsion of peristaltic waves along the length of the intestine; distortions of cell shape are clearly visible during such a contraction (Figure A12-5A–F and Movie S2 [http://www.biolbull.org/content/supplemental]). Typical image acquisition timescales, roughly tens of milliseconds for two-dimensional slices and a few seconds for three-dimensional images, are faster than the periodicity of peristalsis, allowing imaging without motion-induced blurring. Since there are fewer of these cells in the germ-free intestine, and since they produce hormones that regulate peristalsis, we expect to observe interesting changes in the cellular and organ-wide contractile behaviors during colonization.

In addition to the cells of the intestinal epithelial, gut microbes interact with immune cells that actively survey this tissue. Neutrophils are important first responders to intestinal infections, and we have shown that these cells are also recruited to the intestine upon colonization by the microbiota (Bates et al., 2007). Being highly motile and responsive to chemical cues, neutrophils are a model cell for chemotaxis studies, and their in vivo behavior has been characterized extensively in zebrafish by using confocal microscopy (e.g., Feng et al., 2010;

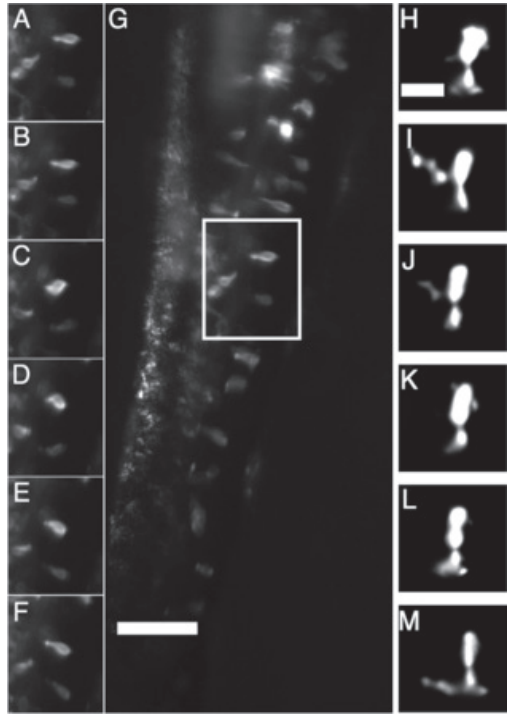


FIGURE A12-5 (A–G) Fluorescently labeled enteroendocrine cells (transgenic line *nkx2.2a:egfp*) in a larval zebrafish gut. Scale bar: 40 µm. (A–F) A time series of the cells within the box in (G). The interval between each panel in (A–F) is 2.8 s. The deformation and movement of cells as the gut undergoes peristalsis during the imaging period is evident. The movie from which these images were taken is provided as Movie S2 (http://www.biolbull.org/content/supplemental). (H–M) A single fluorescently labeled neutrophil (*mpo:gfp*) within the intestinal tissue of a larval zebrafish. Each panel is a two-dimensional optical section from a three-dimensional data set, each separated in time by 2 min. Dynamic rearrangments of the neutrophil's filopodia are evident. Scale bar: 10 µm.

Yoo et al., 2010; Colucci-Guyon et al., 2011). Visualizing their dynamics in internal tissues such as the intestine, however, poses technical challenges that we were able to overcome using light sheet microscopy. We visualized GFP-labeled neutrophils in the *mpo:gfp* transgenic line (Renshaw et al., 2006) at various ages from 4–8 dpf in time-lapse scans of the whole intestine for several hours. We obtained scans spanning 60-µm-thick intestines with optical slices separated by 1 µm in as little as 12 s per three-dimensional data set. The speed and range of imaging allowed us to identify neutrophils undergoing active processes at different depths throughout the intestine, and we were able to visualize

individual cell shape rearrangements such as the extension and retraction of filopodia (Figure A12-5H–M). Simultaneously imaging fluorescently labeled bacteria and neutrophils with light sheet microscopy will enable visualization of neutrophil recruitment to the intestine upon bacterial colonization and will address whether they exhibit different behaviors, such as increased filopodia activity, in the presence of commensal microbes. By examining correlations between local heterogeneities in the microbiota and the positions of neutrophils, we will also be able to explore the possibility that neutrophils influence bacterial population growth dynamics.

Analyzing Cell Population Dynamics in vivo

Live imaging of intestinal bacteria with high spatial and temporal resolution over hours or days produces a vast quantity of image data. The two-color imaging of gut microbes illustrated in Figure A12-4, for example, yielded over 300 GB of images from a single larval zebrafish; this number could easily be increased with finer temporal sampling or a greater number of labeled populations. Like other recently developed biological techniques, such as high-throughput sequencing and proteomic methods, light sheet imaging shares the challenge of extracting comprehensible insights from high volumes of data. It is likely that a variety of approaches to this task will be developed as data sets become available. We suggest here a few promising perspectives.

The raw data output by light sheet imaging of fluorescently labeled intestinal microbes denotes the density of bacteria over time and three-dimensional space. A simple distillation of this information is provided by collapsing this density onto the axis provided by the gut itself, integrating the intensity of image slices perpendicular to the gut center-line at each point along the line and yielding a one-dimensional measure of bacterial population along the anterior-posterior coordinate. This one-dimensional projection can be performed for each measurement time, as illustrated in Figure A12-6 for the data set from which Figure A12-4 was extracted. From this and other observations, it is evident that bacterial populations are not homogeneously distributed along the gut. One can correlate the existence of preferential locations for bacterial growth with anatomical position, noting, for example, high population density in the most anterior and posterior regions of the intestine. Moreover, it is evident that the dynamics of growth are complex; the spatially nonuniform distribution present initially is not simply scaled or magnified as time progresses, but rather varies nonmonotonically over the course of hours.

While projections are easily comprehensible, they necessarily neglect three-dimensional structure and dynamics. Considering interacting bacterial species, it will likely be fruitful to investigate spatial correlation functions that involve different microbial groups. Colocalization, antilocalization, and spatially offset population densities can reveal the functional relationships between species that

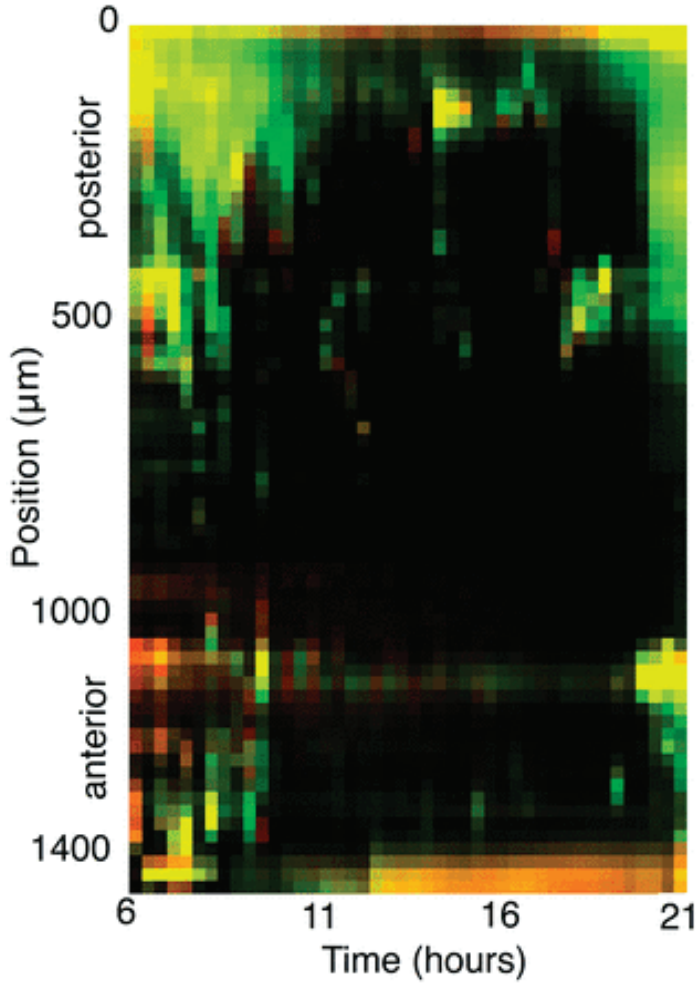


FIGURE A12-6 Population distributions as a function of time for the two coinoculated bacterial populations illustrated in Figure A12-4. Each column indicates fluorescence intensity integrated over the dimensions perpendicular to the gut axis, providing a one-dimensional measure of bacterial density along the gut, the temporal dynamics of which can be visualized in a two-dimensional plot.

cooperate, compete, or interact via exchange of metabolites. Moreover, correlation functions can provide a measure of structure that maps higher-dimensional organization onto visualizable lower-dimensional spaces.

To illustrate this, we first consider the schematic, computer-generated image in Figure A12-7A, in which green spots are distributed randomly in two

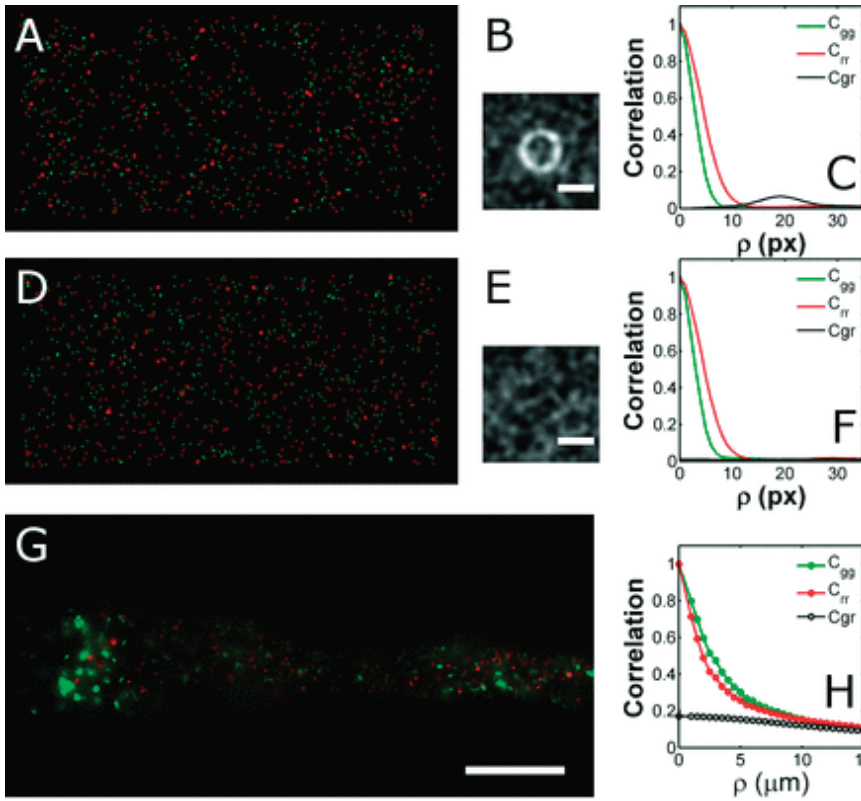


FIGURE A12-7 Image auto- and cross-correlations. (A) A simulated two-dimensional image with randomly positioned green spots and 1.5× larger red spots each placed about 20 pixels from a green spot, randomly oriented. (B) The two-dimensional cross-correlation, C_{gr}, of the green and red channels of (A); a ring at a radius of 20 pixels reveals the constructed correlation. (C) The radial dependence of the auto- and cross-correlation functions; as in (B), C_{gr} shows a peak at a spatial offset of 20 pixels. (D) A simulated two-dimensional image in which both the green and red spots are positioned randomly. (E, F) The correlation functions corresponding to the image in (D). (G) A single optical slice from a three-dimensional data set depicting *Aeromonas veronii* bacteria in a larval zebrafish, as in Figure A12-4, at a single time point. Scale bar: 50 μm. (H) Auto- and cross-correlations of the bacterial intensity distributions calculated from the three-dimensional data set.

dimensions, and slightly larger red spots are positioned such that each is some particular distance (here, 20 ± 1 pixels) from a green spot, in a random direction. By construction, the correlation between red and green is not perfectly sharp. For each green spot, there is a red spot placed 20 ± 1 pixel away. This red spot may be less than or greater than 20 pixels distant from other green spots. This correlation

between the red and green structures in Figure A12-7A is not apparent by eye. Denoting the intensity of pixels in the green and red channels I_g and I_r, respectively, the normalized cross-correlation is given by Gonzalez and Woods (1992):

$$C_{gr}(\vec{\rho}) = \frac{\sum\left(I_g(\vec{r}) - \langle I_g \rangle\right)\left(I_r(\vec{r}-\vec{\rho}) - \langle I_r \rangle\right)}{\left[\sum\left(I_g(\vec{r}) - \langle I_g \rangle\right)^2 \sum\left(I_r(\vec{r}-\vec{\rho}) - \langle I_r \rangle\right)^2\right]^{0.5}},$$

where $\vec{r}$ indicates position $\vec{\rho}$, is the spatial offset, the angle brackets indicate the average intensity, and the sums run over all pixels in an image. If the two intensity distributions are spatially offset by some well-defined amount, the numerator in the above expression will be large when $\vec{\rho}$ equals that amount, and C_{gr} will have a peak at that offset. This is evident in Figure A12-7B, which shows $C_{gr}(\vec{\rho})$ for the image in Figure A12-7A, yielding a ring at a displacement of 20 pixels from the origin. In Figure A12-7C we show C(ρ), the correlation as a function of radial displacement $\rho = |\vec{\rho}|$, that is, averaged over angle. Again, a 20-pixel offset is apparent.

Particular coordinate systems can help characterize particular spatial distributions of bacterial populations. For example, by determining the gut center line (either manually or by computational analysis of the bounded space), one can define a cylinder-like coordinate system, the radial component of which distinguishes luminal and mucosal positions in the gut. Considering cross-correlations in which $\vec{\rho}$ is the radial coordinate, situations in which one population is predominantly mucosal and the other is predominantly luminal would give a peak in C_{gr} at some radial displacement.

The auto-correlation of each intensity function (i.e., its correlation with itself) also provides useful information. $C_{gg}(\vec{\rho})$ and $C_{rr}(\vec{\rho})$ each trivially have a peak at $\vec{\rho} = 0$, as any function is perfectly correlated with itself; these autocorrelations decay with a characteristic scale equal to the typical object size in the intensity images. We see this decay in Figure A12-7C; as expected, the red autocorrelation curve has a larger decay length, as it corresponds to the larger particle size.

In contrast, Figure A12-7D–F shows simulated images and the resulting calculated correlations for images in which the position of both the red and green spots were completely random. For such distributions, the cross-correlation has a low, featureless value.

We provide an example of correlation function analysis of real three-dimensional bacterial images in Figure A12-7G, H. The source data are from the same setup as illustrated in Figure A12-4: two-color images of *A. veronii* HMTn7GFP and HMTn7RFP in the intestine. The intensity correlations shown in Figure A12-7H are calculated from a $166 \times 333 \times 64$ μm^3 volume at a single time point, one optical section of which is depicted in Figure A12-7G. The autocorrelations are similar in each color channel with a characteristic length-scale of a few micrometers. The cross-correlation is small and featureless, as expected, as these two populations are biologically identical and should be randomly situated.

More generally, we can expect different sorts of correlations from more complex sets of species, and a key goal of this article is to encourage progress along these lines. A lack of cross-correlation near $\vec{\rho} = 0$, for example, can indicate competing species that are unlikely to occupy the same spatial niche. Strong correlation at some non-zero distance can reveal, for example, cooperating species whose interactions are mediated by the exchange of metabolic products whose dispersal spans some characteristic range. Of course, using cross-correlations to compare groups requires assessment of the precision of multicolor image registry, which can be compromised by both optical aberrations and by motions of the specimen. Control images can be provided, for example, by larvae that have ingested multicolor fluorescent beads, the images from which should be perfectly correlated. The use of autocorrelations to determine typical colony sizes requires no such multicolor registry, and in itself can reveal features of importance such as the growth of typical colony sizes with time.

It should also be possible to use characterizations of bacterial motility, enabled by the high speed of light sheet microscopy, to examine spatial correlations between differently motile groups. Using either temporal correlations between images or direct tracking of moving microbes, bacterial populations with the same fluorescent reporters can be divided into categories of different motilities, and spatial correlations between these subpopulations could be examined using the same approach described above. We expect that such studies may illuminate connections between microbial behaviors and gut colonization.

Discussion

Advances in genomics, proteomics, and metabolomics have generated an explosion of information about the bacterial associates of humans (Kuczynski et al., 2012). This flood of data is enabling new approaches to modeling the metabolic activities and ecological interactions of host-associated microbial communities (e.g., Borenstein et al., 2008; Greenblum et al., 2012), which will enrich the entire field of symbiosis. These approaches, however, are based on samples that are homogenized mixtures of microbes at a single point in time, and they fail to take into account the complex spatial dynamics of microbial populations (Green et al., 2008).

The study of spatial population dynamics has a long history in ecology (Huffaker, 1958), but the application of these ideas to naturally occurring microbial communities has been hindered by the technical challenges of observing population dynamics on the spatial and temporal scales of microbes. Here we present the methodology of light sheet microscopy and its application to visualizing the dynamics of zebrafish intestinal microbiota and associated host cells. The spatial resolution and nondestructive sampling of light sheet imaging will provide researchers with new opportunities to apply ecological theories and concepts to the study of host-associated microbial communities.

Four-dimensional imaging will afford the opportunity to study the colonization and succession of gut microbial communities (Fierer et al., 2010), determining where in the intestine microorganisms first establish and how this influences the ability of successive colonizers to invade. The mechanisms that drive and control the population dynamics of bacterial species (e.g., dispersal, trophic interactions, environmental pressures) can be inferred by examining patterns of the synchrony of populations across space and time (Bjornstad et al., 1999). Further questions that can be illuminated with this technology include whether succession is driven more by expansion from initial colonizers or from continued migration, how communities respond to disturbances (secondary succession), and where these dynamics occur with relation to host cells and landmarks. Light sheet imaging is particularly useful for visualizing the dynamics of symbiosis in live animals because of the speed of image acquisition and the low photodamage. Light sheet imaging could be employed to image microbial colonization of the internal organs of transparent animals, including many marine organisms and insect larvae, and to record bacterial associations of surface tissues of any animal or plant. We speculate that it will be especially powerful when applied to model symbiosis systems of bacterial associations with the squid light organ, the leech crop, the fruit fly and nematode digestive tracts, and fungal and bacterial interactions with plant roots and rodent skin.

Here we have presented results that demonstrate the feasibility of using light sheet imaging to characterize the bacterial colonization of the zebrafish intestine and the host's response to this colonization at a resolution that informs us about the behaviors of individual cells and the dynamics of entire populations of cells. We discuss the challenges of making sense of the vast amounts of image data generated by this four-dimensional microscopy and present approaches to analyzing and interpreting correlations between different simultaneously imaged cell populations. An important future goal will be to integrate other types of "omics" data with *in vivo* imaging of host-associated microbial communities. Although this will be a formidable challenge, we believe that taking into account spatial organization and temporal dynamics of complex microbial communities resident on animals will be essential for understanding these communities' metabolic activities and the causes and consequences of those activities. In comparison to all other bacterial communities sampled across the globe, the communities found within the vertebrate intestine have a unique phylogenetic composition (Ley et al., 2008). We suspect that vertebrate intestinal microbiota will also possess unique spatial organizations and temporal dynamics that cannot be approximated by models such as the chemostat, which fails to take into account the complex reciprocal interactions between the microbes and their host environment. Direct visualization of these microbe–host interactions is the first important step toward understanding the growth, development, and function of these microbial communities that are integral to animal biology.

Acknowledgments

We thank Jennifer Hampton for contributions to this work, Richard Suhr and Richard Holton for experimental assistance, and Brendan Bohannan for insightful discussions. This work was funded by NSF grant 0922951 to R. P. and NIH grant 1R01GM095385 to K. G; M. T. was supported on NSF GK12 grant 0742540; W. Z. S was supported on NSF IGERT grant DGE 9972830; and J. V. T. was supported on NIH NRSA 1F32DK089716 and American Cancer Society Fellowship 120188-PF-11-272-01-MPC. NIH grant HD22486 provided support for the Oregon Zebrafish Facility.

References

Altarriba, M., S. Merino, R. Gavin, R. Canals, A. Rabaan, J. G. Shaw, and J. M. Tomas. 2003. A polar flagella operon (flg) of *Aeromonas hydrophila* contains genes required for lateral flagella expression. *Microb. Pathog.* 34: 249–259.

Bates, J. M., E. Mittge, J. Kuhlman, K. N. Baden, S. E. Cheesman, and K. Guillemin. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Dev. Biol.* 297: 374– 386.

Bates, J. M., J. Akerlund, E. Mittge, and K. Guillemin. 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* 2: 371–382.

Bjornstad, O. N., R. A. Ims, and X. Lambin. 1999. Spatial population dynamics: analyzing patterns and processes of population synchrony. *Trends Ecol. Evol.* 14: 427–432.

Boettcher, K. J., E. G. Ruby, and M. J. McFall-Ngai. 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J. Comp. Physiol. A* 179: 65–73.

Borenstein, E., M. Kupiec, M. W. Feldman, and E. Ruppin. 2008. Large-scale reconstruction and phylogenetic analysis of metabolic environments. *Proc. Natl. Acad. Sci. USA* 105: 14482–14487.

Butko, M. T., M. Drobizhev, N. S. Makarov, A. Rebane, B. C. Brinkman, and J. G. Gleeson. 2011. Simultaneous multiple-excitation multiphoton microscopy yields increased imaging sensitivity and specificity. *BMC Biotechnol.* 11: 20.

Cheesman, S. E., and K. Guillemin. 2007. We know you are in there: conversing with the indigenous gut microbiota. *Res. Microbiol.* 158: 2–9.

Cheesman, S. E., J. T. Neal, E. Mittge, B. M. Seredick, and K. Guillemin. 2011. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. *Proc. Natl. Acad. Sci. USA* 108 Suppl 1: 4570– 4577.

Colucci-Guyon, E., J. Y. Tinevez, S. A. Renshaw, and P. Herbomel. 2011. Strategies of professional phagocytes in vivo: unlike macrophages, neutrophils engulf only surface-associated microbes. *J. Cell Sci.* 124: 3053–3059.

Davis, J. M., and L. Ramakrishnan. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136: 37–49.

Dethlefsen, L., M. McFall-Ngai, and D. A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449: 811–818.

Feng, Y., C. Santoriello, M. Mione, A. Hurlstone, and P. Martin. 2010. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. *PLoS Biol.* 8: e1000562.

Fierer, N., D. Nemergut, R. Knight, and J. M. Craine. 2010. Changes through time: integrating microorganisms into the study of succession. *Res. Microbiol.* 161: 635–642.

Gonzalez, R. C., and R. E. Woods. 1992. *Digital Image Processing*. Addison-Wesley, Reading, MA.
Graf, J. 1999. Symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech: a novel model for digestive tract associations. *Infect. Immun.* 67: 1–7.
Green, J. L., B. J. Bohannan, and R. J. Whitaker. 2008. Microbial biogeography: from taxonomy to traits. *Science* 320: 1039–1043.
Greenblum, S., P. J. Turnbaugh, and E. Borenstein. 2012. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. *Proc. Natl. Acad. Sci. USA* 109: 594–599.
Grunwald, D. J., and J. S. Eisen. 2002. Headwaters of the zebrafish—emergence of a new model vertebrate. *Nat. Rev. Genet.* 3: 717–724.
Huffaker, C. 1958. Experimental studies of predation: dispersion factors and predator-prey oscillations. *Hilgardia* 21: 795–835.
Huisken, J., J. Swoger, F. Del Bene, J. Wittbrodt, and E. H. Stelzer. 2004. Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305: 1007–1009.
Kanther, M., and J. F. Rawls. 2010. Host-microbe interactions in the developing zebrafish. *Curr. Opin. Immunol.* 22: 10–19.
Keller, P. J., A. D. Schmidt, J. Wittbrodt, and E. H. Stelzer. 2008. Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322: 1065–1069.
Keller, P. J., A. D. Schmidt, A. Santella, K. Khairy, Z. Bao, J. Wittbrodt, and E. H. Stelzer. 2010. Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. *Nat. Methods* 7: 637–642.
Kuczynski, J., C. L. Lauber, W. A. Walters, L. W. Parfrey, J. C. Clemente, D. Gevers, and R. Knight. 2012. Experimental and analytical tools for studying the human microbiome. *Nat. Rev. Genet.* 13: 47–58.
Ley, R. E., C. A. Lozupone, M. Hamady, R. Knight, and J. I. Gordon. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6: 776–788.
Macfarlane, G. T., S. Macfarlane, and G. R. Gibson. 1998. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microb. Ecol.* 35: 180–187.
Milligan-Myhre, K., J. R. Charette, R. T. Phennicie, W. Z. Stephens, J. F. Rawls, K. Guillemin, and C. H. Kim. 2011. Study of hostmicrobe interactions in zebrafish. *Methods Cell Biol.* 105: 87–116.
Ng, A. N., T. A. de Jong-Curtain, D. J. Mawdsley, S. J. White, J. Shin, B. Appel, P. D. Dong, D. Y. Stainier, and J. K. Heath. 2005. Formation of the digestive system in zebrafish. III. Intestinal epithelium morphogenesis. *Dev. Biol.* 286: 114–135.
Nyholm, S. V., and M. J. McFall-Ngai. 2004. The winnowing: establishing the squid-vibrio symbiosis. *Nat. Rev. Microbiol.* 2: 632– 642.
Nyholm, S. V., E. V. Stabb, E. G. Ruby, and M. J. McFall-Ngai. 2000. Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. *Proc. Natl. Acad. Sci. USA* 97: 10231– 10235.
Nyholm, S. V., B. Deplancke, H. R. Gaskins, M. A. Apicella, and M. J. McFall-Ngai. 2002. Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. *Appl. Environ. Microbiol.* 68: 5113–5122.
Planchon, T. A., L. Gao, D. E. Milkie, M. W. Davidson, J. A. Galbraith, C. G. Galbraith, and E. Betzig. 2011. Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination. *Nat. Methods* 8: 417–423.
Rawls, J. F., M. A. Mahowald, R. E. Ley, and J. I. Gordon. 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* 127: 423–433.
Rawls, J. F., M. A. Mahowald, A. L. Goodman, C. M. Trent, and J. I. Gordon. 2007. *In vivo* imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. *Proc. Natl. Acad. Sci. USA* 104: 7622–7627.

Renshaw, S. A., C. A. Loynes, D. M. Trushell, S. Elworthy, P. W. Ingham, and M. K. Whyte. 2006. A transgenic zebrafish model of neutrophilic inflammation. *Blood* 108: 3976–3978.

Roeselers, G., E. K. Mittge, W. Z. Stephens, D. M. Parichy, C. M. Cavanaugh, K. Guillemin, and J. F. Rawls. 2011. Evidence for a core gut microbiota in the zebrafish. *ISME J.* 5: 1595–1608.

Rombough, P. J. 2007. Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (*Danio rerio*) larvae. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 148: 463–469.

Santi, P. A. 2011. Light sheet fluorescence microscopy: a review. *J. Histochem. Cytochem.* 59: 129–138.

Scherz, P. J., J. Huisken, P. Sahai-Hernandez, and D. Y. Stainier. 2008. High-speed imaging of developing heart valves reveals interplay of morphogenesis and function. *Development* 135: 1179–1187.

Singer, J. T., R. T. Phennicie, M. J. Sullivan, L. A. Porter, V. J. Shaffer, and C. H. Kim. 2010. Broad-host-range plasmids for red fluorescent protein labeling of gram-negative bacteria for use in the zebrafish model system. *Appl. Environ. Microbiol.* 76: 3467–3474.

Smith, H. L., and P. Waltman. 1995. *The Theory of the Chemostat: Dynamics of Microbial Competition*. Cambridge University Press, Cambridge.

Sonnenburg, J. L., L. T. Angenent, and J. I. Gordon. 2004. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? *Nat. Immunol.* 5: 569–573.

Spor, A., O. Koren, and R. Ley. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9: 279–290.

Swoger, J., P. Verveer, K. Greger, J. Huisken, and E. H. Stelzer. 2007. Multi-view image fusion improves resolution in three-dimensional microscopy. *Opt. Express* 15: 8029–8042.

Truong, T. V., W. Supatto, D. S. Koos, J. M. Choi, and S. E. Fraser. 2011. Deep and fast live imaging with two-photon scanned lightsheet microscopy. *Nat. Methods* 8: 757–760.

van der Marel, M., V. Schroers, H. Neuhaus, and D. Steinhagen. 2008. Chemotaxis towards, adhesion to, and growth in carp gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio* L. *J. Fish Dis.* 31: 321–330.

Westerfield, M. 2000. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*)*. University of Oregon Press, Eugene.

Yoo, S. K., Q. Deng, P. J. Cavnar, Y. I. Wu, K. M. Hahn, and A. Huttenlocher. 2010. Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. *Dev. Cell* 18: 226–236.

A13

CLINICAL APPLICATION OF FECAL MICROBIOTA TRANSPLANTATION IN *CLOSTRIDIUM DIFFICILE* INFECTION AND BEYOND

Josbert J. ***Keller***[57,*] *and Els van Nood* [58]

Abstract

Fecal microbiota transplantation (FMT or donor feces infusion) is effective against recurrent and antibiotic-refractory *Clostridium difficile* infections (CDI), with a success rate of approximately 90 percent. Restoration of bowel flora and thereby restoration of colonisation resistance is thought to be the mechanism responsible for cure. With the increasing interest for the role of the microbiome in many different disorders, there is also an increasing interest for the application of FMT for other diseases.

Introduction

In 1958, Eiseman first described four patients with severe antibiotic-associated colitis that were cured by enemas with donor feces (Eiseman et al., 1958) Since then, it was reported as an effective treatment against antibiotic-refractory *C. difficile* infection in numerous publications including more than 500 patients (Gough et al., 2011; van Nood et al., 2009). After publication of a randomised controlled trial, showing that donor feces infusion (FMT) is superior compared to vancomycin for recurrent *Clostridium difficile* infection (van Nood et al., 2013), this unconventional treatment approach was also mentioned in guidelines for the treatment of recurrent *Clostridium difficile* infection (Surawicz et al., 2013).

However, wide application of FMT for patients that might benefit from this treatment is still hampered by a lack of experience in many centers, regulatory authorities struggling with the classification of this unstandardized and theoreticaly harmful medical application of stool, and by fear for infectious complications in (severely ill) patients. Interestingly, besides *Clostridium difficile* infection, there are more conditions for which FMT may be beneficial. In

[57] Department of Gastroenterology, Haga Teaching Hospital, The Hague, the Netherlands.
[58] Department of Internal Medicine, Academic Medical Center, University of Amsterdam, the Netherlands.

* Corresponding author email: keller@hagaziekenhuis.nl

particular, disorders that are associated with a disturbed bowel microbiota may be influenced by targetting the microbiome with FMT or powerful probiotics.

The use of donor feces infusion for *Clostridium difficile* infection and potential further applications of this approach will be discussed.

Clostridium difficile Infection

Clostridium difficile infections (CDI) are the most important cause of hospital-acquired infectious diarrhea in the Western world. There is an increasing incidence and severity in the last decade, which seems partly explained by the rise of more virulent strains of *Clostridium difficile* (Kuijper et al., 2006; Loo et al., 2005). *Clostridium difficile* is a spore-forming anaerobic gram-positive rod. Virulent *Clostridium difficile* strains produce the toxins A and B that are able to disrupt colonic epithelial cells, and initiate a local immune response thereby causing diarrhea. The spores formed by Clostridium difficile are able to survive outside an anaerobic environment, and are resistant to many disinfectants (such as hand alcohol) and antibiotics (Hall, 1935; Macleod-Glover and Sadowski, 2010; Viau and Peccia, 2009).

Clostridium difficile infections typically occur in (recently) hospitalised patients that are exposed to antibiotics. This reflects two important observations: (1) contraction of *Clostridium difficile* is strongly associated with hospitalization with an estimated (asymptomatic) carrier rate in the general population of about 3 percent, that gradually increases with (prolonged) hospitalization (Barbut and Petit, 2001); and (2) the outgrowth of *Clostridium difficile* strains to pathogenic levels requires the loss of colonization resistance of the healthy microbiome, which is importantly disturbed by antibiotic use (Dethlefsen et al., 2008; Walters et al., 1983). However, other conditions such as chemotherapy, GI surgery, or PPI use also predispose to *Clostridium difficile* infection, and community acquired *Clostridium difficile* is increasingly reported (Bauer et al., 2008; Kelly, 2012; Kwok et al., 2012; Rodrigues et al., 2010; Tleyjeh et al., 2012).

An initial episode of *Clostridium difficile* infection (although self-limiting in most cases) is usually treated with metronidazol or vancomycin orally. After initial treatment, a recurrence occurs in approximately 25 percent of patients, and those patients are at increased risk to develop subsequent recurrences. With subsequent recurrences, the failure rate of antibiotics gradually increases, and some patients develop chronic relapsing and antibiotic refractory *Clostridium difficile* infection (Maroo and LaMont, 2006). Recently, fidaxomicin was registered for treatment of CDI in the United States and Europe. Fidaxomicin, which relatively preserves the normal bowel flora compared to vancomycin, might by more effective against recurrent *Clostridium difficile* infection, although limited data are available (Cornely et al., 2012; Crook et al., 2012; Louie et al., 2011).

Donor Feces Infusion for Recurrent *Clostridium difficile* Infection

Because antibiotic treatment fails in a subset of patients with recurrent *Clostridium difficile* infection, there is an urgent need for more effective treatment strategies. To date, donor feces infusion (or fecal microbiota transplantation, FMT) seems the only effective alternative available, although unappealing. From 1958 there are more than 500 patients described in case series and case reports reporting successful treatment with FMTs for either antibiotic-associated diarrhea or *Clostridium difficile* infections (Bakken, 2009; van Nood et al., 2009). The majority of publications is published in the past 5 years, explained by the steep increase in the incidence of CDI and the concomitant increase of patients with recurrent disease. Historically, the majority of successfully treated patients received only one infusion with donor feces, but multiple infusions may be required in certain patients.

Recently, the first randomised trial of FMT for recurrent CDI was published. Patients were randomised to either a standard treatment with 14 days vancomycin orally (with or without a whole bowel lavage at day 5) or to a treatment with FMT (van Nood et al., 2013). Patients randomized to FMT were treated with vancomycin orally during 4 or 5 days followed by bowel lavage before infusion of the fresh donor feces (>50 gram) solution through a nasoduodenal tube. The study was stopped prematurely after an interim analysis, showing an overwhelming difference in outcome between treatment groups. Of 16 patients in the infusion group, 13 (81 percent) had resolution of *C. difficile*–associated diarrhea after the first infusion. The three remaining patients received a second infusion with feces from a different donor, with resolution in two patients. Overall, 15 of 16 patients were cured by FMT. In contrast, 4 of 13 patients (31 percent) receiving vancomycin alone and in 3 of 13 patients (23 percent) receiving vancomycin with bowel lavage were cured ($P < 0.001$ for both comparisons with the infusion group). No significant differences in adverse events among the study groups were observed except for short and mild diarrhea and abdominal cramping immediately after infusion of donor feces.

Taken together, FMT is a safe and effective treatment for recurrent *Clostridium difficile* infection. Patients with multiple relapses have a high failure rate (>50 percent) of current antibiotic therapy (Maroo and LaMont, 2006). After a third relapse, FMT should be considered as a serious treatment option (Surawicz et al., 2013). Whether FMT is safe in patients with severe colitis or immune-compromised patients is unknown, although anecdotal reports of the successful use of FMT as rescue treatment in severely ill patients exist (Brandt et al., 2011; Neemann et al., 2012).

Protocol Fecal Microbiota Transplantation (FMT)

Route of Infusion

A donor feces solution can be infused in the lower or upper gastrointestinal tract with fecal enemas, colonoscopy, or via a nasoduodenal or nasogastric tube. Currently, there is no consensus about the preferred route of infusion; both methods seem effective and safe. The overall reported success rate is >90 percent (Gough et al., 2011).

Pretreatment of Patients

Most patients are pretreated with antibiotics prior to FMT. Although this may not be necessary, for logistical reasons initiation of antibiotic therapy before performing FMT seems daily practice. Bowel lavage before infusion of donor feces has the theoretical advantage of clearing the bowel before infusion of healthy microbiota. We usually prescribe 2 liters of a cetomacrogol solution one day before infusion, regardless the route of administration of FMT. However, there is no study that compared donor feces with or without a whole bowel lavage, and FMT seems also effective without bowel lavage (Gough et al., 2011).

Preparation of Feces

We use freshly produced donor stool (>50 gram) that is dissolved in 500 cc of sterile saline and is infused preferably within 6 hours after collection by the donor. Water and other diluents (e.g., yogurt or milk) have also been described as vehiculum. Relatively more failures of FMT were described in patients receiving smaller amounts of feces (van Nood et al., 2009). Recently, standardized frozen stool samples were also reported to be effective (Hamilton et al., 2012). This could importantly simplify the implementation of FMT.

Protocol for Screening of Donors

Most patients receive donor feces from their spouse or relatives, but feces from healthy volunteers can also be used. To date, donor characteristics that predict the success of FMT are not known. Potential donors should not have an increased risk of (sexually) contractible infectious diseases. Even if the potential donor is a relative or spouse, extensive screening is required.

Initial selection of donors prior to screening of blood and feces is important for prevention of diseases in a window phase at the time of screening. We use a questionnaire, adapted from the questionnaire used for potential blood donors, addressing travel history, sexual behaviour, previous operations, blood transfusions, piercings, and all other interventions that might contribute to carriage of an infectious disease. Any risk of a recently contracted infectious disease that is still

in its window phase (HIV, hepatitis) warrants exclusion of the potential donor. Apart from the risk of transmitting an infectious disease, the risk of transmitting a noninfectious disease has to be taken into account. We also exclude individuals with ulcerative colitis, Crohn's disease, irritable bowel syndrome, or a known increased colorectal cancer risk as donor.

In the future, thoroughly screened standardized frozen stool batches might overcome hurdles related to donor selection. Donor screening is summarized in Table A13-1.

The Mechanism of Donor Feces Infusions

The human bowel consists trillions of bacteria, with one gram of feces containing $10e^{11}$ bacteria (Gill et al., 2006). With 16sRNA sequencing, the composition of the microbiota can be assessed. There are four major bacterial phyla identified (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*). In early childhood, the gut microbiota finds an unique equilibrium and stays relatively stable throughout life (Adlerberth and Wold, 2009). The intestinal mucosa and thereby the microbiota is continuously confronted with passing antigens, food, possible toxic substances, and organisms. In a joint effort with the immune system the microbiota needs to balance between protective reactions against harmful pathogens and tolerance of commensal bacteria and dietary antigens to

TABLE A13-1 Donor Screening

1. Initial screening:	Questionnaire addressing risk factors for potentially transmittable disorders
2. Donor screening	Blood tests: Cytomegalovirus (IgG and IgM) Epstein-Barr Virus (VCA IgM, VCA IgG, VCA, antiEBNA) Hepatitis A (total antibodies, *and if positive also hepatitis A IgM*) Hepatitis B (HbsAg, antiHbsAg, antiHBcore) Hepatitis C (anti HCV) HIV type 1 and 2 Human T-lymphotropic virus type I and II (HTLV) *Treponema pallidum* (TPHA) *Entamoeba histolytica* (agglutination and dipstick test) *Strongyloides stercoralis* (ELISA) Fecal tests: Bacteriological evaluation by local standards Parasitological evaluation by local standards (triple feces test) Test for *Clostridium difficile* (toxin ELISA and culture or PCR)
3. One day before donation of feces:	Questionnaire addressing stool frequency and pattern, general health, use of antibiotics, travel history, and (recent) sexual behaviour

maintain intestinal homeostasis (Kootte et al., 2012). Colonisation resistance is the ability of the microbiota to prevent the outgrowth of unwanted pathogens. Antibiotic use is an important disruptive factor of normal homeostasis (Perez-Cobas et al., 2012). If colonisation resistance is disturbed, the outgrowth and differentiation of potential pathogenic bacteria such as *Clostridium difficile* is enabled. Restoration of microbiota seems likely to restore colonisation resistance.

Recent studies have addressed alterations in microbiota composition following FMT. Restoration of disturbed bowel flora is thought to be the principal mechanism responsible for success. The residual colonic microbiota of patients suffering from recurrent *Clostridium difficile* infection shows a reduced diversity and is deficient in members of the bacterial divisions of Firmicutes and Bacteroidetes (Chang et al., 2008; Khoruts et al., 2010; van Nood et al., 2013). FMT importantly alters the composition of the patient's microbiota, which a change towards the fecal bacterial composition of the donor that persists over time, and a normalization of the diversity index (van Nood et al., 2013). In particular, there is a restoration of Bacteroidetes species and clostridium clusters IV and XIVa (Firmicutes), whereas Proteobacteria species decrease (Hamilton et al., 2013; Khoruts et al., 2010; van Nood et al., 2013).

Other Potential Applications for Fecal Microbiota Transplantation

Metabolic Syndrome

Obesity is associated with changed bowel flora composition with a relative abundance of the two dominant bacterial divisions, the Bacteroidetes and the Firmicutes, and colonization of obese mice with "lean microbiota" results in a decrease in total body fat (Bäckhed et al., 2004; Turnbaugh et al., 2006). In addition, Bacteroidetes species are decreased and Firmicutes are increased in feces of obese people compared to lean people. Recently, the results of the FATLOSE trial were published, in which the effect of FMT on insulin resistance (hyperisulinemic clamp with stable isotopes) was assessed in male patients with metabolic syndrome (Vrieze et al., 2012). After 6 weeks, a significant reduction in both periferal and hepatic insulin resistance was observed, implicating substantial effects of whole body glucose metabolism. Moreover, a significant reduction in fasting lipid profiles after allogenic fecal therapy was noted, which is in line with previously published data suggesting a direct effect between duodenal lipid uptake and glucose production orchestrated by a gut microbiota-driven brain-gut axis. The efficacy of FMT may be explained by enhanced production of specific short-chain free fatty acid butyrate produced by the infused lean donor feces, which might restores normal fecal physiology by implantation of missing lean-figure flora components.

Further research is required, and FMT should not be offered to patients with metabolic syndrome outside a strict experimental setting.

FMT for Inflammatory Bowel Disease (IBD)

Several patients with IBD that were successfully treated with FMT are published in case reports (Bennet and Brinkman, 1989; Borody et al., 2003) Currently, there is increasing interest in the role of the microbiota in IBD, and several randomised controlled studies addressing the effect of FMT are recruiting patients. Pending the results of these studies, FMT should not be considered of proven benefit for patients with IBD.

Concluding Remarks

FMT is a treatment modality that is effective in patients with recurrent *Clostridium difficile* infections. FMT seems safe, but careful donor screening is required. Although new drugs such as fidaxomicin demonstrate lower recurrence rates following initial infection, a subgroup of patients will remain dependent on FMT. The refinement of the protocol and selection of patients for FMT requires further investigation. Recently, the FDA has not approved FMTs as a regular treatment and an "investigational new drug application" (NDA) is required, which may result in reluctance of physicians to offer patients FMT.

In the future, further understanding of the optimal composition of the microbiota to prevent *Clostridium difficile* outgrowth or influence other disorders may result in the development of standardized mixtures of bacteria with a true therapeutic efficacy. This might finally eliminate the need for donor feces infusion.

References

Adlerberth, I., and A. E. Wold. 2009. Establishment of the gut microbiota in western infants. *Acta Paediatrica* 98(2):229-238.

Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J.I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences USA* 101(44):15718-15723.

Bakken, J. S. 2009. Fecal bacteriotherapy for recurrent Clostridium difficile infection. *Anaerobe* 15(6):285-289.

Barbut, F., and J. C. Petit. 2001. Epidemiology of Clostridium difficile-associated infections. *Clinical Microbiology and Infection* 7(8):405-410.

Bauer, M. P., A. Goorhuis, T. Koster, S. C. Numan-Ruberg, E. C. Hagen, S. B. Debast, E. J. Kuijper, and J. T. van Dissel. 2008. Community-onset Clostridium difficile-associated diarrhoea not associated with antibiotic usage—two case reports with review of the changing epidemiology of Clostridium difficile-associated diarrhoea. *Netherlands Journal of Medicine* 66(5):207-211.

Bennet, J. D., and M. Brinkman. 1989. Treatment of ulcerative colitis by implantation of normal colonic flora. *Lancet* 1(8630):164.

Borody, T. J., E. F. Warren, S. Leis, R. Surace, and O. Ashman. 2003. Treatment of ulcerative colitis using fecal bacteriotherapy. *Journal of Clinical Gastroenterology* 37(1):42-47.

Brandt, L. J., T. J. Borody, and J. Campbell. 2011. Endoscopic fecal microbiota transplantation: "First-line" treatment for severe Clostridium difficile infection? *Journal of Clinical Gastroenterology* 45(8):655-657.

Chang, J. Y., D. A. Antonopoulos, A. Kalra, A. Tonelli, W. T. Khalife, T. M. Schmidt, and V. B. Young. 2008. Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. *Journal of Infectious Diseases* 197(3):435-438.

Cornely, O. A., M. A. Miller, T. J. Louie, D. W. Crook, and S. L. Gorbach. 2012. Treatment of first recurrence of Clostridium difficile infection: Fidaxomicin versus vancomycin. *Clinical Infectious Diseases* 55 Suppl 2:S154-S161.

Crook, D. W., A. S. Walker, Y. Kean, K. Weiss, O. A. Cornely, M. A. Miller, R. Esposito, T. J. Louie, N. E. Stoesser, B. C. Young, B. J. Angus, S. L. Gorbach, and T. E. Peto. 2012. Fidaxomicin versus vancomycin for Clostridium difficile infection: Meta-analysis of pivotal randomized controlled trials. *Clinical Infectious Diseases* 55 Suppl 2:S93-103.

Dethlefsen, L., S. Huse, M. L. Sogin, and D. A. Relman. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16s rRNA sequencing. *PLoS Biology* 6(11):e280.

Eiseman, B., W. Silen, G. S. Bascom, and A. J. Kauvar. 1958. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery* 44(5):854-859.

Gill, S. R., M. Pop, R. T. Deboy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett, and K. E. Nelson. 2006. Metagenomic analysis of the human distal gut microbiome. *Science* 312(5778):1355-1359.

Gough, E., H. Shaikh, and A. R. Manges. 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. *Clinical Infectious Diseases* 53(10):994-1002.

Hall, I. C., E. O'Toole. 1935. Intestinal flora in new-born infants: With a description of a new pathogenic anaerobe, Bacillus difficilis. *American Journal of Diseases in Children* 49:390.

Hamilton, M. J., A. R. Weingarden, M. J. Sadowsky, and A. Khoruts. 2012. Standardized frozen preparation for transplantation of fecal microbiota for recurrent Clostridium difficile infection. *American Journal of Gastroenterology* 107(5):761-767.

Hamilton, M. J., A. R. Weingarden, T. Unno, A. Khoruts, and M. J. Sadowsky. 2013. High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut Microbes* 4(2):125-135.

Kelly, C. P. 2012. Can we identify patients at high risk of recurrent Clostridium difficile infection? *Clinical Microbiology and Infection* 18 Suppl 6:21-27.

Khoruts, A., J. Dicksved, J. K. Jansson, and M. J. Sadowsky. 2010. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent Clostridium difficile-associated diarrhea. *Journal of Clinical Gastroenterology* 44(5):354-360.

Kootte, R. S., A. Vrieze, F. Holleman, G. M. Dallinga-Thie, E. G. Zoetendal, W. M. de Vos, A. K. Groen, J. B. Hoekstra, E. S. Stroes, and M. Nieuwdorp. 2012. The therapeutic potential of manipulating gut microbiota in obesity and type 2 diabetes mellitus. *Diabetes Obesity and Metabolism* 14(2):112-120.

Kuijper, E. J., B. Coignard, and P. Tull. 2006. Emergence of Clostridium difficile-associated disease in North America and Europe. *Clinical Microbiology and Infection* 12(Suppl 6):2-18.

Kwok, C. S., A. K. Arthur, C. I. Anibueze, S. Singh, R. Cavallazzi, and Y. K. Loke. 2012. Risk of Clostridium difficile infection with acid suppressing drugs and antibiotics: Meta-analysis. *American Journal of Gastroenterology* 107(7):1011-1019.

Loo, V. G., L. Poirier, M. A. Miller, M. Oughton, M. D. Libman, S. Michaud, A. M. Bourgault, T. Nguyen, C. Frenette, M. Kelly, A. Vibien, P. Brassard, S. Fenn, K. Dewar, T. J. Hudson, R. Horn, P. Rene, Y. Monczak, and A. Dascal. 2005. A predominantly clonal multi-institutional outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality. *New England Journal of Medicine* 353(23):2442-2449.

Louie, T. J., M. A. Miller, K. M. Mullane, K. Weiss, A. Lentnek, Y. Golan, S. Gorbach, P. Sears, and Y. K. Shue. 2011. Fidaxomicin versus vancomycin for Clostridium difficile infection. *New England Journal of Medicine* 364(5):422-431.

Macleod-Glover, N., and C. Sadowski. 2010. Efficacy of cleaning products for C. difficile: Environmental strategies to reduce the spread of Clostridium difficile-associated diarrhea in geriatric rehabilitation. *Canadian Family Physician* 56(5):417-423.

Maroo, S., and J. T. LaMont. 2006. Recurrent Clostridium difficile. *Gastroenterology* 130(4):1311-1316.

Neemann, K., D. D. Eichele, P. W. Smith, R. Bociek, M. Akhtari, and A. Freifeld. 2012. Fecal microbiota transplantation for fulminant Clostridium difficile infection in an allogeneic stem cell transplant patient. *Transplant Infectious Disease* 14(6):E161-E165.

Pérez-Cobas, A. E., M. J. Gosalbes, A. Friedrichs, H. Knecht, A. Artacho, K. Eismann, W. Otto, D. Rojo, R. Bargiela, M. von Berger, S. C. Neulinger, C. Däumer, F. A. Heinsen, A. Latorre, C. Barbas, J. Seifert, V. M. Dos Santos, S. J. Ott, M. Ferrer, and A. Moya. 2012. Gut microbiota disturbance during antibiotic therapy: A multi-omic approach. *Gut* doi:10.1136/gutjnl-2012-303184.

Rodrigues, M. A., R. R. Brady, J. Rodrigues, C. Graham, and A. P. Gibb. 2010. Clostridium difficile infection in general surgery patients; Identification of high-risk populations. *International Journal of Surgery* 8(5):368-372.

Surawicz, C. M., L. J. Brandt, D. G. Binion, A. N. Ananthakrishnan, S. R. Curry, P. H. Gilligan, L. V. McFarland, M. Mellow, and B. S. Zuckerbraun. 2013. Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections. *American Journal of Gastroenterology* 108(4):478-498.

Tleyjeh, I. M., A. A. Bin Abdulhak, M. Riaz, F. A. Alasmari, M. A. Garbati, M. AlGhamdi, A. R. Khan, T. M. Al, P. J. Erwin, T. Ibrahim, A. Allehibi, L. M. Baddour, and A. J. Sutton. 2012. Association between proton pump inhibitor therapy and Clostridium difficile infection: A contemporary systematic review and meta-analysis. *PLoS One* 7(12):e50836.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444(7122):1027-1031.

van Nood, E., A. Vrieze, M. Nieuwdorp, S. Fuentes, E. G. Zoetendal, W. M. de Vos, C. E. Visser, E. J. Kuijper, J. F. Bartelsman, J. G. Tijssen, P. Speelman, M. G. Dijkgraaf, and J. J. Keller. 2013. Duodenal infusion of donor feces for recurrent Clostridium difficile. *New England Journal of Medicine* 368(5):407-415.

van Nood, N. E., P. Speelman, E. J. Kuijper, and J. J. Keller. 2009. Struggling with recurrent Clostridium difficile infections: Is donor faeces the solution? *Eurosurveillance* 14(34).

Viau, E., and J. Peccia. 2009. Survey of wastewater indicators and human pathogen genomes in biosolids produced by class a and class b stabilization treatments. *Applied Environmental Microbiology* 75(1):164-174.

Vrieze, A., E. van Nood, F. Holleman, J. Salojärvi, R. S. Kootte, J. F. Bartelsman, G. M. Dallinga-Thie, M. T. Ackermans, M. J. Serlie, R. Oozeer, M. Derrien, A. Druesne, J. E. Van Hylckama Vlieg, V. W. Bloks, A. K. Groen, H. G. Heilig, E. G. Zoetendal, E. S. Stroes, W. M. de Vos, J. B. Hoekstra, and M. Nieuwdorp. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 143(4):913-916.

Walters, B. A., R. Roberts, R. Stafford, and E. Seneviratne. 1983. Relapse of antibiotic associated colitis: Endogenous persistence of Clostridium difficile during vancomycin therapy. *Gut* 24(3):206-212.

A14

CONSUMPTION OF HUMAN MILK GLYCOCONJUGATES BY INFANT-ASSOCIATED BIFIDOBACTERIA: MECHANISMS AND IMPLICATIONS[59]

Daniel Garrido,[60] *David C. Dallas*,[60] *and David A.* ***Mills***[60,*]

Abstract

Human milk is a rich source of nutrients and energy, shaped by mammalian evolution to provide all the nutritive requirements of the newborn. In addition, several molecules in breast milk act as bioactive agents, playing an important role in infant protection and guiding a proper development. While major breast milk nutrients such as lactose, lipids, and proteins are readily digested and consumed by the infant, other molecules, such as human milk oligosaccharides and glycosylated proteins and lipids, can escape intestinal digestion and transit through the gastrointestinal tract. In this environment, these molecules guide the composition of the developing infant intestinal microbiota by preventing the colonization of enteric pathogens and providing carbon and nitrogen sources for other colonic commensals. Only a few bacteria, in particular *Bifidobacterium* species, can gain access to the energetic content of milk as it is displayed in the colon, probably contributing to their predominance in the intestinal microbiota in the first year of life. Bifidobacteria deploy exquisite molecular mechanisms to utilize human milk oligosaccharides, and recent evidence indicates that their activities also target other human milk glycoconjugates. Here, we review advances in our understanding of how these microbes have been shaped by breast milk components and the strategies associated with their consumption of milk glycoconjugates.

Introduction

After birth, the profound and intimate connection between a mother and her newborn continues in several ways. Breast milk represents a physical representation of this relationship: an intriguing fluid synthesized at the mother's expense, shaped throughout evolution to nourish the infant and improve its rate of survival.

[59] Originally published as: D. Garrido, D. C. Dallas, and D. A. Mills. 2013. Consumption of human milk glycoconjugates by infant-associated bifidobacteria: Mechanisms and implications. *Microbiology* 159: 649-664.

[60] Department of Food Science and Technology and Foods for Health Institute, University of California Davis, One Shields Ave, Davis, CA 95616, USA.

* Correspondence: David A. Mills damills@ucdavis.edu

Human milk is perhaps the most personalized food, where the molecular make-up varies from mother to mother and across lactation, providing the infant all the nutrients needed in a concentrated form (Allen et al., 1991; Mitoulas et al., 2002). Breastfeeding is regarded as the "normal way of providing young infants with the nutrients they need for healthy growth and development" (Fewtrell et al., 2007). Exclusive breastfeeding is recommended for up to 6 months of age (American Academy of Pediatrics Section on Breastfeeding, 2012), and its benefits are multifold and some can last beyond childhood (Hernell, 2011; Le Huërou-Luron et al., 2010).

Human milk is a complex food matrix and its composition reflects all the nutritional and physiological demands of the newborn. Essential nutrients in human milk such as lactose, fatty acids, and proteins are absorbed by the small intestine at a rate that is limited by the developing conditions of the gastrointestinal (GI) tract of the infant (Neu, 2007). Other micronutrients such as nucleotides, vitamins and minerals are also highly bioavailable for the infant (Picciano, 2001).

Numerous studies have shown that breastfeeding is associated with a lower risk of infections and diarrhoea. This has been associated with the activity of milk immunoglobulins (Xanthou et al., 1995), antimicrobial agents such as lactoferrin and lysozyme (Håversen et al., 2002; Jollès and Jollès, 1961; Lönnerdal, 2009), and human milk glycoconjugates (Newburg et al., 2005). Several of these molecules are not readily absorbed by the small intestine and transit throughout the GI tract (Dallas et al., 2012), but their impact and biological activities are poorly understood. These bioactive compounds play additional roles in protection and/or stimulate development regardless of their nutritive value (Hamosh, 2001; Lönnerdal, 2010). Bioactives in human milk represent a significant difference between breast milk and bovine milk-based formulas (Hernell, 2011; Le Huërou-Luron et al., 2010).

A common characteristic of these bioactive agents is that they are glycosylated molecules. Glycans in milk can be found as free human milk oligosaccharides (HMO), or conjugated via glycosidic bonds to proteins or lipids. Among other functions, human milk glycans represent the main driving force for bacterial colonization of the distal large intestine of the breast-fed infant (Scholtens et al., 2012). The high concentrations of HMO and conjugated oligosaccharides processed after intestinal digestion are thought to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut. The genome sequences of bifidobacteria show that these micro-organisms are highly adapted to the intestinal environment (Schell et al., 2002), and the genomes of infant gut-associated bifidobacteria have been shaped by complex carbohydrates (Sela and Mills, 2010). In this review, we examine recent advances in our understanding of how milk oligosaccharides and other glycoconjugates influence the dominance of beneficial micro-organisms in the gut microbiota, especially *Bifidobacterium*, and of the mechanisms and strategies that these micro-organisms have devised for using milk components as a carbon source.

HMO

Structures of HMO

A great amount of the energy invested in human milk production is dedicated to synthesize complex free oligosaccharides. These molecules represent the third most abundant component in breast milk after lactose and fatty acids (Petherick, 2010). HMO consist of a pool of soluble carbohydrates with a degree of polymerization of 3 to 15 and linked through a variety of glycosidic bonds (Kunz et al., 2000; Urashima et al., 2012). HMO are composed of five monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc; sialic acid). Figure A14-1 presents a representative HMO structure and the diversity of linkages that can be found. All HMO are characterized by a terminal lactose molecule, modified by fucose or sialic acid in the case of the shorter HMO such as 2′-fucosyllactose (FL), 3FL and sialyllactose (SL), or by repeats of building blocks of lacto-*N*-biose type 1 (LNB; Galβ1-3GlcNAc) or *N*-acetyllactosamine (LacNAc; Galβ1-4GlcNAc). These repeats can be further decorated by fucose or sialic acid in α-linkages, adding more complexity and diversity to these molecules (Bode and Jantscher-Krenn, 2012). HMO can be classified as acidic or neutral depending on the presence of the negatively charged sialic acid. Neutral HMO can be further categorized by the presence of fucose on their structures. Over 200 oligosaccharide structures have been identified in human milk (Wu et al., 2010, 2011).

Significant differences exist in HMO abundance and composition among different mothers and across lactation stages (Coppa et al., 1999; De Leoz et al., 2012; Niñonuevo et al., 2008). An important association also exists between HMO and the blood group type of the mother represented by the Lewis system and the secretor genes, which generates four different groups of milks (Totten et al., 2012). Another important characteristic of human milk is the overabundance of type 1 HMO, containing type 1 LNB, over type 2 HMO containing LacNAc (Urashima et al., 2012). Type 1 predominance and large amounts of fucosylated HMO are characteristic of human milk but much less so for other mammals (Tao et al., 2011; Taufik et al., 2012).

Great efforts have been made to elucidate the composition and structures of HMO. As recently reviewed (Kunz, 2012), milk carbohydrates research started in the early 1900s. Despite recent technological advances, structural elucidation of oligosaccharides from breast milk still remains a challenge, mainly due to the variety of possible isomeric forms of any given composition. MS has become a method of choice for oligosaccharide analysis, and current methods allow isomer differentiation with high resolution (Ruhaak and Lebrilla, 2012).

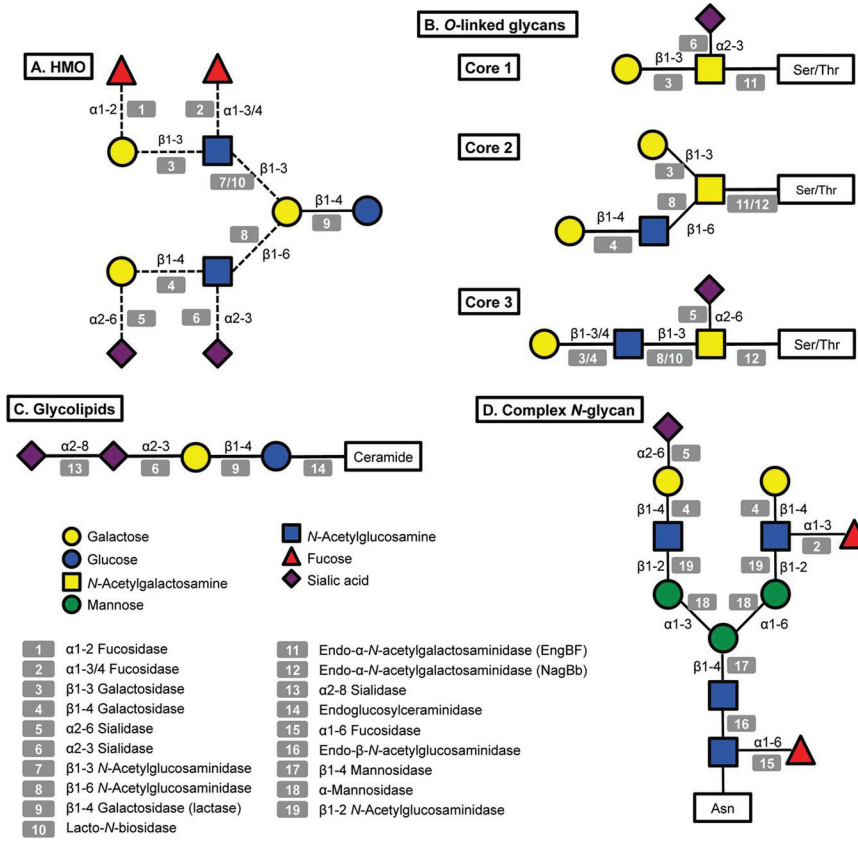


FIGURE A14-1 Structural diversity of glycans in human milk and corresponding glycosyl hydrolases in infant-gut associated bifidobacteria. Legends at the bottom left indicate monosaccharide composition and the corresponding potential glycolytic enzymes in bifidobacteria acting at specific linkages. A: illustrative structure of HMO; B: three different cores found in human *O*-linked glycans; C: glycolipids, the structure of ganglioside GD3 is shown; D: a complex *N*-glycan.

Physiological Effects of HMO

GI enzymes in the infant are not capable of breaking down the diversity of HMO linkages synthesized by glycosyltransferases in the mammary gland (Dallas et al., 2012), which emphasizes the role of milk bioactive molecules in functions beyond nutrition. HMO are minimally affected by transit through the stomach and small intestine, reaching a high concentration in infant faeces (Chaturvedi et al., 2001; Engfer et al., 2000; Gnoth et al., 2000). Excreted faecal HMO in breast-fed infants are a reflection of the mother secretor status, and the

course of oligosaccharide excretion is apparently individual-specific and intermediate degradation products can be observed (Albrecht et al., 2011). Furthermore, small amounts of certain HMO can be found in urine (Rudloff and Kunz, 2012), suggesting that these molecules can exert physiological effects not only locally in the GI tract but also systemically.

HMO are well known for their ability to prevent adherence and invasion of several pathogens (Imberty and Varrot, 2008; Morrow et al., 2005). This is probably due to the structural similarity between HMO and glycoconjugates present in the intestinal mucosa. Fucose- and sialic acid-containing HMO are particularly important in pathogen deflection as they are found at terminal positions in these molecules. Therefore, the abundance of HMO and other milk glycoconjugates can explain in great part how breast milk helps to prevent infant diarrhoea and GI infections in breast-fed infants (Coppa et al., 2006; Hakkarainen et al., 2005; Hong et al., 2009; Martín-Sosa et al., 2002; Morrow et al., 2004; Newburg et al., 2004; Ruiz-Palacios et al., 2003).

Establishment of the Infant Intestinal Microbiota

At birth, the newborn is first exposed to the extrauterine environment, entering a microbial-laden world that results in quick colonization of different body sites, typically in a non-pathogenic fashion (Dominguez-Bello et al., 2010). Of particular interest is how the intestinal microbiota is established, given the potential impact it has on subsequent health and disease (Reinhardt et al., 2009; Scholtens et al., 2012). Patterns of early intestinal colonization can have both short-term and long-term health effects (Bager et al., 2008; Cho et al., 2012; Collado et al., 2012; Kalliomäki et al., 2008; Salvini et al., 2011). Bacterial colonization of the intestine is key in several aspects: bacteria provide essential nutrients for the infant such as vitamins and short-chain fatty acids, they stimulate the development of the immune system, especially adaptive responses, and they provide general protection against pathogen colonization, among several other functions (Hooper et al., 2012; Nicholson et al., 2012). A contribution of the intestinal microbiota has been established in the onset of obesity and type 2 diabetes (Harris et al., 2012; Ley et al., 2005). The establishment of the microbiota in the infant colon has been described as an orchestrated, but chaotic, succession of bacteria (Koenig et al., 2011), wherein the composition depends on a diverse number of factors such as mode of delivery, type of feeding, and genetic, cultural and geographical determinants (Scholtens et al., 2012). The first colonizers of the intestinal tract are facultative anaerobic bacteria, such as *Escherichia coli*, enterococci and streptococci, which predominate in the first days of life. These bacteria will consume the oxygen in the intestinal lumen, creating an anaerobic environment more favourable for strict anaerobes, such as *Bacteroides*, *Clostridium*, and *Bifidobacterium* (Jost et al., 2012).

Mode of delivery is one of the most important factors that dictate the composition of the infant intestinal microbiota in the first months of life. Normal vaginal delivery exposes the infant to the vaginal and faecal microbiota of the mother (Dominguez-Bello et al., 2010; Makino et al., 2011). Human breast milk has been also considered another source of micro-organisms that can potentially contribute to gut colonization (Cabrera-Rubio et al., 2012; Grönlund et al., 2007; Makino et al., 2011); however, this remains controversial, since the microbiota in breast milk can be instead a reflection of the skin or faecal microbiota. In the other hand, the hospital environment (Martirosian et al., 1995) and the skin microbiota (Dominguez-Bello et al., 2010) are considered sources of bacteria for caesarian-born infants. A delay in microbial colonization by prominent members of the intestinal microbiota such as *Bifidobacterium*, *Bacteroides*, and *E. coli* has also been observed (Adlerberth et al., 2006; Mitsou et al., 2008), and bifidobacterial counts are also lower in caesarian-born infants (Chen et al., 2007; Penders et al., 2006).

Significant differences are found in the early composition of the infant intestinal microbiota relative to the type of diet. Infant formulas are traditionally based on bovine milk, and great advances have been made to improve their composition by adding supplements such as minerals, vitamins, and prebiotics, in order to simulate the essential components in breast milk (Hernell, 2011; Koletzko, 2010). Unfortunately, some of the bioactive molecules in human milk are not found in bovine milk, and therefore replicating their effects is challenging (Dewey et al., 1995; Le Huërou-Luron et al., 2010).

Bifidogenic Effect of HMO

Culture-based and current large-scale metagenomic studies show that *Bifidobacterium* is a dominant genus in the intestinal microbiota of breast-fed infants, in some cases representing approximately 75% of total bacteria (Harmsen et al., 2000; Roger and McCartney, 2010; Sakata et al., 2005; Yatsunenko et al., 2012). The overrepresentation of bifidobacteria in this environment is less observed in formula-fed infants, who show a more diverse microbiota (Fallani et al., 2010; Penders et al., 2006). Therefore, differences in bacterial colonization between breast-fed and formula-fed infants can be explained in great part by the non-essential components in human milk.

The predominance of bifidobacteria in breast-fed infant faeces was first noticed over 100 years ago (Moro, 1905). Moro and Tissier suggested that breast milk contained certain molecules that stimulated the growth of these bacteria, defined as *bifidus factors* (Moro, 1905). Gynolactose was later described as a mixture of milk oligosaccharides containing GlcNAc that stimulated the growth of certain *Bifidobacterium* strains (Polonowski and Lespagnol, 1931). These studies first suggested a prebiotic role for oligosaccharides in breast milk.

The ability of these micro-organisms to metabolize HMO might therefore represent an example of co-evolution with their host, and the physiology and

mechanism of this consumption has been addressed. Ward and co-workers first showed that *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) can grow vigorously on HMO in vitro as the sole carbon source (Ward et al., 2006). *B. infantis* displays a preference for shorter HMO but can use larger oligosaccharides as well (LoCascio et al., 2007). The ability to consume HMO in vitro has been demonstrated for additional strains of *B. infantis* and also *Bifidobacterium bifidum*, and to a lesser extent strains of *Bifidobacterium breve* and *Bifidobacterium longum* (Asakuma et al., 2011; LoCascio et al., 2009; Turroni et al., 2010). These four species are usually present in breast-fed infant faeces (Turroni et al., 2012; Yatsunenko et al., 2012; Avershina et al., 2013; Boesten et al., 2011; Matsuki et al., 2002).

Hence, the enrichment in bifidobacteria in breast-fed infant faeces can be explained in great part by their ability to consume and metabolize HMO. Moreover, the prebiotic character of HMO seems to be selective for infant bifidobacteria and a few *Bacteroides* species, and not for adult bifidobacteria or other prominent members of the intestinal microbiota such as *Clostridium* and enterobacteria (Marcobal et al., 2010). Bottle-fed infants display higher numbers of Firmicutes and *Bacteroides* and less of *Bifidobacterium* in their faeces, and the bifidobacteria characteristic of formula-fed infants include additionally *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, which are more commonly associated with the adult intestinal microbiota (Haarman and Knol, 2005; Mangin et al., 2006).

Bifidobacterial Strategies for HMO Consumption

Bifidobacteria possess a fermentative metabolism, and they are almost exclusively associated with the GI tract of animals (Lee et al., 2008; Sela et al., 2010). They are considered to be beneficial for the human host, and several strains of bifidobacteria are commercialized as probiotics. In general they devote a significant portion of their genomes to the consumption of complex oligosaccharides (O'Connell Motherway et al., 2011b; Schell et al., 2002; Sela et al., 2008; Turroni et al., 2010), either of plant origin in the case of adult-associated species or host-derived in the case of species better adapted to the nursing period. Analysis of genome sequences of bifidobacteria isolated from breast-fed infants has enabled predictions regarding the HMO consumption phenotype. In particular, *B. infantis* ATCC 15697 and *B. bifidum* PRL2010 are prototypical members of the infant intestinal microbiota that have possibly co-evolved with their host to consume milk or host oligosaccharides (Sela et al., 2008; Turroni et al., 2010).

Physiologically, *B. infantis* can simultaneously consume distinct classes of HMO in vitro with high efficiency, reaching higher cell densities compared to other infant-associated bifidobacteria (Asakuma et al., 2011; Ward et al., 2006). A hallmark of the genome of this species is a conserved cluster of genes, the HMO cluster I (Figure A14-2), containing several glycosyl hydrolases and ABC

transporters (Sela et al., 2008). Other potentially important clusters for HMO consumption are also conserved among other *B. infantis* strains but are absent in *B. longum* strains (LoCascio et al., 2010). The overall overrepresentation of genes encoding family 1 solute binding proteins (SBPs) and also intracellular glycosyl hydrolases (GHs) with putative affinity for or activity on HMO in the genome of *B. infantis* is suggestive of a consumption strategy based on the import of intact HMO structures and their intracellular enzymic degradation (Zivkovic et al., 2011). Several of these predictions regarding HMO consumption have been addressed and genes encoding functions in HMO import and hydrolysis have been identified (Figure A14-2).

For example, a large array of oligosaccharide-binding SBPs in *B. infantis* ATCC 15697 is biased towards mammalian glycans, especially HMO (Garrido et al., 2011). Their substrate affinities are diverse and include neutral HMO containing either LNB or LacNAc (type 1 or type 2 HMO), or fucosylated HMO such as 2′FL and Lewis epitopes. Chemical blockage of ABC transporters inhibits the ability of *B. infantis* to consume lacto-*N*-tetraose (LNT; type 1 HMO) as the sole carbon source in vitro (Yoshida et al., 2012). In addition, genes encoding SBPs with affinity for HMO are exclusively induced during exponential growth on HMO (Figure A14-2). In addition, some of these proteins are able to bind epithelial surfaces in vitro, probably due to the structural similarities between HMO and epithelial glycoconjugates. These results also suggested physiological differences between *B. infantis* cells growing on either simple lactose or HMO, where epithelium-binding SBPs are induced only during growth on HMO. In agreement with these observations, *B. infantis* cells growing on HMO but not lactose display increased binding to intestinal epithelial cells, and under these conditions they enhance the production of anti-inflammatory cytokines and tight junction proteins (Chichlowski et al., 2012).

Another relevant aspect of bacterial HMO utilization is the enzymic processing of these molecules by glycosyl hydrolases. Interestingly, the microbiome of breast-fed infants is enriched in fucosidases and sialidases (Yatsunenko et al., 2012). The genome of *B. infantis* contains an army of glycosyl hydrolases active on these carbohydrates, including two genes encoding α-sialidases, five α-fucosidases, five β-galactosidases and three β-*N*-acetylglucosaminidases (Garrido et al., 2012a). Recent functional studies on the enzymic properties of these enzymes and their induction by HMO have greatly advanced our understanding of HMO consumption by *B. infantis* (Figure A14-2). Acidic HMO such as 3′SL, 6′SL and sialyl-LNT represent nearly 15% of total HMO. These are probably imported inside the cells by systems different from ABC transporters, and membrane permeases of the major facilitator family are likely candidates. NanH2 is an α-sialidase in *B. infantis* (Blon_2348 in HMO cluster I, Figure A14-2) that removes sialic acid from α2-3 and α2-6 sialyl linkages found in individual HMO such as mono and disialyl-LNT (Sela et al., 2011). The expression of NanH2, but not a second encoded sialidase NanH1, was significantly increased during

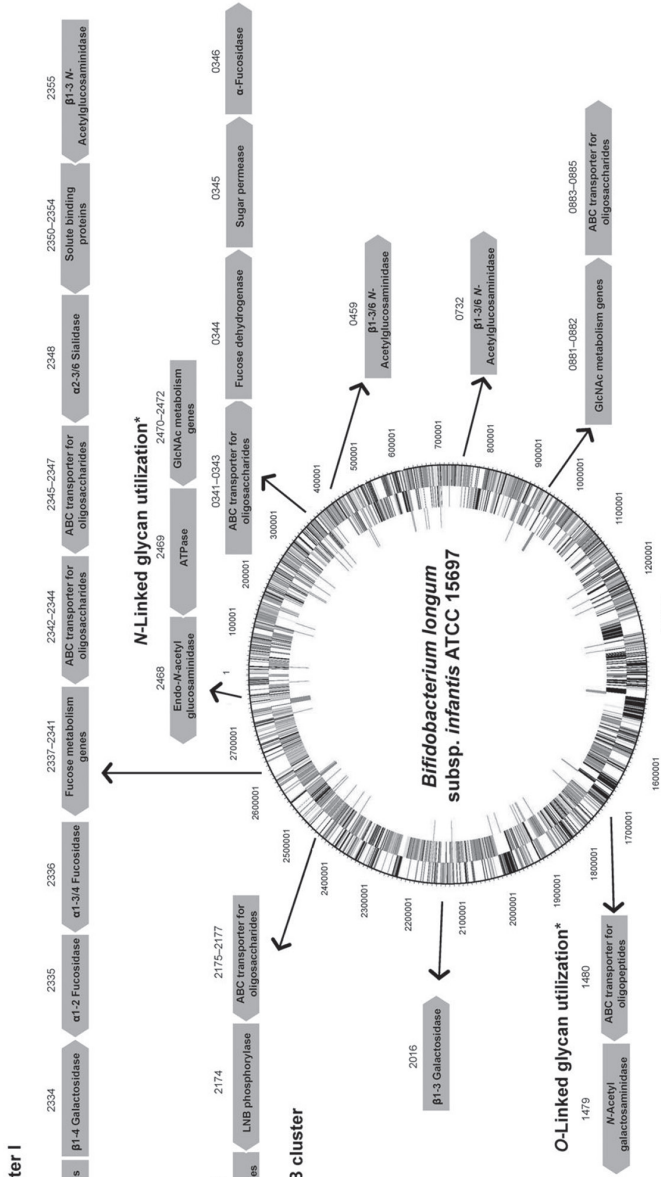


FIGURE A14-2 Clusters of genes in *B. infantis* ATCC 15697 with assigned or putative functions in the utilization of milk glycans. Numbers above the arrows correspond to the respective locus tags (Blon_xxxx). Genes are not drawn to scale, and the genome circle was adapted from Sela et al. (2008). SBPs from ABC transporters with affinities for HMO and expressed during growth on these substrates were identified by Garrido et al. (2011). An α-sialidase and two α-fucosidases were characterized by Sela et al. (2011, 2012). Two β-galactosidases and three β-hexosaminidases active on different linkages in representative HMO are also included (Garrido et al., 2012c; Yoshida et al., 2012). Finally, potential gene clusters for *N*-linked and *O*-linked glycan utilization (*) are depicted (Garrido et al., 2012b; Kiyohara et al., 2012).

bacterial growth on HMO. In addition, two fucosidases in *B. infantis* have significant activity in fucosylated HMO and blood group oligosaccharides. Blon_2335 and Blon_2336 are located in HMO cluster I and belong to GH families 95 and 29, respectively (Figure A14-2). Growth on pooled HMO, LNT, or LNnT (a type 2 HMO) upregulates their gene expression (Sela et al., 2012). Blon_2335 is a highly efficient α1-2 fucosidase that has also considerable activity towards α1-3 and α1-4 fucosyl linkages, contrary to the AfcA fucosidase in *B. bifidum*, which acts exclusively on Fucα1-2 linkages (Ashida et al., 2009). Blon_2335 can release fucose from several HMO such as 2′FL, 3FL and lacto-*N*-fucopentaoses I and III, and also from fucosylated epitopes found in epithelial glycoconjugates such as Lewis^a[Galβ1-3(Fucα1-4)GlcNAc], Lewis^x [Galβ1-4(Fucα1-3)GlcNAc] and the H-disaccharide (Fucα1-2Gal). In the other hand Blon_2336 is specific for α1-3/4 linkages, such as those found on 3FL, lacto-*N*-fucopentaose III and Lewis^x (Sela et al., 2012).

Galactose and *N*-acetylglucosamine constitute the building blocks of HMO, and these monosaccharides are generally fermentable carbon sources for bifidobacteria. Galactose is found in simple carbohydrates such as lactose and complex oligosaccharides of mammalian or plant origin. In *B. infantis*, two β-galactosidases with glycolytic activity on HMO are constitutively expressed (Yoshida et al., 2012). Bga2A, encoded by Blon_2334 in HMO cluster I (Figure A14-2), belongs to GH family 2 and has a preference for lactose, also efficiently removing galactose from type 2 HMO such as LacNAc and LNnT. Complementing this activity is Bga42A (encoded by Blon_2016; Figure A14-2), a GH42 β-galactosidase highly specific for LNT, one of the most abundant HMO (Yoshida et al., 2012). Interestingly, this enzyme has considerably less activity on LNB, suggesting that each residue in LNT is crucial for its enzymic activity. Finally, β-*N*-acetylglucosaminidases participate in this process (Garrido et al., 2012c). Blon_0459, Blon_0732 and also Blon_2355 in the HMO cluster I are expressed mostly during early exponential growth on HMO, and while the three enzymes can cleave the GlcNAcβ1-3Gal linkage found in linear HMO such as LNT or LNnT, only Blon_0459 and Blon_0732 are active on branched HMO, characterized by GlcNAcβ1-6Gal as found in lacto-*N*-hexaose. These results support the concept of sequential hydrolysis of HMO in *B. infantis*, releasing large amounts of monosaccharides that can be fermented in central metabolic pathways.

Parallel studies have provided important details on the mechanisms of HMO utilization by *B. bifidum*, another member of the infant intestinal microbiota. *B. bifidum* and *B. infantis* are competitive in HMO consumption but using different strategies (Garrido et al., 2012a). While *B. infantis* has specialized in the import and intracellular deglycosylation of several HMO, *B. bifidum* uses an array of membrane-associated glycosyl hydrolases, including extracellular α-sialidases (Kiyohara et al., 2011), α-fucosidases (Ashida et al., 2009), β-galactosidases and β-*N*-acetylglucosaminidases (Miwa et al., 2010), which efficiently remove

monosaccharides from complex HMO. Another important difference between these strategies is the presence of membrane lacto-*N*-biosidase activity in *B. bifidum* (Wada et al., 2008). This endoglycosidase generates LNB and lactose from LNT, and some of the mono- and disaccharides released can be internalized and metabolized, especially LNB (Asakuma et al., 2011). The binding protein for this disaccharide is a family 1 SBP, part of a gene cluster found in several bifidobacteria, the LNB/GNB cluster (Kitaoka et al., 2005; Nishimoto and Kitaoka, 2007). Genes encoding ABC permeases, a lacto-*N*-biose phosphorylase that generates galactose 1-phosphate and glucose from LNB, and two other genes in the Leloir pathway for galactose metabolism (Figure A14-2), are adjacent to this SBP. The LNB/GNB cluster is actually conserved across all infant gut-associated bifidobacteria, including *B. bifidum*, *B. infantis*, *B. longum*, and *B. breve* isolates (LoCascio et al., 2010; Xiao et al., 2010).

Relatively little attention has been paid to *B. breve* and *B. longum* regarding HMO consumption, even considering that they are normally dominant in infant faeces, and *B. breve* seems to be found exclusively in this environment (Avershina et al., 2013). *B. longum* ATCC 15707 and *B. breve* ATCC 15700 show only modest growth on pooled HMO and apparently can metabolize solely LNT, leaving other HMO unmodified (Asakuma et al., 2011; LoCascio et al., 2007). An association between the LNB/GNB cluster and HMO consumption in *B. longum* ATCC 15707 was suggested after induction of these genes during growth on human milk (González et al., 2008). Several species of bifidobacteria, including strains of *B. breve* and *B. longum*, are able to grow in vitro using LNB as the sole carbon source (Xiao et al., 2010). This consumption can be explained solely by the presence and activity of the LNB/GNB cluster in these strains that can import and metabolize this disaccharide.

Human Milk Glycoconjugates

The complexity of human milk is far from understood, and one example of this is the multiplicity of functions played by several bioactive agents. While the high concentrations of oligosaccharides in human milk can explain in great part the enrichment in bifidobacteria in breast-fed infant faeces, glycans conjugated to other molecules in milk, such as proteins, peptides or lipids, can also have a prebiotic role. Here we address some of the biological properties of these glycoconjugates and what the mechanisms are that infant bifidobacteria have devised to use these glycoconjugates as a carbon source.

Human Milk Glycolipids

Lipids make up 3–5% of the total volume of human milk, of which 98% are triacylglycerols (Jensen, 1999). A fraction of the remaining fats in human milk consists of glycolipids, mostly associated with the milk fat globule membrane

(Newburg and Chaturvedi, 1992). Glycolipids are composed of a lipid chain of ceramide, a fatty acid linked to a sphingoid base, covalently attached to one or more monosaccharides. Milk glycolipids can be classified as neutral, including galactosylceramide (Galβ1-1Cer) and glucosylceramide (Glcβ1-1Cer) (Bouhours and Bouhours, 1979), or acidic glycosphingolipids (or gangliosides), containing sialic acid (Laegreid et al., 1986). The most abundant gangliosides in human milk are GD3 (Figure A14-1) and GM3 (NeuAcα2-3Galβ1-4Glcβ1-1Cer) (Lee et al., 2011).

The glycan portion of milk glycolipids plays an important role in pathogen deflection, similar to other milk glycoconjugates. Binding the epithelium is the first line of entry for pathogens or their toxins, and this process is usually mediated by glycolipids. Therefore, milk-borne glycolipids associated with milk fat globule membranes can prevent bacterial, viral or toxin binding to the intestine (Lindberg et al., 1987; Miller-Podraza et al., 2005; Newburg, 2009; Otnaess et al., 1983). In addition, several intestinal commensals are able to bind glycolipids in vitro (Mukai et al., 2004; Neeser et al., 2000; Strömberg et al., 1988; Yamamoto et al., 1996).

Milk fat globules are degraded by diverse lipases in the GI tract (Lindquist and Hernell, 2010), releasing lipids that are readily absorbed into the small intestine. However, the fate of milk glycolipids is not yet understood, and it is possible that they transit distal portions of the GI tract. Only a few studies have addressed the degradation of milk or epithelial glycolipids by members of the infant intestinal microbiota, and evidence has indicated that they possess enzymes that can hydrolyse in great part these glycoconjugates (Larson et al., 1988). Right after establishment, the intestinal microbiota is responsible for the degradation of glycolipids observed in breast-fed infant faeces (Gustafsson et al., 1986; Midtvedt et al., 1988). The degree of this hydrolytic activity is however lower than that in adults, but higher compared to newborns or germ-free mice (Larson et al., 1987; Larson and Midtvedt, 1989).

It has been observed in vitro that glycosidases from *Ruminococcus torque*, *B. bifidum*, and *B. infantis* degrade several glycosides containing certain blood group determinants, including the H disaccharide, Lewis^a and Lewis^b (Falk et al., 1991; Larson et al., 1988). Lactosylceramide is a common end product of their reactions (Falk et al., 1990). The ability of bifidobacteria to release sialic acid from predominant milk gangliosides such as GD3 and GM3 has been observed (Falk et al., 1990), suggesting that certain bifidobacteria possess α2-8 and α2-3 sialidase activity (Figure A14-1).

Human Milk Glycoproteins

Protein glycosylation is a post-translational modification in which a glycan is covalently linked to predetermined amino acids in the protein structure. There are two major types of oligosaccharides attached to eukaryotic proteins: *N*-linked

and *O*-linked glycans. These conjugated carbohydrates play several biochemical and physiological roles, for example in protein synthesis, folding, trafficking and secretion, resistance to proteolysis, and prevention of pathogen colonization of epithelial surfaces among several others (Barboza et al., 2012; Gopal and Gill, 2000; Newburg et al., 2005; Peterson et al., 1998; Rudd et al., 1994). In human milk a large number of human milk proteins are glycosylated, including lactoferrin, immunoglobulins and κ-casein among several others (Froehlich et al., 2010).

N-Linked glycans are attached to the protein via specific asparagines in the sequence Asn-xxx-Ser/Thr (Stanley et al., 2009). All *N*-linked glycans have in common a pentasaccharide with the structure $Man_3GlcNAc_2$, where the last GlcNAc is linked to the asparagine via a β-linkage (Figure A14-1). This pentasaccharide can sometimes be modified with core α1-6 fucosylation or a bisecting GlcNAc. *N*-Glycans can be heterogeneous and tissue-specific, but three main classes of *N*-glycans have been described based on further modifications of the pentasaccharide: (a) high mannose, consisting of branches of α-mannose; (b) complex, characterized by core α1-6 fucosylation of the basal GlcNAc and by two or more antennae (Galβ1-4GlcNAc repeats) that can be additionally decorated by fucose or sialic acid in terminal positions (Figure A14-1); and (c) the hybrid type, which consists of a mixture of these two types. The human milk *N*-glycome has been recently described in detail, and in general human milk *N*-glycans are largely fucosylated and present in larger concentrations compared to bovine milk (Dallas et al., 2011; Nwosu et al., 2012). In contrast, bovine *N*-glycans are also more sialylated and characterized by the presence of *N*-glycolylneuraminic acid (NeuGc) instead of *N*-acetylneuraminic acid (Nwosu et al., 2012).

O-Linked glycans differ from *N*-linked glycans by attachment to serine or threonine residues, with no obvious surrounding amino acid consensus sequence. Eight different core structures have been described, each beginning with an α-GalNAc attachment to the amino acid (Brockhausen et al., 2009). Only four of these core structures (cores 1–4) are usually found in humans (Brockhausen et al., 2009). These *O*-linked structures can be further elongated by *N*-acetyllactosamine chains and decorated by fucose, sialic acid or GalNAc at terminal positions (Figure A14-1).

Bifidobacterial Consumption of Human Milk Glycoproteins

In milk, protein glycosylation increases the resistance of proteins to proteolysis (van Berkel et al., 1995), probably contributing to the excretion of considerable amounts of intact or partially degraded milk proteins in breast-fed infant faeces (Davidson and Lönnerdal, 1987; Prentice et al., 1989). Milk proteins vary in their digestibility (Le et al., 2012; Ye et al., 2011), and non-glycosylated proteins such as β-casein and α-lactoglobulin are more digested in comparison to lactoferrin, IgA and milk mucins (Jakobsson et al., 1982; Lindh, 1975; Prentice et al., 1989).

Therefore, breast milk glycoproteins, in conjunction with mucosal secretions and shed epithelial cells, transit the GI tract of the breast-fed infant and can play a role in shaping the developing intestinal microbiota. Evidence indicates that these microbes largely modify host glycoconjugates (Hoskins et al., 1985; Variyam and Hoskins, 1981). Germ-free mice secrete intact mucins in their faeces, while conventionalized animals are able to degrade and metabolize mucins completely (Corfield et al., 1992; Midtvedt et al., 1987). In addition, bacteria extracted en masse from adult and infant stools produce a variety of extracellular glycosidases that degrade the glycans of hog gastric mucin under anaerobic conditions (Midtvedt et al., 1988; Variyam and Hoskins, 1981). In addition, individual members of the infant and adult intestinal microbiota have been well studied for their ability to deglycosylate mucins in order to gain access to the bound oligosaccharides as a carbon source (Derrien et al., 2010; Wright et al., 2000). Several species of *Bacteroides* deploy exquisite mechanisms for mucin glycan degradation based on membrane-bound glycosyl hydrolases and importers that are crucial for the survival and predominance of these species in the adult microbiota (Bäckhed et al., 2005; Martens et al., 2009). Interestingly, certain *Bacteroides* species can utilize HMO (Marcobal et al., 2010), and the transcriptional responses elicited during growth in vitro on mucin are highly similar to those witnessed on HMO for *Bacteroides thetaiotaomicron*, suggesting that these substrates are energetically similar for this species (Marcobal et al., 2011).

Some bifidobacteria are also well-known mucin degraders (Crociani et al., 1994; Hoskins et al., 1985). To date, this phenotype seems to be exclusive to *B. bifidum* and certain isolates of *B. longum* (Ruas-Madiedo et al., 2008). Pivotal to the release of *O*-linked glycans from mucins are endo-α-*N*-acetylgalactosaminidases (EngBF, glycosyl hydrolase family 101). Functional studies have shown that these extracellular enzymes cleave Core 1*O*-linked glycans (Galβ1-3GalNAcα-Ser/Thr) found in mucins (Figure A14-1; Ashida et al., 2008; Fujita et al., 2005). This hydrolysis releases galacto-*N*-biose (Galβ1-3GalNAc; GNB), a disaccharide structurally similar to LNB from HMO that can be directly used as a carbon source by *B. longum* via an ABC importer and enzymes in the LNB/GNB cluster (Kitaoka et al., 2005; Nishimoto and Kitaoka, 2007). Since EngBFs are highly specific, GNB release and import probably require the previous action of several glycosyl hydrolases on mucin glycans, such as α-fucosidases, α-sialidases and lacto-*N*-biosidase. These enzymes are also active on HMO, and the genes encoding these activities are highly expressed during growth in vitro on hog gastric mucin as well as on HMO (Turroni et al., 2010).

An alternative mucin utilization pathway has been recently described in bifidobacteria, which might represent a more accurate representation of intestinal mucin degradation by these micro-organisms in vivo (Kiyohara et al., 2012). The glycans on colonic mucins contain mostly Core 3 *O*-linked glycans based on the structure GlcNAcβ1-3GalNAcα1-Ser/Thr (Figure A14-1), which

are inaccessible for EngBF. A novel endo-α-*N*-acetylgalactosaminidase (NagBb, GH129) in *B. bifidum* is specific for the Tn antigen (GalNAcα-Ser/Thr). This structure is potentially produced after extracellular degradation by glycosyl hydrolases. While this mechanism remains to be validated, this novel enzyme was shown to be present in several genomes of infant gut-associated bifidobacteria including *B. infantis* (Figure A14-2), *B. breve* and *B. longum* (Kiyohara et al., 2012), suggesting a common route to degradation of Core 3 *O*-linked glycans (Figure A14-1).

The ability of bifidobacteria to access *O*-linked glycans in human or bovine milk proteins has been less explored; however, we hypothesize that similar mechanisms to those described above are prevalent for human milk mucins. As mentioned above, milk mucins contain a majority of Core 2 *O*-glycans (Figure A14-1). It is possible that after gastric and intestinal digestive processes a higher concentration of mucin-derived glycopeptides is available for infant-associated bifidobacteria. This is not a new concept, as the bovine κ-casein-derived GMP is a highly sialylated glycopeptide that has been suggested to have prebiotic effects (Gomes et al., 1998; Janer et al., 2004; Petschow and Talbott, 1990).

We recently explored the ability of bifidobacteria to gain access to *N*-glycans from host glycoproteins using a representative panel of 76 strains of these micro-organisms isolated from infant faeces (Garrido et al., 2012b). Endo-β-*N*-acetylglucosaminidases (EC 3.2.1.96; endoglycosidases) hydrolyse the *N*, *N*′-diacetyl-chitobiose core common to all *N*-glycans (Figure A14-1). Genes encoding these enzymes were found in several isolates of *B. longum*, *B. infantis*, and *B. breve*, and their presence correlated with the ability of these micro-organisms to release the *N*-linked glycan of bovine RNase B. Among these enzymes, those belonging to glycosyl hydrolase family 18 (GH18) were able to remove the *N*-glycans from bovine and human lactoferrin (Garrido et al., 2012b), containing high mannose and complex *N*-glycans, respectively (Nwosu et al., 2011; Yu et al., 2011). Further characterization by MALDI-Fourier transform ion cyclotron resonance (FTICR) MS of endoglycosidase EndoBI-1 from *B. infantis* ATCC 15697 (Blon_2468, Figure A14-2) revealed that this enzyme can deglycosylate common host glycoproteins such as IgA and IgG in their native forms in addition to human and bovine lactoferrin. Surprisingly, EndoBI-1 cleavage specificity was wide, releasing *N*-glycans with a variety of structures including high mannose *N*-linked glycans, or complex glycans with core α1-6 fucosylation, chain sialylation or fucosylation, and bi- and tri-antennary structures (Figure A14-1). Furthermore, incubation of the enzyme with fresh breast milk samples led to a complete removal of milk protein *N*-glycans.

Replicating the Bifidogenic Effect of Breast Milk

For some mothers, breastfeeding is not possible, and therefore there is an increased need for human milk substitutes. Commercial production of synthetic

mimics of HMO or other milk glycoconjugates is challenging, given the diversity of complex glycans involved. However, commercial production of more simple HMO species such as LNnT, 2′FL and 6′SL is now possible, as is the ability to test these molecules for bifidogenicity in animal (Marcobal et al., 2011) and human trials. Other prebiotics such as fructooligosaccharides (FOS), galacto-oligosaccharides (GOS) and inulin (Gibson et al., 2004; Torres et al., 2010) are commonly included in infant formula. GOS are synthetic substrates derived enzymically from the transglycosylation of lactose, with a degree of polymerization of 3–15 (Barboza et al., 2009). It has been suggested that GOS resemble galactan chains found in plant oligosaccharides (O'Connell Motherway et al., 2011a). We recently observed that the consumption of GOS with a large degree of polymerization is strain-dependent in *B. infantis* isolates (Garrido et al., 2013), and discrete mechanisms for import and intracellular degradation are active in *B. infantis* strain ATCC 15697. On the other hand, FOS and inulin are naturally found in chicory plants. The wide availability of FOS and GOS has enabled numerous in vitro, animal and human studies of their prebiotic effects, and their bifidogenic effect is currently accepted (Bakker-Zierikzee et al., 2005; Brunser et al., 2006; Davis et al., 2011).

Analysis of the milk of other mammals indicates that they do not possess the high concentration of oligosaccharides combined with a high level of fucosylation witnessed in human milks. For example, mature bovine milk is very low in free oligosaccharides, which are mainly sialylated (Sundekilde et al., 2012; Tao et al., 2008). More efficient analytical tools have recently revealed the presence of low concentrations of several neutral fucosylated oligosaccharides that resemble derivatives of lacto-*N*-neohexaose present in early milk HMO (Barile et al., 2010; Sundekilde et al., 2012). At present, new approaches are being applied to use dairy streams from cheese production to recover bovine milk oligosaccharides (BMO) in larger quantities (Zivkovic and Barile, 2011).

Conclusion and Future Directions

The complexity of breast milk is intriguing and still far from being understood. The fundamental role of human milk as a nutrient source for the infant has been the focus of study for decades, with the critical goal of understanding and improving nutritional deficiencies during the neonatal period. However, the influence of breastfeeding beyond nutrition is increasingly being revealed, demonstrating that milk provides much more than protection against pathogens. Breastfeeding has been associated with a variety of long-term health impacts including lowered incidence of obesity (Kalliomäki et al., 2008), diabetes (Mayer et al., 1988; Owen et al., 2006; Pettitt et al., 1997) and allergies (Gdalevich et al., 2001; Snijders et al., 2007). A future challenge will be to identify a mechanistic basis for these benefits.

An infant gut microbiota dominated by bifidobacteria has long been associated with health; however, our understanding of this process is still unclear.

Recently the protective role of production of short-chain fatty acids by certain species of bifidobacteria against pathogenic *E. coli* challenge (Fukuda et al., 2011) has been demonstrated. This work highlighted the importance of in situ metabolism of complex carbohydrates by bifidobacteria in host–microbe interactions. However, the amounts of protective acetate and lactate produced by bifidobacteria can be different depending on the growth substrate, which might have direct consequences for the host (Garrido et al., 2013). Consumption of certain HMO can also be a selective colonization factor; for example, *B. infantis* grows vigorously on lacto-*N*-neotetraose, and this ability enables the bacterium to outcompete *Bacteroides thetaiotamicron* in a mouse model, emphasizing the selectivity and bifidogenic activities of these unique glycans (Marcobal et al., 2011).

A clear benefit of mechanistic research is the rapid nature by which this information can be translated to address a range of intestinal maladies (Gordon et al., 2012). The ability to purify, or synthesize, HMO-like oligosaccharides and/or glycoconjugates at commercial scales is increasingly becoming a reality. This ability, combined with an expanding number of well-characterized bifidobacterial strains that grow on these complex milk glycans, will help to design tailored synbiotic formulations to target specific at-risk populations such as premature and malnourished infants. Given that milk is the product of millions of years of mammalian evolution, it is not surprising that it displays a constellation of health benefits for the infant. With the advances in nanotechnology and systems biology perhaps this "constellation" will become more comprehensible and inspire new opportunities for protective modulation of the human GI tract.

Acknowledgements

We acknowledge all the researchers in the UC Davis Foods for Health Institute and the Milk Bioactives Program for their enthusiasm, imagination and collective contribution to this subject matter. Work by the Milk Bioactives Program has been supported by University of California Discovery Grant Program, the California Dairy Research Foundation, the Bill and Melinda Gates Foundation, and National Institutes of Health awards R01HD059127,R01HD065122, R01HD061923, R21AT006180, R01AT007079. D.G. has been supported in part by a Fulbright-Conicyt Chile scholarship and a National Milk Producers Federation scholarship. D.A.M. acknowledges support as the Peter J. Shields Endowed Chair in Dairy Food Science.

R01HD059127R01HD065122R01HD061923R21AT006180R01AT007079
National Institutes of Health
University of California Discovery Grant Program
California Dairy Research Foundation
Bill and Melinda Gates Foundation

Fulbright-Conicyt Chile
National Milk Producers Federation

References

Adlerberth I., Lindberg E., Aberg N., Hesselmar B., Saalman R., Strannegård I. L., Wold A. E. (2006). Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? Pediatr Res 59, 96–101.

Albrecht S., Schols H. A., van den Heuvel E. G., Voragen A. G., Gruppen H. (2011). Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. Carbohydr Res 346, 2540–2550.

Allen J. C., Keller R. P., Archer P., Neville M. C. (1991). Studies in human lactation: milk composition and daily secretion rates of macronutrients in the first year of lactation. Am J Clin Nutr 54, 69–80.

American Academy of Pediatrics Section on Breastfeeding (2012). Breastfeeding and the use of human milk. Pediatrics 129, e827–e841.

Asakuma S., Hatakeyama E., Urashima T., Yoshida E., Katayama T., Yamamoto K., Kumagai H., Ashida H., Hirose J., Kitaoka M. (2011). Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J Biol Chem 286, 34583–34592.

Ashida H., Maki R., Ozawa H., Tani Y., Kiyohara M., Fujita M., Imamura A., Ishida H., Kiso M., Yamamoto K. (2008). Characterization of two different endo-α-N-acetylgalactosaminidases from probiotic and pathogenic enterobacteria, *Bifidobacterium longum* and *Clostridium perfringens*. Glycobiology 18, 727–734.

Ashida H., Miyake A., Kiyohara M., Wada J., Yoshida E., Kumagai H., Katayama T., Yamamoto K. (2009). Two distinct α-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology 19, 1010–1017.

Avershina E., Storrø O., Oien T., Johnsen R., Wilson R., Egeland T., Rudi K. (2013). Bifidobacterial succession and correlation networks in a large unselected cohort of mothers and their children. Appl Environ Microbiol 79, 497–507.

Bäckhed F., Ley R. E., Sonnenburg J. L., Peterson D. A., Gordon J. I. (2005). Host-bacterial mutualism in the human intestine. Science 307, 1915–1920.

Bager P., Wohlfahrt J., Westergaard T. (2008). Caesarean delivery and risk of atopy and allergic disease: meta-analyses. Clin Exp Allergy 38, 634–642.

Bakker-Zierikzee A. M., Alles M. S., Knol J., Kok F. J., Tolboom J. J., Bindels J. G. (2005). Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the intestinal microflora during the first 4 months of life. Br J Nutr 94, 783–790.

Barboza M., Sela D. A., Pirim C., Locascio R. G., Freeman S. L., German J. B., Mills D. A., Lebrilla C. B. (2009). Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans. Appl Environ Microbiol 75, 7319–7325.

Barboza M., Pinzon J., Wickramasinghe S., Froehlich J. W., Moeller I., Smilowitz J. T., Ruhaak L. R., Huang J., Lönnerdal B. & other authors (2012). Glycosylation of human milk lactoferrin exhibits dynamic changes during early lactation enhancing its role in pathogenic bacteria-host interactions. Mol Cell Proteomics 11, 015248.

Barile D., Marotta M., Chu C., Mehra R., Grimm R., Lebrilla C. B., German J. B. (2010). Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. J Dairy Sci 93, 3940–3949.

Bode L., Jantscher-Krenn E. (2012). Structure-function relationships of human milk oligosaccharides. Adv Nutr 3, 383S–391S.

Boesten R., Schuren F., Ben Amor K., Haarman M., Knol J., de Vos W. M. (2011). *Bifidobacterium* population analysis in the infant gut by direct mapping of genomic hybridization patterns: potential for monitoring temporal development and effects of dietary regimens. Microb Biotechnol 4, 417–427.

Bouhours J. F., Bouhours D. (1979). Galactosylceramide is the major cerebroside of human milk fat globule membrane. Biochem Biophys Res Commun 88, 1217–1222.

Brockhausen I., Schachter H., Stanley P. (2009). O-GalNAc glycans. In *Essentials of Glycobiology*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Brunser O., Gotteland M., Cruchet S., Figueroa G., Garrido D., Steenhout P. (2006). Effect of a milk formula with prebiotics on the intestinal microbiota of infants after an antibiotic treatment. Pediatr Res 59, 451–456.

Cabrera-Rubio R., Collado M. C., Laitinen K., Salminen S., Isolauri E., Mira A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. Am J Clin Nutr 96, 544–551.

Chaturvedi P., Warren C. D., Buescher C. R., Pickering L. K., Newburg D. S. (2001). Survival of human milk oligosaccharides in the intestine of infants. Adv Exp Med Biol 501, 315–323.

Chen J., Cai W., Feng Y. (2007). Development of intestinal bifidobacteria and lactobacilli in breast-fed neonates. Clin Nutr 26, 559–566.

Chichlowski M., De Lartigue G., German J. B., Raybould H. E., Mills D. A. (2012). Bifidobacteria isolated from infants and cultured on human milk oligosaccharides affect intestinal epithelial function. J Pediatr Gastroenterol Nutr 55, 321–327.

Cho I., Yamanishi S., Cox L., Methé B. A., Zavadil J., Li K., Gao Z., Mahana D., Raju K. & other authors (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature 488, 621–626.

Collado M. C., Laitinen K., Salminen S., Isolauri E. (2012). Maternal weight and excessive weight gain during pregnancy modify the immunomodulatory potential of breast milk. Pediatr Res 72, 77–85.

Coppa G. V., Pierani P., Zampini L., Carloni I., Carlucci A., Gabrielli O. (1999). Oligosaccharides in human milk during different phases of lactation. Acta Paediatr Suppl 88, 89–94.

Coppa G. V., Zampini L., Galeazzi T., Facinelli B., Ferrante L., Capretti R., Orazio G. (2006). Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatr Res 59, 377–382.

Corfield A. P., Wagner S. A., Clamp J. R., Kriaris M. S., Hoskins L. C. (1992). Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect Immun 60, 3971–3978.

Crociani F., Alessandrini A., Mucci M. M., Biavati B. (1994). Degradation of complex carbohydrates by *Bifidobacterium* spp. Int J Food Microbiol 24, 199–210.

Dallas D. C., Martin W. F., Strum J. S., Zivkovic A. M., Smilowitz J. T., Underwood M. A., Affolter M., Lebrilla C. B., German J. B. (2011). N-Linked glycan profiling of mature human milk by high-performance microfluidic chip liquid chromatography time-of-flight tandem mass spectrometry. J Agric Food Chem 59, 4255–4263.

Dallas D. C., Sela D., Underwood M. A., German J. B., Lebrilla C. B. (2012). Protein-linked glycan degradation in infants fed human milk. J Glycomics Lipidomics S1, 002.

Davidson L. A., Lönnerdal B. (1987). Persistence of human milk proteins in the breast-fed infant. Acta Paediatr Scand 76, 733–740.

Davis L. M., Martínez I., Walter J., Goin C., Hutkins R. W. (2011). Barcoded pyrosequencing reveals that consumption of galactooligosaccharides results in a highly specific bifidogenic response in humans. PLoS ONE 6, e25200.

De Leoz M. L., Gaerlan S. C., Strum J. S., Dimapasoc L. M., Mirmiran M., Tancredi D. J., Smilowitz J. T., Kalanetra K. M., Mills D. A. & other authors (2012). Lacto-*N*-tetraose, fucosylation, and secretor status are highly variable in human milk oligosaccharides from women delivering preterm. J Proteome Res 11, 4662–4672.

Derrien M., van Passel M. W., van de Bovenkamp J. H., Schipper R. G., de Vos W. M., Dekker J. (2010). Mucin-bacterial interactions in the human oral cavity and digestive tract. Gut Microbes 1, 254–268.

Dewey K. G., Heinig M. J., Nommsen-Rivers L. A. (1995). Differences in morbidity between breast-fed and formula-fed infants. J Pediatr 126, 696–702.

Dominguez-Bello M. G., Costello E. K., Contreras M., Magris M., Hidalgo G., Fierer N., Knight R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 107, 11971–11975.

Engfer M. B., Stahl B., Finke B., Sawatzki G., Daniel H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71, 1589–1596.

Falk P., Hoskins L. C., Larson G. (1990). Bacteria of the human intestinal microbiota produce glycosidases specific for lacto-series glycosphingolipids. J Biochem 108, 466–474.

Falk P., Hoskins L. C., Larson G. (1991). Enhancing effects of bile salts on the degradation of glycosphingolipids by glycosidases from bacteria of the human fecal flora. Biochim Biophys Acta 1084, 139–148.

Fallani M., Young D., Scott J., Norin E., Amarri S., Adam R., Aguilera M., Khanna S., Gil A. & other authors (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 51, 77–84.

Fewtrell M. S., Morgan J. B., Duggan C., Gunnlaugsson G., Hibberd P. L., Lucas A., Kleinman R. E. (2007). Optimal duration of exclusive breastfeeding: what is the evidence to support current recommendations? Am J Clin Nutr 85, 635S–638S.

Froehlich J. W., Dodds E. D., Barboza M., McJimpsey E. L., Seipert R. R., Francis J., An H. J., Freeman S., German J. B., Lebrilla C. B. (2010). Glycoprotein expression in human milk during lactation. J Agric Food Chem 58, 6440–6448.

Fujita K., Oura F., Nagamine N., Katayama T., Hiratake J., Sakata K., Kumagai H., Yamamoto K. (2005). Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-α*N*-acetylgalactosaminidase from *Bifidobacterium longum*. J Biol Chem 280, 37415–37422.

Fukuda S., Toh H., Hase K., Oshima K., Nakanishi Y., Yoshimura K., Tobe T., Clarke J. M., Topping D. L. & other authors (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547.

Garrido D., Kim J. H., German J. B., Raybould H. E., Mills D. A. (2011). Oligosaccharide binding proteins from *Bifidobacterium longum* subsp. infantis reveal a preference for host glycans. PLoS ONE 6, e17315.

Garrido D., Barile D., Mills D. A. (2012a). A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. Adv Nutr 3, 415S–421S.

Garrido D., Nwosu C., Ruiz-Moyano S., Aldredge D., German J. B., Lebrilla C. B., Mills D. A. (2012b). Endo-β-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex *N*-glycans from human milk glycoproteins. Mol Cell Proteomics 11, 775–785.

Garrido D., Ruiz-Moyano S., Mills D. A. (2012c). Release and utilization of *N*-acetyl-D-glucosamine from human milk oligosaccharides by *Bifidobacterium longum* subsp. *infantis*. Anaerobe 18, 430–435.

Garrido D., Ruiz-Moyano S., Jimenez-Espinoza R., Eom H. J., Block D. E., Mills D. A. (2013). Utilization of galactooligosaccharides by Bifidobacterium longum subsp. infantis isolates. Food Microbiol 33, 262–270.

Gdalevich M., Mimouni D., David M., Mimouni M. (2001). Breast-feeding and the onset of atopic dermatitis in childhood: a systematic review and meta-analysis of prospective studies. J Am Acad Dermatol 45, 520–527.

Gibson G. R., Probert H. M., Loo J. V., Rastall R. A., Roberfroid M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17, 259–275.

Gnoth M. J., Kunz C., Kinne-Saffran E., Rudloff S. (2000). Human milk oligosaccharides are minimally digested in vitro. J Nutr 130, 3014–3020.

Gomes A. M., Malcata F. X., Klaver F. A. (1998). Growth enhancement of *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki by milk hydrolyzates. J Dairy Sci 81, 2817–2825.

González R., Klaassens E. S., Malinen E., de Vos W. M., Vaughan E. E. (2008). Differential transcriptional response of *Bifidobacterium longum* to human milk, formula milk, and galactooligosaccharide. Appl Environ Microbiol 74, 4686–4694.

Gopal P. K., Gill H. S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br J Nutr 84 (Suppl. 1), S69–S74.

Gordon J. I., Dewey K. G., Mills D. A., Medzhitov R. M. (2012). The human gut microbiota and undernutrition. Sci Transl Med 4, 37ps12.

Grönlund M. M., Gueimonde M., Laitinen K., Kociubinski G., Grönroos T., Salminen S., Isolauri E. (2007). Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the *Bifidobacterium* microbiota in infants at risk of allergic disease. Clin Exp Allergy 37, 1764–1772.

Gustafsson B. E., Karlsson K. A., Larson G., Midtvedt T., Strömberg N., Teneberg S., Thurin J. (1986). Glycosphingolipid patterns of the gastrointestinal tract and feces of germ-free and conventional rats. J Biol Chem 261, 15294–15300.

Haarman M., Knol J. (2005). Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 71, 2318–2324.

Hakkarainen J., Toivanen M., Leinonen A., Frängsmyr L., Strömberg N., Lapinjoki S., Nassif X., Tikkanen-Kaukanen C. (2005). Human and bovine milk oligosaccharides inhibit *Neisseria meningitidis* pili attachment in vitro. J Nutr 135, 2445–2448.

Hamosh M. (2001). Bioactive factors in human milk. Pediatr Clin North Am 48, 69–86.

Harmsen H. J., Wildeboer-Veloo A. C., Raangs G. C., Wagendorp A. A., Klijn N., Bindels J. G., Welling G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 30, 61–67.

Harris K., Kassis A., Major G., Chou C. J. (2012). Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? J Obes 2012, 879151.

Håversen L., Ohlsson B. G., Hahn-Zoric M., Hanson L. A., Mattsby-Baltzer I. (2002). Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB. Cell Immunol 220, 83–95.

Hernell O. (2011). Human milk vs. cow's milk and the evolution of infant formulas. Nestle Nutr Workshop Ser Pediatr Program 67, 17–28.

Hong P., Ninonuevo M. R., Lee B., Lebrilla C., Bode L. (2009). Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN). Br J Nutr 101, 482–486.

Hooper L. V., Littman D. R., Macpherson A. J. (2012). Interactions between the microbiota and the immune system. Science 336, 1268–1273.

Hoskins L. C., Agustines M., McKee W. B., Boulding E. T., Kriaris M., Niedermeyer G. (1985). Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. J Clin Invest 75, 944–953.

Imberty A., Varrot A. (2008). Microbial recognition of human cell surface glycoconjugates. Curr Opin Struct Biol 18, 567–576.

Jakobsson I., Lindberg T., Benediktsson B. (1982). In vitro digestion of cow's milk proteins by duodenal juice from infants with various gastrointestinal disorders. J Pediatr Gastroenterol Nutr 1, 183–192.

Janer C., Pelaez C., Requena T. (2004). Caseinomacropeptide and whey protein concentrate enhance *Bifidobacterium lactis* growth in milk. Food Chem 86, 263–267.

Jensen R. G. (1999). Lipids in human milk. Lipids 34, 1243–1271.

Jollès P., Jollès J. (1961). Lysozyme from human milk. Nature 192, 1187–1188.

Jost T., Lacroix C., Braegger C. P., Chassard C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. PLoS ONE 7, e44595.

Kalliomäki M., Collado M. C., Salminen S., Isolauri E. (2008). Early differences in fecal microbiota composition in children may predict overweight. Am J Clin Nutr 87, 534–538.

Kitaoka M., Tian J., Nishimoto M. (2005). Novel putative galactose operon involving lacto-*N*-biose phosphorylase in *Bifidobacterium longum*. Appl Environ Microbiol 71, 3158–3162.

Kiyohara M., Tanigawa K., Chaiwangsri T., Katayama T., Ashida H., Yamamoto K. (2011). An exo-α-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. Glycobiology 21, 437–447.

Kiyohara M., Nakatomi T., Kurihara S., Fushinobu S., Suzuki H., Tanaka T., Shoda S., Kitaoka M., Katayama T. & other authors (2012). α-*N*-Acetylgalactosaminidase from infant-associated bifidobacteria belonging to novel glycoside hydrolase family 129 is implicated in alternative mucin degradation pathway. J Biol Chem 287, 693–700.

Koenig J. E., Spor A., Scalfone N., Fricker A. D., Stombaugh J., Knight R., Angenent L. T., Ley R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 108 (Suppl. 1), 4578–4585.

Koletzko B. (2010). Innovations in infant milk feeding: from the past to the future. Nestle Nutr Workshop Ser Pediatr Program 66, 1–17.

Kunz C. (2012). Historical aspects of human milk oligosaccharides. Adv Nutr 3, 430S–439S.

Kunz C., Rudloff S., Baier W., Klein N., Strobel S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 20, 699–722.

Laegreid A., Kolstø Otnaess A. B., Bryn K. (1986). Purification of human milk gangliosides by silica gel chromatography and analysis of trifluoroacetate derivatives by gas chromatography. J Chromatogr A 377, 59–67.

Larson G., Midtvedt T. (1989). Glycosphingolipids in feces of germ-free rats as a source for studies of developmental changes of intestinal epithelial cell surface carbohydrates. Glycoconj J 6, 285–292.

Larson G., Watsfeldt P., Falk P., Leffler H., Koprowski H. (1987). Fecal excretion of intestinal glycosphingolipids by newborns and young children. FEBS Lett 214, 41–44.

Larson G., Falk P., Hoskins L. C. (1988). Degradation of human intestinal glycosphingolipids by extracellular glycosidases from mucin-degrading bacteria of the human fecal flora. J Biol Chem 263, 10790–10798.

Le T. T., Van de Wiele T., Do T. N., Debyser G., Struijs K., Devreese B., Dewettinck K., Van Camp J. (2012). Stability of milk fat globule membrane proteins toward human enzymatic gastrointestinal digestion. J Dairy Sci 95, 2307–2318.

Le Huërou-Luron I., Blat S., Boudry G. (2010). Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. Nutr Res Rev 23, 23–36.

Lee J. H., Karamychev V. N., Kozyavkin S. A., Mills D., Pavlov A. R., Pavlova N. V., Polouchine N. N., Richardson P. M., Shakhova V. V. & other authors (2008). Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. BMC Genomics 9, 247.

Lee H., An H. J., Lerno L. A. Jr., German J. B., Lebrilla C. B. (2011). Rapid profiling of bovine and human milk gangliosides by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. Int J Mass Spectrom 305, 138–150.

Ley R. E., Bäckhed F., Turnbaugh P., Lozupone C. A., Knight R. D., Gordon J. I. (2005). Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 102, 11070–11075.

Lindberg A. A., Brown J. E., Strömberg N., Westling-Ryd M., Schultz J. E., Karlsson K. A. (1987). Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J Biol Chem 262, 1779–1785.

Lindh E. (1975). Increased resistance of immunoglobulin A dimers to proteolytic degradation after binding of secretory component. J Immunol 114, 284–286.

Lindquist S., Hernell O. (2010). Lipid digestion and absorption in early life: an update. Curr Opin Clin Nutr Metab Care 13, 314–320.

LoCascio R. G., Niñonuevo M. R., Freeman S. L., Sela D. A., Grimm R., Lebrilla C. B., Mills D. A., German J. B. (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J Agric Food Chem 55, 8914–8919.

LoCascio R. G., Niñonuevo M. R., Kronewitter S. R., Freeman S. L., German J. B., Lebrilla C. B., Mills D. A. (2009). A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microb Biotechnol 2, 333–342.

LoCascio R. G., Desai P., Sela D. A., Weimer B., Mills D. A. (2010). Broad conservation of milk utilization genes in *Bifidobacterium longum* subsp. *infantis* as revealed by comparative genomic hybridization. Appl Environ Microbiol 76, 7373–7381.

Lönnerdal B. (2009). Nutritional roles of lactoferrin. Curr Opin Clin Nutr Metab Care 12, 293–297.

Lönnerdal B. (2010). Bioactive proteins in human milk: mechanisms of action. J Pediatr 156 (Suppl.), S26–S30.

Makino H., Kushiro A., Ishikawa E., Muylaert D., Kubota H., Sakai T., Oishi K., Martin R., Ben Amor K. & other authors (2011). Transmission of intestinal *Bifidobacterium longum* subsp. *longum* strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. Appl Environ Microbiol 77, 6788–6793.

Mangin I., Suau A., Magne F., Garrido D., Gotteland M., Neut C., Pochart P. (2006). Characterization of human intestinal bifidobacteria using competitive PCR and PCR-TTGE. FEMS Microbiol Ecol 55, 28–37.

Marcobal A., Barboza M., Froehlich J. W., Block D. E., German J. B., Lebrilla C. B., Mills D. A. (2010). Consumption of human milk oligosaccharides by gut-related microbes. J Agric Food Chem 58, 5334–5340.

Marcobal A., Barboza M., Sonnenburg E. D., Pudlo N., Martens E. C., Desai P., Lebrilla C. B., Weimer B. C., Mills D. A. & other authors (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe 10, 507–514.

Martens E. C., Roth R., Heuser J. E., Gordon J. I. (2009). Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. J Biol Chem 284, 18445–18457.

Martín-Sosa S., Martín M. J., Hueso P. (2002). The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic *Escherichia coli* human strains. J Nutr 132, 3067–3072.

Martirosian G., Kuipers S., Verbrugh H., van Belkum A., Meisel-Mikolajczyk F. (1995). PCR ribotyping and arbitrarily primed PCR for typing strains of *Clostridium difficile* from a Polish maternity hospital. J Clin Microbiol 33, 2016–2021.

Matsuki T., Watanabe K., Fujimoto J., Miyamoto Y., Takada T., Matsumoto K., Oyaizu H., Tanaka R. (2002). Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68, 5445–5451.

Mayer E. J., Hamman R. F., Gay E. C., Lezotte D. C., Savitz D. A., Klingensmith G. J. (1988). Reduced risk of IDDM among breast-fed children. The Colorado IDDM Registry. Diabetes 37, 1625–1632.

Midtvedt T., Carlstedt-Duke B., Höverstad T., Midtvedt A. C., Norin K. E., Saxerholt H. (1987). Establishment of a biochemically active intestinal ecosystem in ex-germfree rats. Appl Environ Microbiol 53, 2866–2871.

Midtvedt A. C., Carlstedt-Duke B., Norin K. E., Saxerholt H., Midtvedt T. (1988). Development of five metabolic activities associated with the intestinal microflora of healthy infants. J Pediatr Gastroenterol Nutr 7, 559–567.

Miller-Podraza H., Lanne B., Angström J., Teneberg S., Milh M. A., Jovall P. A., Karlsson H., Karlsson K. A. (2005). Novel binding epitope for *Helicobacter pylori* found in neolacto carbohydrate chains: structure and cross-binding properties. J Biol Chem 280, 19695–19703.

Mitoulas L. R., Kent J. C., Cox D. B., Owens R. A., Sherriff J. L., Hartmann P. E. (2002). Variation in fat, lactose and protein in human milk over 24 h and throughout the first year of lactation. Br J Nutr 88, 29–37.

Mitsou E. K., Kirtzalidou E., Oikonomou I., Liosis G., Kyriacou A. (2008). Fecal microflora of Greek healthy neonates. Anaerobe 14, 94–101.

Miwa M., Horimoto T., Kiyohara M., Katayama T., Kitaoka M., Ashida H., Yamamoto K. (2010). Cooperation of β-galactosidase and β-*N*-acetylhexosaminidase from bifidobacteria in assimilation of human milk oligosaccharides with type 2 structure. Glycobiology 20, 1402–1409.

Moro E. (1905). Morphologische und biologische Untersuchung über die Darmbakterien des Säuglings. Jahrb f Kinderh 61, 687–734.

Morrow A. L., Ruiz-Palacios G. M., Altaye M., Jiang X., Guerrero M. L., Meinzen-Derr J. K., Farkas T., Chaturvedi P., Pickering L. K., Newburg D. S. (2004). Human milk oligosaccharide blood group epitopes and innate immune protection against *Campylobacter* and calicivirus diarrhea in breastfed infants. Adv Exp Med Biol 554, 443–446.

Morrow A. L., Ruiz-Palacios G. M., Jiang X., Newburg D. S. (2005). Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. J Nutr 135, 1304–1307.

Mukai T., Kaneko S., Matsumoto M., Ohori H. (2004). Binding of *Bifidobacterium bifidum* and *Lactobacillus reuteri* to the carbohydrate moieties of intestinal glycolipids recognized by peanut agglutinin. Int J Food Microbiol 90, 357–362.

Neeser J. R., Granato D., Rouvet M., Servin A., Teneberg S., Karlsson K. A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 10, 1193–1199.

Neu J. (2007). Gastrointestinal development and meeting the nutritional needs of premature infants. Am J Clin Nutr 85, 629S–634S.

Newburg D. S. (2009). Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J Anim Sci 87 (Suppl.), 26–34.

Newburg D. S., Chaturvedi P. (1992). Neutral glycolipids of human and bovine milk. Lipids 27, 923–927.

Newburg D. S., Ruiz-Palacios G. M., Altaye M., Chaturvedi P., Meinzen-Derr J., Guerrero M. L., Morrow A. L. (2004). Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. Glycobiology 14, 253–263.

Newburg D. S., Ruiz-Palacios G. M., Morrow A. L. (2005). Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr 25, 37–58.

Nicholson J. K., Holmes E., Kinross J., Burcelin R., Gibson G., Jia W., Pettersson S. (2012). Host-gut microbiota metabolic interactions. Science 336, 1262–1267.

Niñonuevo M. R., Perkins P. D., Francis J., Lamotte L. M., LoCascio R. G., Freeman S. L., Mills D. A., German J. B., Grimm R., Lebrilla C. B. (2008). Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry. J Agric Food Chem 56, 618–626.

Nishimoto M., Kitaoka M. (2007). Identification of *N*-acetylhexosamine 1-kinase in the complete lacto-*N*-biose I/galacto-*N*-biose metabolic pathway in *Bifidobacterium longum*. Appl Environ Microbiol 73, 6444–6449.

Nwosu C. C., Seipert R. R., Strum J. S., Hua S. S., An H. J., Zivkovic A. M., German B. J., Lebrilla C. B. (2011). Simultaneous and extensive site-specific *N*- and *O*-glycosylation analysis in protein mixtures. J Proteome Res 10, 2612–2624.

Nwosu C. C., Aldredge D. L., Lee H., Lerno L. A., Zivkovic A. M., German J. B., Lebrilla C. B. (2012). Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. J Proteome Res 11, 2912–2924.

O'Connell Motherway M., Fitzgerald G. F., van Sinderen D. (2011a). Metabolism of a plant derived galactose-containing polysaccharide by *Bifidobacterium breve* UCC2003. Microb Biotechnol 4, 403–416.

O'Connell Motherway M., Zomer A., Leahy S. C., Reunanen J., Bottacini F., Claesson M. J., O'Brien F., Flynn K., Casey P. G. & other authors (2011b). Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc Natl Acad Sci U S A 108, 11217–11222.

Otnaess A. B., Laegreid A., Ertresvåg K. (1983). Inhibition of enterotoxin from *Escherichia coli* and *Vibrio cholerae* by gangliosides from human milk. Infect Immun 40, 563–569.

Owen C. G., Martin R. M., Whincup P. H., Smith G. D., Cook D. G. (2006). Does breastfeeding influence risk of type 2 diabetes in later life? A quantitative analysis of published evidence. Am J Clin Nutr 84, 1043–1054.

Penders J., Thijs C., Vink C., Stelma F. F., Snijders B., Kummeling I., van den Brandt P. A., Stobberingh E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511–521.

Peterson J. A., Patton S., Hamosh M. (1998). Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections. Biol Neonate 74, 143–162.

Petherick A. (2010). Development: mother's milk: a rich opportunity. Nature 468, S5–S7.

Petschow B. W., Talbott R. D. (1990). Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. J Clin Microbiol 28, 287–292.

Pettitt D. J., Forman M. R., Hanson R. L., Knowler W. C., Bennett P. H. (1997). Breastfeeding and incidence of non-insulin-dependent diabetes mellitus in Pima Indians. Lancet 350, 166–168.

Picciano M. F. (2001). Nutrient composition of human milk. Pediatr Clin North Am 48, 53–67.

Polonowski M., Lespagnol A. (1931). Sur deux nouveaux sucres du lait de femme, le gynolactose et l'allolactose. C R Acad Sci 192, 1319.

Prentice A., MacCarthy A., Stirling D. M., Vasquez-Velasquez L., Ceesay S. M. (1989). Breast-milk IgA and lactoferrin survival in the gastrointestinal tract – a study in rural Gambian children. Acta Paediatr Scand 78, 505–512.

Reinhardt C., Reigstad C. S., Bäckhed F. (2009). Intestinal microbiota during infancy and its implications for obesity. J Pediatr Gastroenterol Nutr 48, 249–256.

Roger L. C., McCartney A. L. (2010). Longitudinal investigation of the faecal microbiota of healthy full-term infants using fluorescence in situ hybridization and denaturing gradient gel electrophoresis. Microbiology 156, 3317–3328.

Ruas-Madiedo P., Gueimonde M., Fernández-García M., de los Reyes-Gavilán C. G., Margolles A. (2008). Mucin degradation by *Bifidobacterium* strains isolated from the human intestinal microbiota. Appl Environ Microbiol 74, 1936–1940.

Rudd P. M., Joao H. C., Coghill E., Fiten P., Saunders M. R., Opdenakker G., Dwek R. A. (1994). Glycoforms modify the dynamic stability and functional activity of an enzyme. Biochemistry 33, 17–22.

Rudloff S., Kunz C. (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr 3, 398S–405S.

Ruhaak L. R., Lebrilla C. B. (2012). Advances in analysis of human milk oligosaccharides. Adv Nutr 3, 406S–414S.

Ruiz-Palacios G. M., Cervantes L. E., Ramos P., Chavez-Munguia B., Newburg D. S. (2003). *Campylobacter jejuni* binds intestinal H(O) antigen (Fucα1, 2Galβ1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J Biol Chem 278, 14112–14120.

Sakata S., Tonooka T., Ishizeki S., Takada M., Sakamoto M., Fukuyama M., Benno Y. (2005). Culture-independent analysis of fecal microbiota in infants, with special reference to *Bifidobacterium* species. FEMS Microbiol Lett 243, 417–423.

Salvini F., Riva E., Salvatici E., Boehm G., Jelinek J., Banderali G., Giovannini M. (2011). A specific prebiotic mixture added to starting infant formula has long-lasting bifidogenic effects. J Nutr 141, 1335–1339.

Schell M. A., Karmirantzou M., Snel B., Vilanova D., Berger B., Pessi G., Zwahlen M. C., Desiere F., Bork P. & other authors (2002). The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 99, 14422–14427.

Scholtens P. A., Oozeer R., Martin R., Amor K. B., Knol J. (2012). The early settlers: intestinal microbiology in early life. Annu Rev Food Sci Technol 3, 425–447.

Sela D. A., Mills D. A. (2010). Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. Trends Microbiol 18, 298–307.

Sela D. A., Chapman J., Adeuya A., Kim J. H., Chen F., Whitehead T. R., Lapidus A., Rokhsar D. S., Lebrilla C. B. & other authors (2008). The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 105, 18964–18969.

Sela D. A., Price N. P., Mills D. A. (2010). Metabolism of bifidobacteria. In Bifidobacteria: Genomics and Molecular Aspects, pp. 45–70. Edited by Mayo B., van Sinderen D. . Norwich, UK: Caister Academic Press.

Sela D. A., Li Y., Lerno L., Wu S., Marcobal A. M., German J. B., Chen X., Lebrilla C. B., Mills D. A. (2011). An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides. J Biol Chem 286, 11909–11918.

Sela D. A., Garrido D., Lerno L., Wu S., Tan K., Eom H. J., Joachimiak A., Lebrilla C. B., Mills D. A. (2012). Bifidobacterium longum subsp. infantis ATCC 15697 α-fucosidases are active on fucosylated human milk oligosaccharides. Appl Environ Microbiol 78, 795–803.

Snijders B. E., Thijs C., Dagnelie P. C., Stelma F. F., Mommers M., Kummeling I., Penders J., van Ree R., van den Brandt P. A. (2007). Breast-feeding duration and infant atopic manifestations, by maternal allergic status, in the first 2 years of life (KOALA study). J Pediatr 151, 347–351, e1–e2.

Stanley P., Schachter H., Taniguchi N. (2009). N-Glycans. In Essentials of Glycobiology, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Strömberg N., Ryd M., Lindberg A. A., Karlsson K.-A. (1988). Studies on the binding of bacteria to glycolipids. Two species of Propionibacterium apparently recognize separate epitopes on lactose of lactosylceramide. FEBS Lett 232, 193–198.

Sundekilde U. K., Barile D., Meyrand M., Poulsen N. A., Larsen L. B., Lebrilla C. B., German J. B., Bertram H. C. (2012). Natural variability in bovine milk oligosaccharides from Danish Jersey and Holstein-Friesian breeds. J Agric Food Chem 60, 6188–6196.

Tao N., DePeters E. J., Freeman S., German J. B., Grimm R., Lebrilla C. B. (2008). Bovine milk glycome. J Dairy Sci 91, 3768–3778.

Tao N., Wu S., Kim J., An H. J., Hinde K., Power M. L., Gagneux P., German J. B., Lebrilla C. B. (2011). Evolutionary glycomics: characterization of milk oligosaccharides in primates. J Proteome Res 10, 1548–1557.

Taufik E., Fukuda K., Senda A., Saito T., Williams C., Tilden C., Eisert R., Oftedal O., Urashima T. (2012). Structural characterization of neutral and acidic oligosaccharides in the milks of strepsirrhine primates: greater galago, aye-aye, Coquerel's sifaka and mongoose lemur. Glycoconj J 29, 119–134.

Torres D. P. M., Gonçalves M. P. F., Teixeira J. A., Rodrigues L. R. (2010). Galacto-oligosaccharides: production, properties, applications, and significance as prebiotics. Comprehensive Reviews in Food Science and Food Safety 9, 438–454.

Totten S. M., Zivkovic A. M., Wu S., Ngyuen U., Freeman S. L., Ruhaak L. R., Darboe M. K., German J. B., Prentice A. M., Lebrilla C. B. (2012). Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. J Proteome Res 11, 6124–6133.

Turroni F., Bottacini F., Foroni E., Mulder I., Kim J. H., Zomer A., Sánchez B., Bidossi A., Ferrarini A. & other authors (2010). Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 107, 19514–19519.

Turroni F., Peano C., Pass D. A., Foroni E., Severgnini M., Claesson M. J., Kerr C., Hourihane J., Murray D. & other authors (2012). Diversity of bifidobacteria within the infant gut microbiota. PLoS ONE 7, e36957.

Urashima T., Asakuma S., Leo F., Fukuda K., Messer M., Oftedal O. T. (2012). The predominance of type I oligosaccharides is a feature specific to human breast milk. Adv Nutr 3, 473S–482S.

van Berkel P. H., Geerts M. E., van Veen H. A., Kooiman P. M., Pieper F. R., de Boer H. A., Nuijens J. H. (1995). Glycosylated and unglycosylated human lactoferrins both bind iron and show identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibilities towards tryptic proteolysis. Biochem J 312, 107–114.

Variyam E. P., Hoskins L. C. (1981). Mucin degradation in human colon ecosystems. Degradation of hog gastric mucin by fecal extracts and fecal cultures. Gastroenterology 81, 751–758.

Wada J., Ando T., Kiyohara M., Ashida H., Kitaoka M., Yamaguchi M., Kumagai H., Katayama T., Yamamoto K. (2008). Bifidobacterium bifidum lacto-*N*-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure. Appl Environ Microbiol 74, 3996–4004.

Ward R. E., Niñonuevo M., Mills D. A., Lebrilla C. B., German J. B. (2006). In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*. Appl Environ Microbiol 72, 4497–4499.

Wright D. P., Rosendale D. I., Robertson A. M. (2000). *Prevotella* enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. FEMS Microbiol Lett 190, 73–79.

Wu S., Tao N., German J. B., Grimm R., Lebrilla C. B. (2010). Development of an annotated library of neutral human milk oligosaccharides. J Proteome Res 9, 4138–4151.

Wu S., Grimm R., German J. B., Lebrilla C. B. (2011). Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res 10, 856–868.

Xanthou M., Bines J., Walker W. A. (1995). Human milk and intestinal host defense in newborns: an update. Adv Pediatr 42, 171–208.

Xiao J. Z., Takahashi S., Nishimoto M., Odamaki T., Yaeshima T., Iwatsuki K., Kitaoka M. (2010). Distribution of in vitro fermentation ability of lacto-*N*-biose I, a major building block of human milk oligosaccharides, in bifidobacterial strains. Appl Environ Microbiol 76, 54–59.

Yamamoto K., Miwa T., Taniguchi H., Nagano T., Shimamura K., Tanaka T., Kumagai H. (1996). Binding specificity of *Lactobacillus* to glycolipids. Biochem Biophys Res Commun 228, 148–152.

Yatsunenko T., Rey F. E., Manary M. J., Trehan I., Dominguez-Bello M. G., Contreras M., Magris M., Hidalgo G., Baldassano R. N. & other authors (2012). Human gut microbiome viewed across age and geography. Nature 486, 222–227.

Ye A., Cui J., Singh H. (2011). Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. J Dairy Sci 94, 2762–2770.

Yoshida E., Sakurama H., Kiyohara M., Nakajima M., Kitaoka M., Ashida H., Hirose J., Katayama T., Yamamoto K., Kumagai H. (2012). *Bifidobacterium longum* subsp. *infantis* uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. Glycobiology 22, 361–368.

Yu T., Guo C., Wang J., Hao P., Sui S., Chen X., Zhang R., Wang P., Yu G. & other authors (2011). Comprehensive characterization of the site-specific *N*-glycosylation of wild-type and recombinant human lactoferrin expressed in the milk of transgenic cloned cattle. Glycobiology 21, 206–224.

Zivkovic A. M., Barile D. (2011). Bovine milk as a source of functional oligosaccharides for improving human health. Adv Nutr 2, 284–289.

Zivkovic A. M., German J. B., Lebrilla C. B., Mills D. A. (2011). Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc Natl Acad Sci U S A 108 (Suppl. 1), 4653–4658.

A15

BACTERIOPHAGE ADHERING TO MUCUS PROVIDE A NON–HOST-DERIVED IMMUNITY[61]

Jeremy J. Barr,[62,63] *Rita Auro*,[62] *Mike Furlan*,[62] *Katrine L. Whiteson*,[62] *Marcella L. Erb*,[64] *Joe Pogliano*,[64] *Aleksandr Stotland*,[62] *Roland Wolkowicz*,[62] *Andrew S. Cutting*,[62] *Kelly S. Doran*,[62] *Peter Salamon*,[65] *Merry Youle*,[66] *and Forest* ***Rohwer***[62]

Abstract

Mucosal surfaces are a main entry point for pathogens and the principal sites of defense against infection. Both bacteria and phage are associated with this mucus. Here we show that phage-to-bacteria ratios were increased, relative to the adjacent environment, on all mucosal surfaces sampled, ranging from cnidarians to humans. In vitro studies of tissue culture cells with and without surface mucus demonstrated that this increase in phage abundance is mucus dependent and protects the underlying epithelium from bacterial infection. Enrichment of phage in mucus occurs via binding interactions between mucin glycoproteins and Ig-like protein domains exposed on phage capsids. In particular, phage Ig-like domains bind variable glycan residues that coat the mucin glycoprotein component of mucus. Metagenomic analysis found these Ig-like proteins present in the phages sampled from many environments, particularly from locations adjacent to mucosal surfaces. Based on these observations, we present the bacteriophage adherence to mucus model that provides a ubiquitous, but non–host-derived, immunity

[61] Reprinted with permission from *Proceedings of the National Academy of Sciences*.

[62] Department of Biology, San Diego State University, San Diego, CA 92182.
[63] To whom correspondence should be addressed. E-mail: jeremybarr85@gmail.com.
[64] Division of Biological Sciences, University of California, San Diego, CA 92093.
[65] Department of Mathematics and Statistics, San Diego State University, San Diego, CA 92182.
[66] Rainbow Rock, Ocean View, HI 96737.

Author contributions: J.J.B. and F.R. designed research; J.J.B., R.A., K.L.W., M.L.E., J.P., A.S.C., and P.S. performed research; J.J.B., K.L.W., M.L.E., J.P., A.S., R.W., A.S.C., and K.S.D. contributed new reagents/analytic tools; J.J.B., R.A., M.F., K.L.W., A.S., R.W., P.S., M.Y., and F.R. analyzed data; and J.J.B., M.Y., and F.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: Raw glycan array data are available from the Consortium for Functional Glycomics (accession no. 2621).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10. 1073/pnas.1305923110/-/DCSupplemental.

applicable to mucosal surfaces. The model suggests that metazoan mucosal surfaces and phage co-evolve to maintain phage adherence. This benefits the metazoan host by limiting mucosal bacteria, and benefits the phage through more frequent interactions with bacterial hosts. The relationships shown here suggest a symbiotic relationship between phage and metazoan hosts that provides a previously unrecognized antimicrobial defense that actively protects mucosal surfaces.

Mucosal surfaces are the primary zones where animals meet their environment, and thus also the main points of entry for pathogenic microorganisms. The mucus layer is heavily colonized by bacteria, including many symbionts that contribute additional genetic and metabolic potential to the host (Bäckhed et al., 2005; Dethlefsen et al., 2007). Bacterial symbionts associated with a variety of other host surfaces also provide goods and services such as nutrients (Clay and Holah, 1999; Douglas, 1989; Hooper et al., 2002; Hosokawa et al., 2010), bioluminescence (Nyholm and McFall-Ngai, 2004; Ruby, 1996), and antibiotics (Currie et al., 1999; Kaltenpoth et al., 2005). These resident symbionts benefit from increased nutrient availability (Berry et al., 2013; Hooper et al., 2002; Martens et al., 2008; Sonnenburg et al., 2005), as well as the opportunity for both vertical transmission and increased dissemination (Chow et al., 2010; Sachs et al., 2011; Stouthamer et al., 1999).

Within the mucus, the predominant macromolecules are the large (up to 10^6–10^9 Da) mucin glycoproteins. The amino acid backbone of these proteins incorporates tandem repeats of exposed hydrophobic regions alternating with blocks bearing extensive O-linked glycosylation (Cone, 2009). Hundreds of variable, branched, negatively charged glycan chains extend 0.5–5 nm from the peptide core outward into the surrounding environment (Cone, 209; Linden et al., 2008). Other proteins, DNA, and cellular debris also are present. Continual secretion and shedding of mucins maintain a protective mucus layer from 10–700 μm thick depending on species and body location (Button et al., 2012; Clunes and Boucher, 2007; Garren and Azam, 2012; Strugala et al., 2003).

By offering both structure and nutrients, mucus layers commonly support higher bacterial concentrations than the surrounding environment (Martens et al., 2008; Poulsen et al., 1994). Of necessity, hosts use a variety of mechanisms to limit microbial colonization (Phalipon et al., 2002; Raj and Dentino, 2002; Schluter and Foster 2012; Vaishnava et al., 2011). Secretions produced by the underlying epithelium influence the composition of this microbiota (Hooper et al., 1999; Schluter and Foster, 2012; Sonnenburg et al., 2005). When invaded by pathogens, the epithelium may respond by increased production of antimicrobial agents, hypersecretion of mucin, or alteration of mucin glycosylation patterns to subvert microbial attachment (Gerken, 2004; Jentoft, 1990; Schulz et al., 2007).

Also present in the mucus environment are bacteriophage (phage), the most common and diverse biological entities. As specific bacterial predators,

they increase microbial diversity through Red Queen/kill-the-winner dynamics (Rodriguez-Brito et al., 2010; Thingstad and Lignell, 1997). Many phages establish conditional symbiotic relationships with their bacterial hosts through lysogeny. As integrated prophages, they often express genes that increase host fitness or virulence (Groisman and Ochman, 1993; Johansen et al., 2001; Willner et al., 2011) and protect their host from lysis by related phages. As free phage, they aid their host strain by killing related competing strains (Dierkop et al., 2012; Furuse et al., 1983; Weinbauer, 2004). Phages participate, along with their bacterial hosts, in tripartite symbioses with metazoans that affect metazoan fitness (Clokie et al., 2011; Moran et al., 2005; Oliver et al., 2009; Roossinck, 2011). However, no direct symbiotic interactions between phage and metazoans are known.

Recently, Minot et al. (2012) showed that phages in the human gut encode a population of hypervariable proteins. For 29 hypervariable regions, evidence indicated that hypervariability was conferred by targeted mutagenesis through a reverse transcription mechanism (McMahon et al., 2005; Minot et al., 2012). Approximately half of these encoded proteins possessed the C-type lectin fold previously found in the major tropism determinant protein at the tip of the *Bordetella* phage BPP-1 tail fibers (Medhekar and Miller 2007); six others contained Ig-like domains. These Ig-like proteins, similar to antibodies and T-cell receptors, can accommodate large sequence variation ($>10^{13}$ potential alternatives) (Halaby and Mornon, 1998). Ig-like domains also are displayed in the structural proteins of many phage (Fraser et al., 2006, 2007). That most of these displayed Ig-like domains are dispensable for phage growth in the laboratory (Fraser et al., 2007; McMahon et al., 2005) led to the hypothesis that they aid adsorption to their bacterial prey under environmental conditions (Fraser et al., 2007). The possible role and function of these hypervariable proteins remain to be clarified.

Here, we show that phage adhere to mucus and that this association reduces microbial colonization and pathology. In vitro studies demonstrated that this adherence was mediated by the interaction between displayed Ig-like domains of phage capsid proteins and glycan residues, such as those in mucin glycoproteins. Homologs of these Ig-like domains are encoded by phages from many environments, particularly those adjacent to mucosal surfaces. We propose the bacteriophage adherence to mucus (BAM) model whereby phages provide a non–host-derived antimicrobial defense on the mucosal surfaces of diverse metazoan hosts.

Results

Phage Adhere to Mucus

Our preliminary investigations of mucosal surfaces suggested that phage concentrations in the mucus layer were elevated compared with the surrounding

environment. Here, we used epifluorescence microscopy to count the phage and bacteria in mucus sampled from a diverse range of mucosal surfaces (e.g., sea anemones, fish, human gum), and in each adjacent environment (SI Materials and Methods and Fig. S1). Comparing the calculated phage-to-bacteria ratios (PBRs) showed that PBRs in metazoan-associated mucus layers were on average 4.4-fold higher than those in the respective adjacent environment (Figure A15-1A). The PBRs on these mucus surfaces ranged from 21:1 to 87:1 (average, 39:1), compared with 3:1 to 20:1 for the surrounding milieus (average, 9:1; $n = 9$, $t = 4.719$; $^{***}P = 0.0002$). Earlier investigations of phage abundance in marine environments reported that phage typically outnumber bacteria by an order of magnitude (Breitbart et al., 2002; Danovaro and Serresi, 2000; Fuhrman, 1999), but here we demonstrate that this margin was significantly larger in metazoan-associated mucus surface layers.

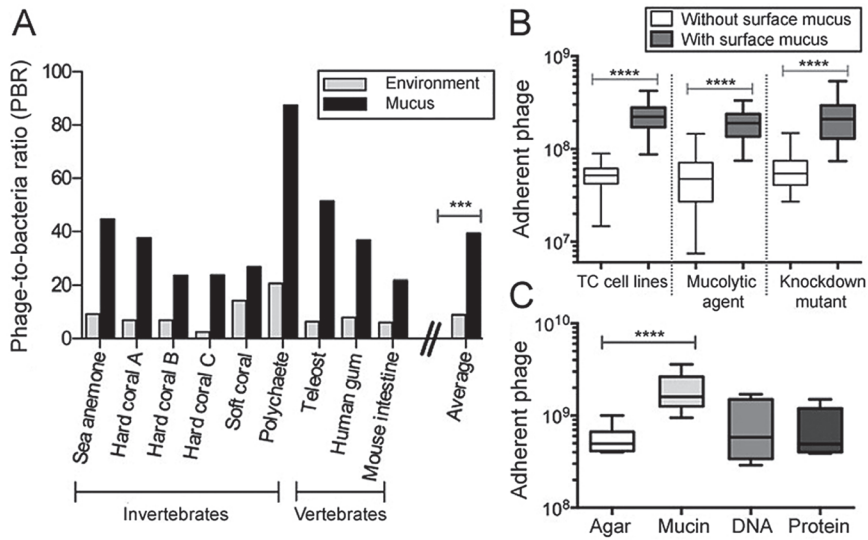


FIGURE A15-1 Phage adhere to cell-associated mucus layers and mucin glycoprotein. (A) PBR for diverse mucosal surfaces and the adjacent environment. On average, PBRs for mucosal surfaces were 4.4-fold greater than for the adjacent environment ($n = 9$, $t = 4.719$, $^{***}P = 0.0002$, unpaired t test). (B) Phage adherence to TC cell monolayers, with and without surface mucus (unpaired t tests). (Left) Non–mucus-producing Huh-7 liver hepatocyte cells and mucus-producing T84 colon epithelial cells ($n > 18$, $t = 8.366$, $^{****}P < 0.0001$). (*Center*) Mucus-producing A549 lung epithelial cells with and without treatment with NAC, a mucolytic agent ($n > 40$, $t = 9.561$, $^{****}P < 0.0001$). (Right) Mucus-producing shRNA control A549 cells (*shControl*) and mucus knockdown (MUC^{-}) A549 cells ($n > 37$, $t = 7.673$, $^{****}P < 0.0001$). (C) Phage adherence to uncoated agar plates and agar coated with mucin, DNA, or protein ($n = 12$, $t = 5.306$, $^{****}P < 0.0001$, unpaired t test).

To determine whether this enrichment was dependent on the presence of mucus rather than some general properties of the cell surface (e.g., charge), phage adherence was tested with tissue culture (TC) cells with and without surface mucus (SI Materials and Methods). In these assays, T4 phage were washed across confluent cell monolayers for 30 min, after which nonadherent phage were removed by repeated washings and the adherent phage quantified by epifluorescence microscopy. Two mucus-producing cell lines were used: T84 (human colon epithelial cells) and A549 (human lung epithelial cells). For these cells, mucin secretion was stimulated by pretreatment with phorbol 12-myristate 13-acetate (Hong et al., 1999; Forstner et al., 1993). Comparison of the T84 cells with the non–mucus-producing Huh-7 human hepatocyte cell line showed that T4 phage adhered significantly more to the mucus-producing T84 cells (Figure A15-1B; $n > 18$, $t = 8.366$; $^{****}P < 0.0001$). To demonstrate the mucus dependence of this adherence, the mucus layer was chemically removed from A549 cells by N-acetyl-L-cysteine (NAC) treatment (Alemka et al., 2010) (Fig. S2). This significantly reduced the number of adherent phage to levels similar to those observed with non–mucus-producing cell lines (Figure A15-1B; $n > 40$, $t = 9.561$; $^{****}P < 0.0001$). We also created an A549 shRNA mucus knockdown cell line (MUC–), reducing mucus production in A549, and a nonsense shRNA control (shControl; Figs. S3 and S4). Again, T4 phage adhered significantly more to the mucus-producing cells (Figure A15-1B; $n > 37$, $t = 7.673$; $^{****}P < 0.0001$).

Although mucin glycoproteins are the predominant component of mucus, other macromolecular components also are present, any of which might be involved in the observed phage adherence. We developed a modified top agar assay to determine whether phage adhered to a specific macromolecular component of mucus. Plain agar plates and agar plates coated with 1% (wt/vol) mucin, DNA, or protein were prepared. That concentration was chosen because it is at the low end of the range of physiological mucin concentrations (Lieleg et al., 2010). T4 phage suspensions were incubated on the plates for 30 min, after which the phage suspension was decanted to remove unbound particles. The plates then were overlaid with a top agar containing *Escherichia coli* hosts and incubated overnight. The number of adherent phage was calculated from the number of plaque-forming units (pfu) observed. Significantly more T4 phage adhered to the 1% mucin-coated agar surface (Figure A15-1C; $n = 12$, $t = 5.306$; $^{****}P < 0.0001$). Combined, these three assays show that phage adhere to mucin glycoproteins.

Phage Adherence and Bacterial Infection

The mucus layer is an optimal environment for microbial growth, providing structure as well as nutrients in the form of diverse, mucin-associated glycans. To limit this growth, the metazoan host retards microbial colonization by diverse antimicrobial mechanisms (Phalipon et al., 2002; Raj and Dentino, 2002; Schluter and Foster, 2012; Vaishnava et al., 2011). Does the increased number of adherent

phage found on mucosal surfaces also reduce microbial colonization? To answer this, bacterial attachment to mucus-producing and non–mucus-producing TC cells was assayed both with and without pretreatment of the cells with the mucus-adherent phage T4. Here confluent monolayers of TC cells were overlaid with T4 phage for 30 min, washed to remove nonadherent phage, and then incubated with *E. coli* for 4 h. Cells then were scraped from the plates and the attached bacteria were fluorescently stained and counted by epifluorescence microscopy. Phage pretreatment of mucus-producing TC cell lines (T84, A549) significantly decreased subsequent bacterial attachment (Figure A15-2A; T84: $n > 30$, $t = 32.05$, $^{****}P < 0.0001$; A549: $n > 30$, $t = 36.85$, $^{****}P < 0.0001$). Phage pretreatment of non–mucus-producing cells (Huh-7; *MUC*$^{-}$, an A549 mucus knockdown strain) had a much smaller effect on bacterial attachment.

To determine whether this reduced bacterial attachment depended on bacterial lysis and the production of progeny viruses, we repeated these experiments using an amber mutant T4 phage (T4 *am43*$^{-}$*44*$^{-}$). When infecting wild-type *E. coli*, this phage produces no infective progeny virions, but infection of the *E. coli* amber suppressor strain *SupD* yields infective virions. For these experiments, mucus-producing A549 cells were pretreated with amber mutant T4 *am43*$^{-}$*44*$^{-}$ phage and then incubated with either wild-type or the amber suppressor strain *E. coli* (Figure A15-2B). Bacterial attachment was reduced by more than four orders of magnitude when phage could replicate and thereby increase the number of infective virions within the mucus ($n = 8$, $^{****}P < 0.0001$). Comparatively, when no phage replication occurred in the mucus, there was no observable change in bacterial colonization and fewer phages were detected ($n = 8$, $^{*}P = 0.0227$). These results show that pretreatment of a mucosal surface with phage reduces adherence of a bacterial pathogen and that this protection is mediated by continued phage replication in the mucus.

To test whether the observed reduced bacterial adherence was accompanied by reduced pathology of the underlying TC cells, mucus-producing A549 and non–mucus-producing *MUC*$^{-}$ TC cells were exposed to *E. coli* overnight, either with or without a 30-min pretreatment with T4 phage. Infection was quantified as the percentage of cell death. Adherence of phage effectively protected the mucus-producing cells against the subsequent bacterial challenge (Figure A15-2C; $n = 12$, $^{****}P < 0.0001$). Phage pretreatment showed a reduced protection to the non–mucus-producing *MUC*$^{-}$ cells, decreasing cell death only twofold. Evaluating the importance of mucus production for effective protection, we found that phage pretreatment of mucus-producing A549 cells resulted in a 3.6-fold greater reduction in cell death ($n = 12$, $^{*}P = 0.0181$) than the same pretreatment of the mucin knockdown *MUC*$^{-}$ cells.

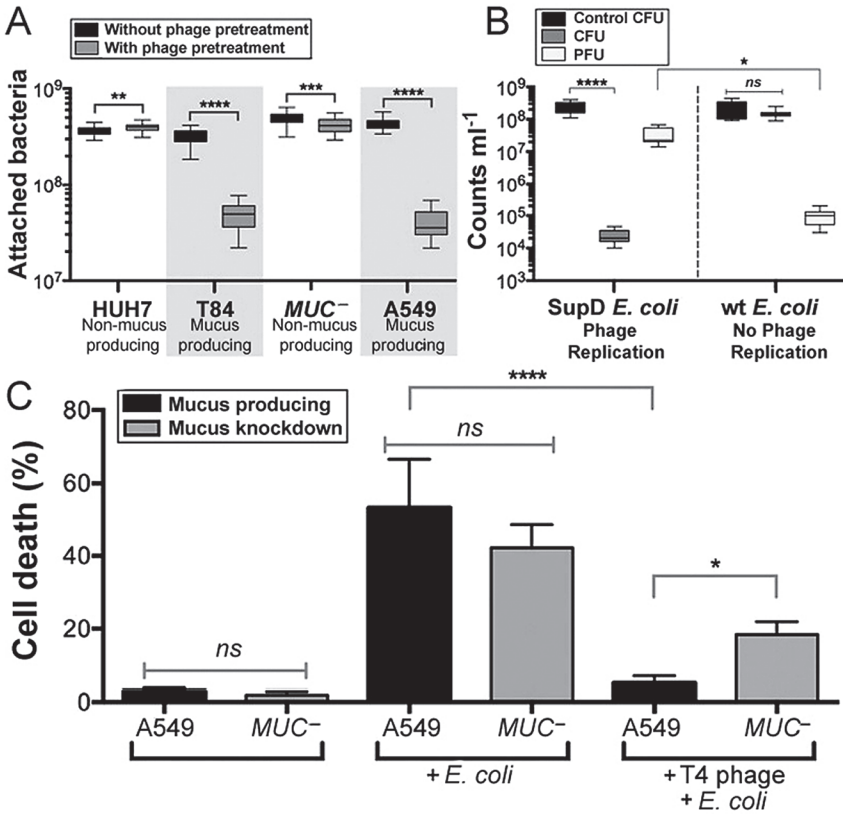


FIGURE A15-2 Effect of phage adsorption on subsequent bacterial infection of epithelial cells. (A) Bacterial attachment to mucus-producing (T84 and A549) and non–mucus-producing (Huh-7, *MUC*⁻) TC cells, with and without phage pretreatment. T4 phage pretreatment significantly decreased subsequent bacterial adherence to mucus-producing TC cell lines (T84: $n > 30$, $t = 32.05$, $^{****}P < 0.0001$; A549: $n > 30$, $t = 36.85$, $^{****}P < 0.0001$; unpaired t tests). Less dramatic shifts were seen for non–mucus-producing cells (Huh-7: $n > 30$, $t = 2.72$, $^{**}P = 0.0098$; *MUC*⁻: $n > 30$, $t = 3.52$, $^{***}P = 0.0007$; unpaired t tests). (B) Mucus-producing A549 cells were pretreated with T4 *am43⁻44⁻* phage (Materials and Methods) and then incubated for 4 h with either wildtype (wt) or amber-suppressor (SupD) *E. coli*. Phage replication in the SupD E. coli strain significantly reduced bacterial colony-forming units (CFU) in the mucus ($n = 8$, $^{****}P < 0.0001$, Tukey's two-way ANOVA) and increased phage-forming units (PFU) relative to the no-phage replication wt *E. coli* ($n = 8$, $^{*}P = 0.0227$). (C) Mortality of mucus-producing (A549) and mucus knockdown (MUC⁻) A549 lung epithelial cells following overnight incubation with *E. coli.* Phage pretreatment completely protected mucus-producing A549 cells from bacterial challenge ($n = 12$, $^{****}P < 0.0001$, Tukey's one-way ANOVA); protection of *MUC*⁻ cells was 3.1-fold less ($n = 12$, $^{*}P = 0.0181$). *ns*, not significant.

Role of Capsid Ig-Like Domains in Phage Adherence

Minot et al. (2012) recently reported that phage communities associated with the human gut encode a diverse array of hypervariable proteins, including some with hypervariable Ig-like domains. Four Ig-like domains are found in highly antigenic outer capsid protein (Hoc), a T4 phage structural protein of which 155 copies are displayed on the capsid surface (Fokine et al., 2011; Sathaliyawala et al., 2010). Based on this, and given that most Ig-like domains function in recognition and adhesion processes, we hypothesized that the T4 Hoc protein might mediate the adherence of T4 phage to mucus. To test this, we performed three experiments. First, we compared the adherence of *hoc*$^+$ T4 phage and a *hoc*$^-$ mutant to mucin-, DNA-, and protein-coated agar plates to an uncoated agar control using the modified top agar assay (see above). Relative to plain agar, the adherence of *hoc*$^+$ T4 phage increased 4.1-fold for mucin-coated agar ($n > 11$, $t = 3.977$, $^{***}P = 0.0007$), whereas adherence increased only slightly for agar coated with DNA (1.1-fold) or protein (1.2-fold; Figure A15-3A). Unlike the *hoc*$^+$ T4 phage, the *hoc*$^-$ phage did not adhere preferentially to the mucin-coated agar, but instead showed 1.2-, 1.2-, and 1.1-fold increased adherence but instead showed 1.2-, 1.2-, and 1.1-fold increased adherence for mucin, DNA, and protein coatings, respectively. To ensure that none of the macromolecules directly affected phage infectivity, *hoc*$^+$ and *hoc*$^-$ T4 phage were incubated in 1% (wt/vol) solutions of mucin, DNA, or protein. Phage suspensions were combined with *E. coli* top agar as described above and layered over uncoated agar plates. The results confirmed that the macromolecules did not alter phage infectivity (Fig. S5). To provide further evidence that the mucin adherence was dependent on the capsid displayed Ig-like domains rather than some other property of T4 phage, we repeated the modified top agar assay using Ig$^+$ and Ig$^-$ T3 phage. As with T4, the Ig-like domains of T3 are displayed on the surface of the major capsid protein (Fraser et al., 2007). Results indicated a similar increase in adherence to mucin for the Ig$^+$, but not the Ig$^-$, T3 phage (Fig. S6). Thus, adherence of these phage to mucus requires the Ig-like protein domains.

Second, a competition assay using *hoc*$^+$ and *hoc*$^-$ T4 phage and mucus-producing TC cells was performed to demonstrate the role of mucin in phage adherence. Phage suspended in mucin solutions ranging from 0% to 5% (wt/vol) were washed over confluent layers of mucus-producing A549 TC cells; phage adherence then was assayed as above. Adherence of *hoc*$^+$ T4 phage, but not of *hoc*$^-$ T4 phage, was reduced by mucin competition in a concentration-dependent manner (Figure A15-3B).

Third, interaction of the Hoc protein domains displayed on the capsid surface with mucin glycoproteins was hypothesized to affect the rate of diffusion of T4 virions in mucus. To evaluate this, we used multiple-particle tracking (MPT) to quantify transport rates of phage particles in buffer and in mucin suspensions. The ensemble average effective diffusivity (Deff) calculated at a time scale of 1 s for both *hoc*$^+$ and *hoc*$^-$ T4 phage in buffer was compared against that in 1% (wt/vol)

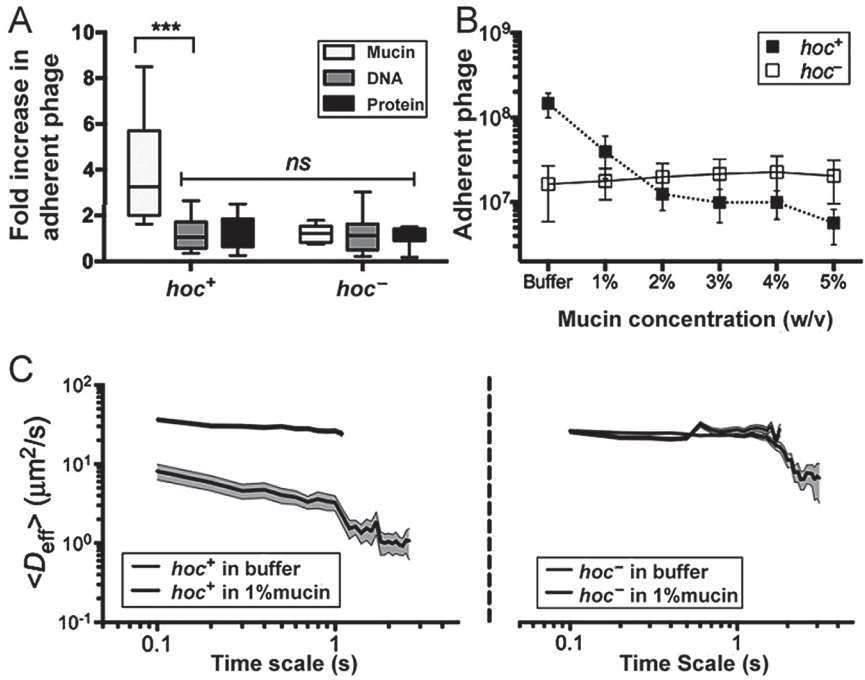


FIGURE A15-3 Effect of Hoc protein on phage–mucin interactions. (A) Adherence of *hoc*$^+$ and *hoc*$^-$ T4 phage to agar coated with mucin, DNA, or protein reported as an increase relative to plain agar controls ($n > 11$, $t = 3.977$, ***$P = 0.0007$, unpaired t test). (B) Competitive effect of mucin on phage adherence when *hoc*$^+$ and *hoc*$^-$ T4 phage in 0–5% (wt/vol) mucin solution (1 × PBS) were washed over mucus-producing A549 cells ($n = 25$ per sample). (C) Diffusion of fluorescence-labeled *hoc*$^+$ (Left) and *hoc*$^-$ (Right) T4 phage in buffer and 1% mucin as determined by MPT. Mucin hindered diffusion of *hoc*$^+$ T4 phage but not *hoc*$^-$ phage (10 analyses per sample, trajectories of $n > 100$ particles for each analysis; error bars represent SE).

mucin suspensions (SI Materials and Methods). Both *hoc*$^+$ and *hoc*$^-$ phage diffuse rapidly through buffer (Figure A15-3C). Whereas *hoc*$^-$ phage diffused in 1% mucin at the same rate as in buffer, the mucin decreased the diffusion rate for *hoc*$^+$ phage particles eightfold. Thus, all three of these experimental approaches supported our hypothesis that the Hoc proteins displayed on the T4 phage capsid interact with mucin.

Phage Capsid Ig-Like Domains Interact with Glycans

It is known that ~25% of sequenced tailed dsDNA phages (Caudovirales) encode structural proteins with predicted Ig-like domains (Fraser et al., 2006).

A search of publicly available viral metagenomes for homologs of the Ig-like domains of the T4 Hoc protein yielded numerous viral Ig-like domains from a variety of environments (Figure A15-4A). These domains were more likely to be found in samples collected directly from mucus (e.g., sputum samples) or from an environment adjacent to a mucosal surface (e.g., intestinal lumen, oral cavity). All homologs displayed high structural homology (Phyre2 confidence score average, 96 ± 5%) with a plant-sugar binding domain known for its promiscuous carbohydrate binding specificity (SI Materials and Methods and Table S1), suggesting an interaction between these Ig-like domains and glycans.

Mucins are complex glycoproteins with highly variable glycan groups exposed to the environment. To investigate whether Hoc interacts with glycans and, if so, to determine whether it interacts with a specific glycan or with a diverse array of glycans, we assayed phage adherence to microarrays printed with 610 mammalian glycans. The *hoc*$^{+}$ T4 phage adhered to many diverse glycans and showed a preference for the O-linked glycan residues typically found in mucin glycoproteins (Figure A15-4B, SI Materials and Methods, and Table S2). The *hoc*$^{-}$ T4 phage exhibited significantly lower affinity for all tested glycans. This indicates that Hoc mediates interactions between T4 phage and varied glycan residues.

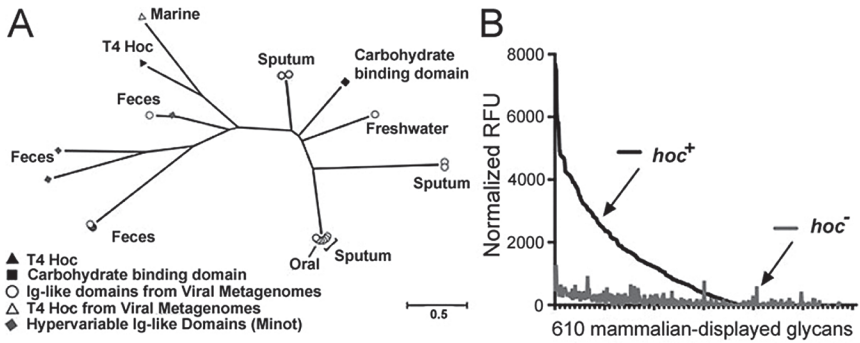


FIGURE A15-4 Hoc-mediated glycan binding and Hoc-related phylogeny. (A) Phylogenetic tree of sequences from viral metagenomes with high-sequence homology to Ig-like domains. Many of the identified homologs are from mucus-associated environments (e.g., human feces, sputum). Also included are the Hoc protein of T4 phage and the hypervariable Ig-like domains previously obtained by deep sequencing of phage DNA from the human gut (Minot et al., 2012). The scale bar represents an estimated 0.5 amino acid substitutions per site. See SI Materials and Methods for methods. (B) Binding of fluorescence-stained *hoc*$^{+}$ and *hoc*$^{-}$ T4 phage to a microarray of 610 mammalian glycans. Normalized relative fluorescence units (RFU) were calculated from mean fluorescence minus background binding.

Discussion

In diverse metazoans, body surfaces that interact with the environment are covered by a protective layer of mucus. Because these mucus layers provide favorable habitats for bacteria, they serve as the point of entry for many pathogens and support large populations of microbial symbionts. Also present are diverse phages that prey on specific bacterial hosts. Moreover, phage concentrations in mucus are elevated relative to the surrounding environment (an average 4.4-fold increase for a diverse sample of invertebrate and vertebrate metazoans; Figure A15-1A). The increased concentration of lytic phage on mucosal surfaces provides a previously unrecognized metazoan immune defense affected by phage lysis of incoming bacteria.

Working with a model system using T4 phage and various TC cell lines, we demonstrated that the increased concentration of phage on mucosal surfaces is mediated by weak binding interactions between the variable Ig-like domains on the T4 phage capsid and mucin-displayed glycans. The Ig protein fold is well known for its varied but essential roles in the vertebrate immune response and cell adhesion. Ig-like domains also are present in approximately one quarter of the sequenced genomes of tailed DNA phages, the Caudovirales (Fraser et al., 2006). Notably, these domains were found only in virion structural proteins and typically are displayed on the virion surface. Thus, they were postulated to bind to bacterial surface carbohydrates during infection (Fraser et al., 2006, 2007). However, mucin glycoproteins, the predominant macromolecular constituent of mucus, display hundreds of variable glycan chains to the environment that offer potential sites for binding by phage Ig-like proteins. Furthermore, we speculate that phage use the variability of the Ig-like protein scaffold (supporting $>10^{13}$ potential alternatives) to adapt to the host's ever-changing patterns of mucin glycosylation.

The presence of an Ig-like protein (Hoc) displayed on the capsid of T4 phage significantly slowed the diffusion of the phage in mucin solutions. In vivo, similar phage binding to mucin glycans would increase phage residence time in mucus layers. Because bacterial concentrations typically are enriched in mucus (Fig. S1), we predict that mucus-adherent phage are more likely to encounter bacteria, potentially increasing their replicative success. If so, phage Ig-like domains that bind effectively to the mucus layer would be under positive selection. Likely, Hoc and other phage proteins with Ig-like domains interact with other glycans with different ramifications, as well (Fokine et al., 2011; Fraser et al., 2007).

Previous metagenomic studies documented the ubiquity and diversity of bacteria and phage within mucus-associated environments (e.g., human gut, human respiratory tract, corals) (Breitmart et al., 2002; Eckburg et al., 2005; Marhaver et al., 2008; Reyes et al., 2010; Wegley et al., 2007; Willner et al., 2009, 2012). Known also were some of the essential but adaptable services provided by symbiotic bacteria in these environments (McFall-Ngai, 2013). However, only recently have efforts been made to investigate the dynamic influences of phage within

host-associated ecosystems (Duerkop et al., 2012; Minot et al., 2012; Weitz et al., 2013). In this work, we used an in vitro model system to demonstrate a mechanism of phage adherence to the mucus layers that shield metazoan cells from the environment. Furthermore, adherent phage protected the underlying epithelial cells from bacterial infection. Based on these observations and previous research, we proposed the BAM model of immunity, in which the adherence of phage to mucosal surfaces yields a non-host-derived, antimicrobial defense. According to this model (summarized in Figure A15-5), the mucus layer, already considered part of the innate immune system and known to provide physical and biochemical antimicrobial defenses (Lieleg et al., 2012; Linden et al., 2008; Schluter and Foster et al., 2012), also accumulates phage.

The model system we used involved a single lytic phage and host bacterium; the situation in vivo undoubtedly is more complex. Within the mucosal layer reside diverse bacterial lineages and predictably an even greater diversity of phage strains, both enmeshed within complex phage–bacterial infection networks and engaged in a dynamic arms race (Labrie et al., 2010; Weitz et al., 2013). These and other factors lower the probability that any given phage–bacterium encounter

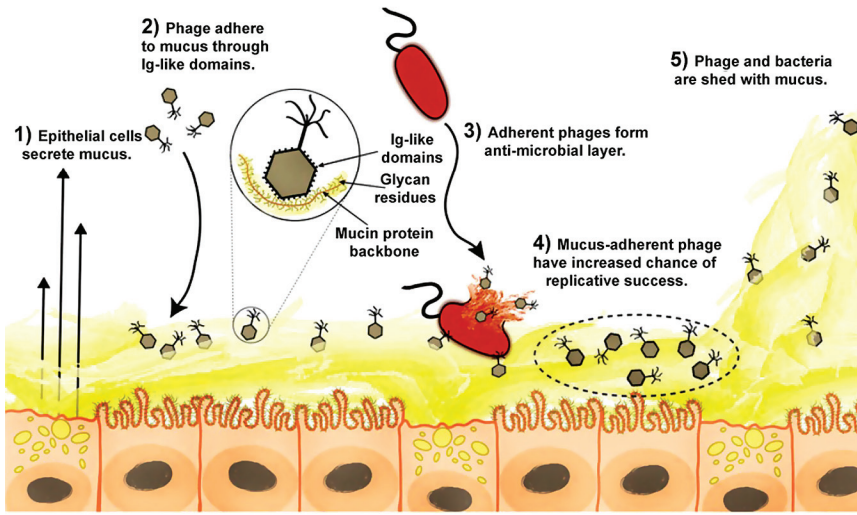


FIGURE A15-5 The BAM model. (1) Mucus is produced and secreted by the underlying epithelium. (2) Phage bind variable glycan residues displayed on mucin glycoproteins via variable capsid proteins (e.g., Ig-like domains). (3) Phage adherence creates an antimicrobial layer that reduces bacterial attachment to and colonization of the mucus, which in turn lessens epithelial cell death. (4) Mucus-adherent phage are more likely to encounter bacterial hosts, thus are under positive selection for capsid proteins that enable them to remain in the mucus layer. (5) Continual sloughing of the outer mucus provides a dynamic mucosal environment.

will result in a successful infection. The time dimension adds further complexity. The mucus layer is dynamic. Mucins are secreted continually by the underlying epithelium while mucus is sloughed continually from the outer surface. As a result, there is an ongoing turnover of both the bacterial and phage populations in the mucus layer. Driven by kill-the-winner dynamics, the population of phage types that can infect the dominant bacterial types present will cycle along with the populations of their hosts. Through such mechanisms, we envision that adherent lytic phages provide a dynamic and adaptable defense for their metazoan hosts—a unique example of a metazoan–phage symbiosis.

We posit that BAM immunity reduces bacterial pathogenesis and provides a previously unrecognized, mucosal immunity. This has far-reaching implications for numerous fields, such as human immunity, gastroenterology, coral disease, and phage therapy. Meanwhile, key questions remain. For instance, what role do temperate phages play in the dynamics of BAM immunity? When integrated into the bacterial chromosome as prophages, they protect their bacterial hosts from infection by related phages; as free phages, they infect and kill sensitive related bacterial strains that compete with their bacterial hosts (Duerkop et al., 2012; Furuse et al., 1983; Weinbauer, 2004). Both mechanisms may benefit their metazoan host by contributing to the maintenance of a selected commensal mucosal microbiota. These possibilities remain to be investigated. Likewise, in vivo investigations are needed to characterize the bacterial and phage diversity present and the consequent effects on BAM immunity. As of now, the relationships shown here open an arena for immunological study, introduce a phage–metazoan symbiosis, and recognize the key role of the world's most abundant biological entities in the metazoan immune system.

Materials and Methods

Bacterial Strains, Phage Stocks, TC Cell Lines, and Growth Conditions

E. coli 1024 strain was used for all *E. coli* experiments and was grown in LB (10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 L dH_2O) at 37°C overnight. *E. coli* amber-suppressor strain *SupD* strain CR63 was used as a host for amber mutant phage and grown as above. Bacteriophage T4 was used at ~109 pfu·mL^{-1}. *Hoc*$^-$ T4 phage were kindly supplied by Prof. Venigalla Rao (Fokine et al., 2011), The Catholic University of America, Washington, D.C. T3 *am10* Ig$^-$ amber mutant phage were kindly supplied by Prof. Ian J. Molineux (Condreay et al., 1989), University of Texas, Austin, TX. T4 replication-negative *43*$^-$ (*am*E4332: DNA polymerase) *44*$^-$ (*am*N82: subunit of polymerase clamp holder) amber mutant phage were kindly supplied by Prof. Kenneth Kreuzer (Benson and Kreuzer, 1992), Duke University School of Medicine, Durham, NC. The human tumorigenic colon epithelial cell line, T84, was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM/F12-K media with 5% FBS

and 100 μg·mL^{-1} penicillin–streptomycin (PS). The human tumorigenic lung epithelial cell line A549 was kindly supplied by Prof. Kelly Doran, San Diego State University, San Diego, CA and cultured in F12-K media with 10% FBS, 100 μg·mL^{-1} PS. The human tumorigenic liver epithelial cell line Huh7 was kindly supplied by Prof. Roland Wolkowicz, San Diego State University, San Diego, CA, and cultured in F12-K media with 10% FBS, 100 μg·mL^{-1} PS. All TC cell lines initially were grown in 50 mL Primaria Tissue Culture Flasks (Becton Dickinson) at 37°C and 5% CO_2.

Phage Adherence to Mucus-Associated Macromolecules

LB agar plates were coated with 1 mL of 1% (wt/vol) of one of the following in 1 × PBS: type III porcine stomach mucin, DNA from salmon testes, or BSA (all three from Sigma–Aldrich) and then allowed to dry. Stocks of *hoc*$^+$ and *hoc*$^-$ T4 phage (10^9 pfu·mL^{-1}) were serially diluted to 1×10^{-7} and 1×10^{-8} per milliliter in LB, and a 5-mL aliquot of each dilution was washed across the plates for 30 min at 37°C on an orbital shaker. After the phage suspensions were decanted from the plates, the plates were shaken twice to remove excess liquid and dried. Each plate then was layered with 1 mL of overnight *E. coli* culture (10^9 mL^{-1}) in 3 mL of molten top agar and incubated overnight at 37°C. The number of adherent phage was calculated from the number of plaque-forming units observed multiplied by the initial phage dilution. To determine whether mucus macromolecules directly affected phage infectivity, *hoc*$^+$ and *hoc*$^-$ T4 phage (10^9 pfu·mL^{-1}) were serially diluted as described above into 1 mL LB solutions containing 1% (wt/vol) mucin, DNA, or BSA. After incubation for 30 min at 37°C, the phage suspensions were combined with *E. coli* top agar as described above and layered over uncoated agar plates (Fig. S5).

Phage Treatment of TC Cells

TC cells were washed twice with 5 mL of serum-free media to remove residual antibiotics, layered with 2 mL of serum-free media containing T4 phage (10^7 or 10^9 mL^{-1}), and incubated at 37°C and 5% CO_2 for 30 min. Cells then were washed five times with 5 mL of serum-free media to remove nonadherent phage.

Phage Adherence to TC Cells

TC cells were treated with phage (10^9 mL^{-1}; see above), then scraped from plates using Corning Cell Scrapers (Sigma–Aldrich). Adherent phage were counted by epifluorescence microscopy as described above.

Bacterial Adherence to TC Cells With/Without Phage Pretreatment

TC cells with or without pretreatment with T4 phage (10^7 mL^{-1}) were layered with 2 mL serum-free media containing *E. coli* (10^7 mL^{-1}), incubated at 37°C and 5% CO_2 for 4 h, and then washed five times with 5 mL serum-free medium to remove nonadherent phage and bacteria. Cells were scraped from plates, and adherent phage and bacteria were counted by either epifluorescence microscopy, as described above, or colony-forming and plaque-forming units. Then, 100 µL of a relevant dilution was spread onto an agar plate and incubated overnight at 37°C, and the number of adherent bacteria was calculated from the colony-forming units observed multiplied by the initial dilution. Plaque-forming units were counted by a top agar assay as described above.

TC Cell Death from Bacterial Infection

Mucus-producing A549 and *MUC*$^-$ A549 TC cells were grown to confluence. T4 phage were cleaned using Amicon 50-kDa centrifugal filters (Millipore) and saline magnesium buffer (SM) to remove bacterial lysis products. Cells, with or without T4 phage pretreatment (10^7 mL^{-1}), were incubated with *E. coli* (10^7 mL^{-1}) overnight. Afterward, TC cells were recovered from the plates by trypsin/EDTA solution (Invitrogen). Cells were pelleted by centrifugation and resuspended in 1 × PBS. Dead cells were identified by staining with 1 mg/mL of propidium iodide (Invitrogen). Samples then were analyzed on a FACSCanto II flow cytometer (BD Biosciences) with excitation at 488 nm and emission detected through a 670 long pass filter. The forward scatter threshold was set at 5,000, and a total of 10,000 events were collected for each sample.

Mucin Competition Assay

Mucus-producing A549 TC cells were grown to confluence. *Hoc*$^+$ and *hoc*$^-$ T4 phage (109 mL^{-1}) were diluted into mucin solutions ranging between 0% and 5% (wt/vol) in 1 × PBS then washed over TC cells for 30 min at 37°C and 5% CO_2. Cells were washed five times with 5 mL serum-free media to remove nonadherent phage, scraped from plates, and adherent phage were quantified as described above.

Graphing and Statistics

Graphing and statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). All error bars represent 5–95% confidence intervals. The midline represents the median and the mean for box plots and bar plots, respectively.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grants R01: GM095384, GM073898, and R21: AI094534 from the National Institute of General Medical Sciences. The authors thank the Protein-Glycan Interaction Resource at Emory University School of Medicine, Atlanta, GA (funded by NIH Grant GM98791), for support of the glycan microarray analyses. The authors acknowledge the San Diego State University (SDSU) Flow Cytometry Core Facility and the SDSU Electron Microscopy Facility for assistance with sample analysis.

References

Alemka A, et al. (2010) Probiotic colonization of the adherent mucus layer of HT29MTXE12 cells attenuates Campylobacter jejuni virulence properties. Infect Immun 78(6):2812–2822.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307(5717):1915–1920.

Benson KH, Kreuzer KN (1992) Plasmid models for bacteriophage T4 DNA replication: Requirements for fork proteins. J Virol 66(12):6960–6968.

Berry D, et al. (2013) Host-compound foraging by intestinal microbiota revealed by single-cell stable isotope probing. Proc Natl Acad Sci USA 110(12):4720–4725.

Breitbart M, et al. (2002) Genomic analysis of uncultured marine viral communities. Proc Natl Acad Sci USA 99(22):14250–14255.

Button B, et al. (2012) A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. Science 337(6097):937–941.

Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK (2010) Host-bacterial symbiosis in health and disease. Adv Immunol 107:243–274.

Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Science 285(5434):1742–1745.

Clokie MR, Millard AD, Letarov AV, Heaphy S (2011) Phages in nature. Bacteriophage 1(1):31–45.

Clunes MT, Boucher RC (2007) Cystic fibrosis: The mechanisms of pathogenesis of an inherited lung disorder. Drug Discov Today Dis Mech 4(2):63–72.

Condreay JP, Wright SE, Molineux IJ (1989) Nucleotide sequence and complementation studies of the gene 10 region of bacteriophage T3. J Mol Biol 207(3):555–561.

Cone RA (2009) Barrier properties of mucus. Adv Drug Deliv Rev 61(2):75–85.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398(6729):701–704.

Danovaro R, Serresi M (2000) Viral density and virus-to-bacterium ratio in deep-sea sediments of the Eastern Mediterranean. Appl Environ Microbiol 66(5):1857–1861.

Dethlefsen L, McFall-Ngai M, Relman DA (2007) An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449(7164):811–818.

Douglas AE (1989) Mycetocyte symbiosis in insects. Biol Rev Camb Philos Soc 64(4):409–434.

Duerkop BA, Clements CV, Rollins D, Rodrigues JLM, Hooper LV (2012) A composite bacteriophage alters colonization by an intestinal commensal bacterium. Proc Natl Acad Sci USA 109(43):17621–17626.

Eckburg PB, et al. (2005) Diversity of the human intestinal microbial flora. Science 308(5728):1635–1638.

Fokine A, et al. (2011) Structure of the three N-terminal immunoglobulin domains of the highly immunogenic outer capsid protein from a T4-like bacteriophage. J Virol 85(16):8141–8148.

Forstner G, Zhang Y, McCool D, Forstner J (1993) Mucin secretion by T84 cells: Stimulation by PKC, Ca2+, and a protein kinase activated by Ca2+ ionophore. Am J Physiol 264(6 Pt 1):G1096–G1102.

Fraser JS, Maxwell KL, Davidson AR (2007) Immunoglobulin-like domains on bacteriophage: Weapons of modest damage? Curr Opin Microbiol 10(4):382–387.

Fraser JS, Yu Z, Maxwell KL, Davidson AR (2006) Ig-like domains on bacteriophages: A tale of promiscuity and deceit. J Mol Biol 359(2):496–507.

Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399(6736):541–548.

Furuse K, et al. (1983) Bacteriophage distribution in human faeces: Continuous survey of healthy subjects and patients with internal and leukaemic diseases. J Gen Virol 64(Pt 9):2039–2043.

Garren M, Azam F (2012) Corals shed bacteria as a potential mechanism of resilience to organic matter enrichment. ISME J 6(6):1159–1165.

Gerken TA (2004) Kinetic modeling confirms the biosynthesis of mucin core 1 (β-Gal(1-3) α-GalNAc-O-Ser/Thr) O-glycan structures are modulated by neighboring glycosylation effects. Biochemistry 43(14):4137–4142.

Groisman EA, Ochman H (1993) Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. EMBO J 12(10):3779–3787.

Halaby DM, Mornon JPE (1998) The immunoglobulin superfamily: an insight on its tissular, species, and functional diversity. J Mol Evol 46(4):389–400.

Hong DH, Petrovics G, Anderson WB, Forstner J, Forstner G (1999) Induction of mucin gene expression in human colonic cell lines by PMA is dependent on $PKC_{-\varepsilon}$. Am J Physiol 277(5 Pt 1):G1041–G1047.

Hooper LV, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22(1):283–307.

Hooper LV, Xu J, Falk PG, Midtvedt T, Gordon JI (1999) A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc Natl Acad Sci USA 96(17):9833–9838.

Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T (2010) Wolbachia as a bacteriocyte-associated nutritional mutualist. Proc Natl Acad Sci USA 107(2):769–774.

Jentoft N (1990) Why are proteins O-glycosylated? Trends Biochem Sci 15(8):291–294. 31. Schulz BL, et al. (2007) Glycosylation of sputum mucins is altered in cystic fibrosis patients. Glycobiology 17(7):698–712.

Johansen BK, Wasteson Y, Granum PE, Brynestad S (2001) Mosaic structure of Shigatoxin-2-encoding phages isolated from Escherichia coli O157:H7 indicates frequent gene exchange between lambdoid phage genomes. Microbiology 147(Pt 7):1929–1936.

Kaltenpoth M, Göttler W, Herzner G, Strohm E (2005) Symbiotic bacteria protect wasp larvae from fungal infestation. Curr Biol 15(5):475–479.

Labrie SJ, Samson JE, Moineau S (2010) Bacteriophage resistance mechanisms. Nat Rev Microbiol 8(5):317–327.

Lieleg O, Lieleg C, Bloom J, Buck CB, Ribbeck K (2012) Mucin biopolymers as broadspectrum antiviral agents. Biomacromolecules 13(6):1724–1732.

Lieleg O, Vladescu I, Ribbeck K (2010) Characterization of particle translocation through mucin hydrogels. Biophys J 98(9):1782–1789.

Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008) Mucins in the mucosal barrier to infection. Mucosal Immunol 1(3):183–197.

Marhaver KL, Edwards RA, Rohwer F (2008) Viral communities associated with healthy and bleaching corals. Environ Microbiol 10(9):2277–2286.

Martens EC, Chiang HC, Gordon JI (2008) Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe 4(5):447–457.

McFall-Ngai M, et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci USA 110(9):3229–3236.

McMahon SA, et al. (2005) The C-type lectin fold as an evolutionary solution for massive sequence variation. Nat Struct Mol Biol 12(10):886–892.

Medhekar B, Miller JF (2007) Diversity-generating retroelements. Curr Opin Microbiol 10(4):388–395.

Minot S, et al. (2011) The human gut virome: Inter-individual variation and dynamic response to diet. Genome Res 21(10):1616–1625.

Minot S, Grunberg S, Wu GD, Lewis JD, Bushman FD (2012) Hypervariable loci in the human gut virome. Proc Natl Acad Sci USA 109(10):3962–3966.

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. Proc Natl Acad Sci USA 102(47):16919–16926.

Nyholm SV, McFall-Ngai MJ (2004) The winnowing: Establishing the squid-vibrio symbiosis. Nat Rev Microbiol 2(8):632–642.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325(5943):992–994.

Phalipon A, et al. (2002) Secretory component: A new role in secretory IgA-mediated immune exclusion in vivo. Immunity 17(1):107–115.

Poulsen LK, et al. (1994) Spatial distribution of Escherichia coli in the mouse large intestine inferred from rRNA in situ hybridization. Infect Immun 62(11):5191–5194.

Raj PA, Dentino AR (2002) Current status of defensins and their role in innate and adaptive immunity. FEMS Microbiol Lett 206(1):9–18.

Reyes A, et al. (2010) Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466(7304):334–338.

Rodriguez-Brito B, et al. (2010) Viral and microbial community dynamics in four aquatic environments. ISME J 4(6):739–751.

Roossinck MJ (2011) The good viruses: Viral mutualistic symbioses. Nat Rev Microbiol 9(2):99–108.

Ruby EG (1996) Lessons from a cooperative, bacterial-animal association: The Vibrio fischeri-Euprymna scolopes light organ symbiosis. Annu Rev Microbiol 50(1):591–624.

Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis. Proc Natl Acad Sci USA 108(Suppl 2):10800–10807.

Sathaliyawala T, et al. (2010) Functional analysis of the highly antigenic outer capsid protein, Hoc, a virus decoration protein from T4-like bacteriophages. Mol Microbiol 77(2):444–455.

Schluter J, Foster KR (2012) The evolution of mutualism in gut microbiota via host epithelial selection. PLoS Biol 10(11):e1001424.

Sonnenburg JL, et al. (2005) Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science 307(5717):1955–1959.

Stouthamer R, Breeuwer JA, Hurst GD (1999) Wolbachia pipientis: Microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53(1):71–102.

Strugala V, Allen A, Dettmar PW, Pearson JP (2003) Colonic mucin: Methods of measuring mucus thickness. Proc Nutr Soc 62(1):237–243.

Thingstad T, Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquat Microb Ecol 13:19–27.

Vaishnava S, et al. (2011) The antibacterial lectin RegIII{gamma} promotes the spatial segregation of microbiota and host in the intestine. Sci STKE 334(6053):255.

Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F (2007) Metagenomic analysis of the microbial community associated with the coral Porites astreoides. Environ Microbiol 9(11):2707–2719.

Weinbauer MG (2004) Ecology of prokaryotic viruses. FEMS Microbiol Rev 28(2):127–181.

Weitz JS, et al. (2013) Phage-bacteria infection networks. Trends Microbiol 21(2):82–91.

Willner D, et al. (2009) Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. PLoS ONE 4(10):e7370.

Willner D, et al. (2011) Metagenomic detection of phage-encoded platelet-binding factors in the human oral cavity. Proc Natl Acad Sci USA 108(Suppl 1):4547–4553.

Willner D, et al. (2012) Case studies of the spatial heterogeneity of DNA viruses in the cystic fibrosis lung. Am J Respir Cell Mol Biol 46(2):127–131.

A16

TOPOGRAPHIC DIVERSITY OF FUNGAL AND BACTERIAL COMMUNITIES IN HUMAN SKIN[67]

Keisha Findley,[68] *Julia Oh*,[68] *Joy Yang*,[68] *Sean Conlan*,[68]
Clayton Deming,[68] *Jennifer A. Meyer*,[68] *Deborah Schoenfeld*,[69]
Effie Nomicos,[69] *Morgan Park*,[70]
NIH Intramural Sequencing Center Comparative Sequencing Program,[71]
Heidi H. Kong,[69,*] *and Julia A.* ***Segre***[68,*]

Traditional culture-based methods have incompletely defined the microbial landscape of common recalcitrant human fungal skin diseases, including athlete's foot and toenail infections. Skin protects humans from invasion by pathogenic microorganisms and provides a home for diverse commensal microbiota (Marples, 2012). Bacterial genomic sequence data have generated novel hypotheses about species and community structures underlying human disorders (Grice and Segre, 2012; Human Microbiome Project Consortium, 2012; Pflughoeft and Versalovic, 2012). However, microbial diversity is not limited to bacteria; microorganisms such as fungi also have major roles in microbial community stability, human health and disease (Peleg et al., 2010). Genomic methodologies to identify fungal species and communities have been limited compared with those that are available for bacteria (Ollive et al., 2012). Fungal evolution can be reconstructed with phylogenetic markers, including ribosomal RNA gene regions and other highly conserved genes (James et al., 2006). Here we sequenced and analysed fungal communities of 14 skin sites in 10 healthy adults. Eleven core-body and arm sites were dominated by fungi of the genus Malassezia, with only species-level classifications revealing fungal-community composition differences between sites. By contrast, three foot sites—plantar heel, toenail and toe web—showed high fungal diversity. Concurrent analysis of bacterial and fungal communities

[67] Reprinted by permission from Macmillan Publishers Ltd. Nature. Findley, K. F. et al. 2013. Topographic diversity of fungal and bacterial communities in human skin. *Nature* 498(7454):367-370. Copyright 2013.

[68] Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

[69] Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

[70] Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

[71] NIH Intramural Sequencing Center, National Human Genome Research Institute, National Institutes of Health, Rockville, Maryland 20852, USA.

* These authors contributed equally to this work.

demonstrated that physiologic attributes and topography of skin differentially shape these two microbial communities. These results provide a framework for future investigation of the contribution of interactions between pathogenic and commensal fungal and bacterial communities to the maintenance of human health and to disease pathogenesis.

Since Hippocrates first described oral candidiasis in 400 BC, scientists have sought to explore the roles that commensal and pathogenic fungi and microbial communities have in human health and disease (Ghannoum et al., 2010; Paulino et al., 2006). Culture-based studies have reported *Malassezia*, *Rhodotorula*, *Debaromyces*, *Cryptococcus*, and, in some sites, *Candida* as fungal skin commensals (Roth and James, 1988). Cutaneous fungal infections affect 29 million North Americans, but the role of dermatophytes in common toenail infections can be difficult to characterize using culture-based studies (Bickers et al., 2006). For other common skin disorders, such as seborrheic dermatitis (dandruff), fungal involvement remains incompletely understood (Gaitanis et al., 2012; Saunders et al., 2012). Difficulty in establishing growth conditions (Larone, 2002; St-Germain and Summerbell, 2011) contribute to challenges to rapidly identify and direct treatment against pathogenic fungi.

To compare culture- and DNA-sequence-based identification of human skin-associated fungi, we obtained parallel samples from four skin sites of adult healthy volunteers (Supplementary Fig. 1 and Supplementary Table 1). We characterized isolates by morphological features and molecular markers. In total, we cultured more than 130 fungal isolates: 62 *Malassezia* (species *globosa*, *restricta*, and *sympodialis* [Gioti et al., 2013; Saunders et al., 2012]), 25 *Penicillium* (species *chrysogenum* and *lanosum*), and 19 *Aspergillus* (species *candidus*, *terreus*, and *versicolor*) (Supplementary Table 2). Five or fewer *Alternaria*, *Candida*, *Chaetomium*, *Chrysosporium*, *Cladosporium*, *Mucor*, *Rhodotorula*, and *Trichophyton* isolates were cultured.

To explore fungal diversity with culture-independent methods, we prepared DNA from clinical swabs, and polymerase chain reaction (PCR)-amplified and sequenced two phylogenetic markers within the ribosomal RNA region: 18S rRNA and the intervening internal transcribed spacer 1 (ITS1) region (Bruns et al., 1992; James et al., 2006; Schoch et al., 2012). We generated a custom ITS1 database based on sequences deposited in GenBank to classify sequences to genus level with greater than 97% accuracy (Supplementary Table 3). 18S rRNA sequences were classified using the SILVA database (Quast et al., 2013). We determined the relative abundance of fungal genera of the occiput (back of head), nare (nostril), plantar heel and retroauricular crease (behind the ear). The genus *Malassezia* predominated in the retroauricular crease, nare and occiput; this was consistent across 18S rRNA and ITS1-characterized samples. Plantar heel was the most diverse site with representation of *Malassezia*, *Aspergillus*, *Cryptococcus*, *Rhodotorula*, *Epicoccum*, and others (Supplementary Fig. 2). ITS1 sequencing

enabled greater genus-level taxonomic resolution, reflecting the specificity of the genomic region and richness of the molecular database. Based on technical and analytic advantages, we selected the ITS1 region for subsequent sequencing and analyses of fungal diversity.

We generated more than 5 million ITS1 sequences from 10 healthy volunteers (from each of whom 14 skin-site samples were taken) (Supplementary Table 4). Both Ascomycetous and Basidiomycetous fungi were identified as normal skin flora. The genus *Malassezia* predominated at all 11 core-body and arm sites: antecubital fossa, back, external auditory canal, glabella, hypothenar palm, inguinal crease, manubrium, nare, occiput, retroauricular crease and volar forearm (Figure A16-1). We explored *Malassezia* species-level resolution with a taxonomic data set that we developed based on reference ITS1 sequences and our human-associated *Malassezia* isolates. Pairwise comparisons of these *Malassezia* ITS1 sequences showed sequence identity within species to be greater than 91%, and identity between species to be 70 to 88%. These *Malassezia* sequences served as references within the phylogenetic placer (Matsen et al., 2010) program to classify approximately 80 to 90% of *Malassezia* sequences per skin site to species level. Species-level identification revealed fungal specificity between body sites (Figure A16-1). *M. restricta* predominated in external auditory canal, retroauricular crease and glabella, and *M. globosa* predominated on back, occiput and inguinal crease. Sites such as nares, antecubital fossa, volar forearm and hypothenar palm were characterized by multiple species (*M. restricta*, *M. globosa*, and *M. sympodialis*). In total, we identified 11 of the 14 known *Malassezia* species among skin sites, suggesting that human skin is colonized with a wide range of *Malassezia*. Based on species-level resolution, we observed that fungal diversity is more dependent on body site than individual subject. ITS1 sequences also matched *Candida* species *tropicalis*, *parapsilosis*, and *orthopsilosis*, and *Cryptococcus* species *flavus*, *dimennae*, and *diffluens*, which are considered to be part of the normal human flora and to be possible pathogens in wounds or immunocompromised patients (Larone et al., 2002).

Substantially greater diversity was observed on three foot sites (plantar heel, toenail and toe web), in both the number of genera observed and the variation between individuals (Supplementary Fig. 3 and Supplementary Table 5). The fungal profile of one of the subjects (who we will refer to as healthy volunteer 7) was notably more diverse than other participants (Figure A16-1). Healthy volunteer 7 completed a 2-month course of oral antifungal medication for a toenail infection 7 months before sampling. The remaining healthy volunteers reported no use of either oral or topical antifungal medication for at least 2 years before sampling. Healthy volunteer 7 is an outlier, but the additional genera that were identified (for example, *Aspergillus* and *Saccharomyces*) show that skin is capable of harbouring high fungal diversity. Although microbial sequencing is unable to determine causation, these data may suggest either that fungal community imbalance is associated with recurrent toenail infections or that alterations in fungal skin

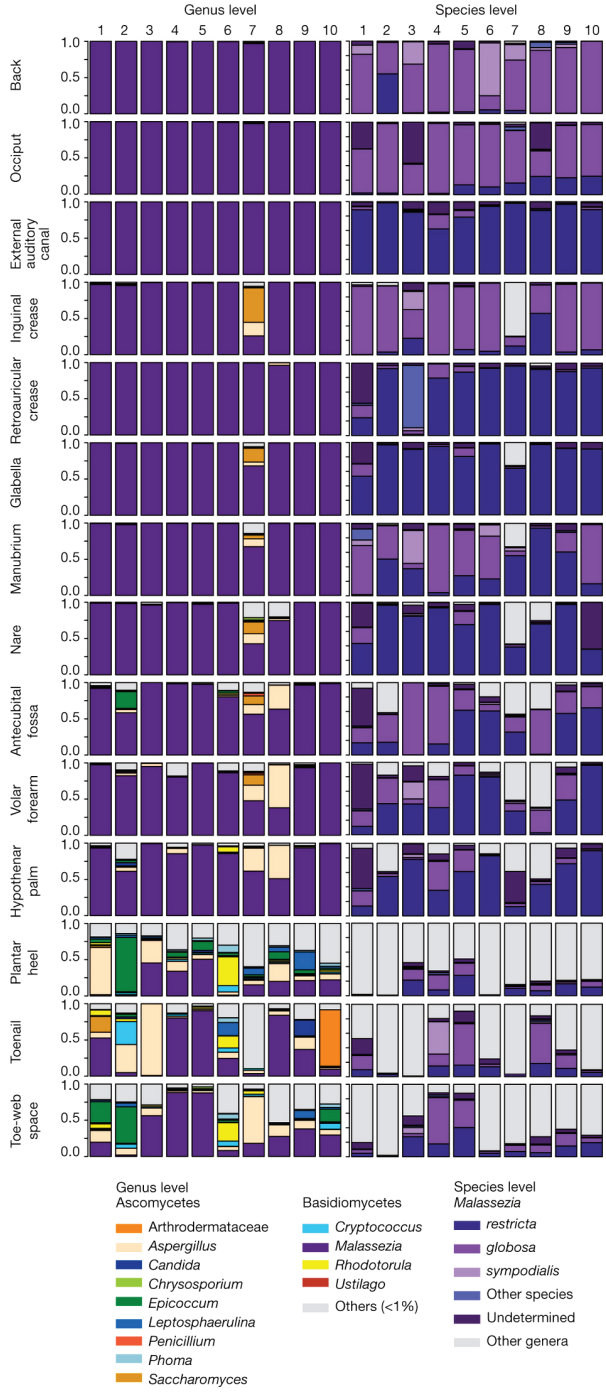


FIGURE A16-1 Relative abundance of fungal genera and *Malassezia* species at different human skin sites. Fungal diversity of individual body sites of healthy volunteers (1–10) was taxonomically classified at the genus level, with further resolution of *Malassezia* species. For all body sites, the left side of the body was used, except for the right toenail of healthy volunteer 7.

communities persist even 7 months after discontinuing antifungal medications. In comparison with culture-based analysis, ITS1 sequencing can provide a more complete view of the diversity of commensal microbiota, and also of potentially pathogenic microbiota.

To quantify and compare community similarity and taxonomic richness of skin sites, we assigned fungal sequences to taxonomic units based on genus-level phylogeny rather than percent sequence identity to obviate the high variation noted between species (Schloss et al., 2009) with the latter metric. Plantar heel was the most complex fungal site (median richness of approximately 80 genera), and other foot sites had the next highest diversity (toe web and toenail, with approximately 60 and 40 genera, respectively; Figure A16-2, Supplementary Table 6). Arm sites showed intermediate richness, ranging from 18 to 32 genera and core-body sites exhibited much lower richness, ranging from 2 to approximately 10 genera. Thus, regional location is a strong determinant of fungal richness. As observed in skin bacterial studies, left–right similarity within an individual was greater than between different individuals at the same body site (Supplementary Fig. 4 and Supplementary Table 7). To determine the temporal stability of the fungal microbiome, 6 healthy volunteers returned 1 to 3 months after initial sampling. Sites that showed *Malassezia* predominance at initial sampling displayed the same genus- and species-level predominance with strong community structure stability (Supplementary Figs. 5 and 6, and Supplementary Table 8). Foot sites continued to show high diversity, perhaps reflecting frequent environmental exposure.

To explore the relationship between skin-associated fungi and bacteria, we sequenced the 16S rRNA gene from the same clinical samples. Consistent with previous studies (Costello et al., 2009; Grice et al., 2009), bacteria on healthy human skin were predominantly *Propionibacterium*, *Corynebacterium*, and *Staphylococcus* (Supplementary Fig. 7). Similar to other moist skin sites, the toenail bacteria (not surveyed previously) were predominantly *Corynebacterium* and *Staphylococcus*. Interestingly, although healthy volunteer 7 was an outlier in terms of fungal diversity and membership, the bacterial profile was normal with respect to taxonomic and ecological measures of diversity (Supplementary Fig. 7). Directed studies may help elucidate how antibacterial and antifungal therapies perturb fungal and bacterial communities.

Bacterial and fungal richness were not linearly correlated, but were instead grouped into discrete clusters of sites from the same region; arm, foot and core-body (sites from the same regions had similar bacterial and fungal richness) (Figure A16-2 and Supplementary Fig. 8). Arm sites displayed markedly greater bacterial diversity and lower fungal diversity than the foot and core-body sites. In contrast, foot sites displayed markedly greater fungal diversity with lower bacterial diversity than the arm and core-body sites. Core-body sites clustered together, and showed both lower bacterial and lower fungal diversity. These data reveal that

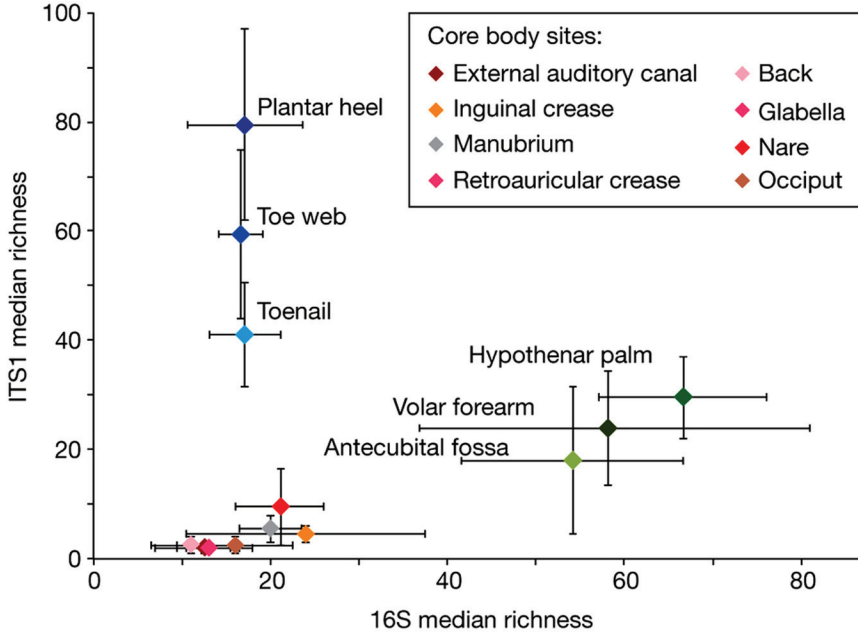


FIGURE A16-2 Median richness of fungal and bacterial genera. Median taxonomic richness (or number of observed genera) of fungal and bacterial genera at 14 body sites. Error bars represent the median absolute deviation. The values for the core body sites retroauricular crease and glabella are identical and are therefore represented by a single data point on the graph and a shared colour in the key.

the skin microbiome is complex and suggest that different characteristics shape skin bacterial and fungal communities.

Using principal coordinates analysis of community structure, we explored properties that may shape bacterial and fungal communities differentially. Consistent with previous studies (Peleg et al., 2010), bacterial communities varied in the proportion of lipophilic bacteria (*Corynebacterium*, *Propionibacterium*, and *Turicella*) and staphylococcal species, and grouped based on skin physiology into sebaceous, moist and dry sites (Figure A16-3 and Supplementary Fig. 9). In contrast, fungal communities were segregated more clearly by site location than physiology, with foot, arm, head and torso sites forming discrete groups. Different *Malassezia* species drive variation in arms, torso and head, whereas a wide range of fungal genera drive variation in feet (Figure A16-3 and Supplementary Fig. 9). Co-occurrence analysis of foot sites, based on Spearman correlation of fungal and bacterial taxonomic relative abundances (Supplementary Fig. 10), provided a preliminary evaluation of major fungal–bacterial associations. For

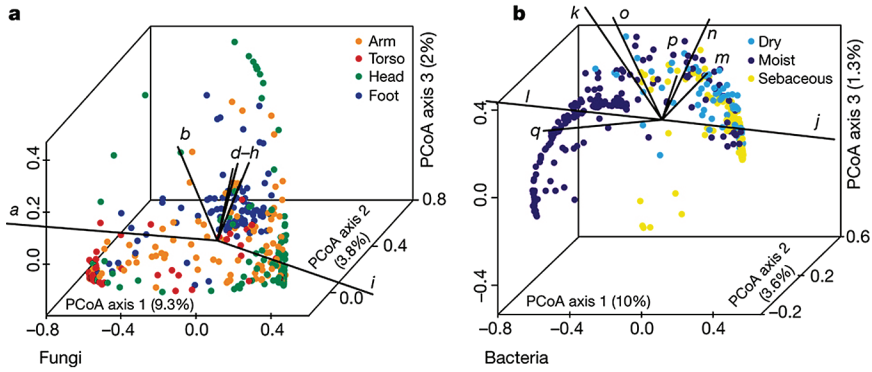


FIGURE A16-3 Forces that shape fungal and bacterial communities. a, b, Principal coordinates analysis (PCoA) of the degree of fungal- and bacterial-community similarity at 14 body sites, based on predominant genera and species. Variation in fungal communities segregated strongly according to site location, with arm, foot, head and core-body sites forming discrete groups (a). Bacterial community structure was more dependent on site physiology (b). Axes that most significantly contribute to variation and their relative lengths are shown (these are defined in Supplementary Fig. 10 legend). For fungi, *M. restricta* (*i*; ρ = 0.92) and *M. globosa* (*a*; ρ = –0.79) are primary and opposing drivers of variation based on Spearman correlation with PCoA axis 1. For bacteria, *Propionibacterium* (*j*; ρ = 0.95) contributes to sebaceous site variation, whereas *Corynebacterium* (*l*; ρ = –0.74) and *Turicella* (*q*; ρ = –0.56) are the greatest contributors at moist sites based on Spearman correlation with PCoA axis 1.

example, a group of primarily Actinobacteria was anti-correlated with resident Ascomycota and Basidiomycota in contrast to a group of primarily Firmicutes and Proteobacteria that was positively correlated with these fungal taxa.

We observed that 20% (12 out of 60) of our study participants had clinical involvement (plantar-heel scaling, toe web scaling, or toenail changes), consistent with possible fungal infections (Supplementary Table 1). Of the subjects with observed clinical involvement, positive mycological cultures were obtained from toenails (two samples) and plantar heel (one sample) (*Trichophyton*, *Penicillium*, and *Aspergillus*). These observations are similar to the results of larger studies, which report signs of clinical involvement in up to 60% of feet of healthy individuals, and find 2 to 25% of cultures to be positive for fungi. The wide variation in prevalence of clinical involvement and positive fungal cultures is dependent on several factors, including population and climate (Cohen et al., 2005; Perea et al., 2000). As an initial inquiry into the aetiology of foot fungal disorders, we examined how observed clinical status (involved or uninvolved) at plantar heel, toe web or toenail affected fungal community structure. For uninvolved sites, interpersonal variation in community structure was highly consistent across all

foot sites. In contrast, for sites with observed clinical involvement, similarity of community structure was much higher for plantar heel but much lower for toenail (Figure A16-4). These data may suggest that there is a common fungal community shared among individuals with plantar-heel involvement, and high fungal diversity underlying toenail infections, but further studies are needed. These data sets can now be used to inform future clinical studies that examine microbial community shifts associated with fungal infections.

This systematic study clearly demonstrates that human skin surfaces are complex ecosystems, providing diverse environments for microorganisms that inhabit our bodies. Different factors determine bacterial and fungal communities, depending on the physiological properties of the skin. *Malassezia* species predominate on all core-body and arm sites. In contrast, foot sites display tremendous fungal diversity and markedly lower stability over time. Microbial community instability may provide an opportunity for potentially pathogenic microbes to establish disease. Plantar heels, toe webs and toenails are common sites of recurrent human fungal disease, which can be recalcitrant to treatment.

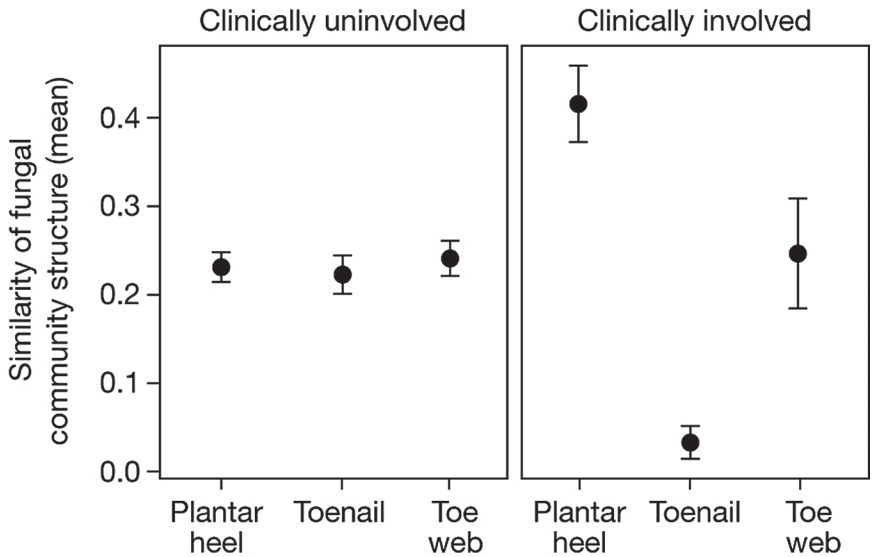


FIGURE A16-4 Clinical involvement alters shared fungal community structure. Community structure measures the type and relative abundance of each genus. A value of 1 implies identical community structure and 0 implies dissimilar structures. Among uninvolved foot sites, community structure is fairly consistent at plantar heel, toenail and toe web sites. For involved sites, plantar heel has much greater shared community structure and toenails have much lower shared community structure. Error bars represent the s.e.m.

This study also investigated fungal diversity at sites of predilection for other skin disorders, including seborrheic dermatitis, tinea cruris, and subtypes of atopic dermatitis. With genomic advances, such as shotgun metagenomic sequencing, it is possible to begin to address interactions between microbes (bacterial–fungal, bacterial–bacterial, fungal–fungal) residing in these complex environments. The role of fungal commensals in educating the human immune system is gaining new appreciation in intestinal disease (Iliev et al., 2012). Further studies of healthy skin and dermatologic disorders are needed to explore these host–microbe interactions. In addition, antifungal medications, including azoles, echinocandins and amphotericin B, have potentially serious side effects such as liver or kidney damage (Perfect et al., 1992). Therefore, new treatment approaches are required to strategically target microbial dysbiosis and to combat the increasingly observed resistance against our current arsenal of antimicrobial therapies.

Methods Summary

Subject Recruitment and Sampling

This study was approved by the Institutional Review Board of the National Human Genome Research Institute (http://www.clinicaltrials.gov/ct2/show/NCT00605878) and all subjects provided written informed consent before participation. For fungal culturing studies, skin was scraped with a surgical blade and placed directly in media. For sequencing studies, swabs from skin and environmental negative controls were placed in MasterPure Yeast DNA purification kit (Epicentre) lysis buffer augmented with lysozyme. Proteinase K (Invitrogen) was added to pre-digest toenail clippings and incubated overnight with shaking at 55 uC. Steel beads (5 mm in diameter) were added to mechanically disrupt fungal cell walls using Tissuelyser (Qiagen) for 2 min at 30 Hz and then using the PureLink Genomic DNA Kit (Invitrogen). For 18S rRNA sequencing, each DNA was amplified with SR6 (59-TACCTGG TTGATTCTGC) and SR1R (59-TGTTACGACTTTTACTT) primers. For ITS1 sequencing, each DNA was amplified with adaptor plus 18SF (59-GTAA AAGTCGTAACAAGGTTTC) and 5.8S1R plus barcode primers (59-GTTCA AAGAYTCGATGATTCAC). For 16S rRNA sequencing, each DNA was amplified with adaptor plus V1_27F (59-AGAGTTTGATCCTGGCTCAG) and V3_534R plus barcode primers (59-CAGCACGCATTACCGCGGCTGCTGG).

Sequence Classification and Analyses

Sequences were pre-processed to remove primers and barcodes. Possible chimaeras were identified with UCHIME in mother (Edgar et al., 2011; Schloss et al., 2009). ITS1 sequences were classified to genus level with BLAST (basic local-alignment search tool) and the k-nearest neighbour algorithminmothur.

18S rRNA sequences were classified using the SILVA v108 database (Quast et al., 2013). 16S rRNA sequences were classified to genus level using the RDP-classifier and training set 6 (Wang et al., 2007).We curated and aligned a reference library of Malassezia ITS1 type-strain sequences retrieved from GenBank augmented with those from our human-associated fungal cultures. This curated library was used as a reference to phylogenetically place and classify ITS1 sequences to species level within pplacer (Matsen et al., 2010). Sequence placement on the reference tree was visualized in Archaeopteryx using the "guppy" command for classifications with a likelihood score of greater than or equal to 0.65. Full methods are found in the Supplementary Information.

Competing Financial Interests

The authors declare no competing financial interests.

Acknowledgements

We thank J. Heitman, A. Amend, Y. Shea, M. Turner, I. Brownell, and M. Udey for helpful discussions. We thank J. Fekecs for assistance with the figures. This work was supported by the US National Institutes of Health (NIH) NHGRI and NCI Intramural Research Programs, and in part by NIH grant no. 1K99AR059222 (to H.H.K.). Sequencing was funded by grants from the NIH (1UH2AR057504-01 and 4UH3AR057504-02).

Author Contributions

K.F., H.H.K., and J.A.S. designed the outline of the study. D.S. and E.N. recruited human subjects and assisted H.H.K. in sample collection for the experiment. K.F. and J.Y. assembled and curated the fungal database. J.A.M. and C.D. prepared the clinical samples for sequencing. The members of the NIH Intramural Sequencing Center Comparative Sequencing program carried out sequencing. K.F., J.O., S.C. and M.P. analysed sequence data. K.F., H.H.K., and J.A.S. drafted the manuscript with specific contributions from J.O., J.Y., and S.C. All authors read and approved the final version of the manuscript.

Author Information

Sequence data from this study have been submitted to GenBank/EMBL/DDBJ under accession numbers KC669797–KC675175, and the Sequence Read Archive,and can be accessed through BioProject identification no.46333. Patient and sample metadata have been deposited in the controlled-access Database of Genotypes and Phenotypes (dbGaP) under study accession phs000266.v1.p1. Reprints and permissions information is available at www.nature.com/reprints. The

authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to H.H.K. (konghe@mail.nih.gov) or J.A.S. (jsegre@nhgri.nih.gov).

References

Bickers, D. R. *et al.* The burden of skin diseases: 2004 a joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology. *J. Am. Acad. Dermatol.* 55, 490–500 (2006).

Bruns, T. D. *et al.* Evolutionary relationships within the fungi: analyses of nuclear small subunit rRNA sequences. *Mol. Phylogenet. Evol.* 1, 231–241 (1992).

Cohen, A. D., Wolak, A., Alkan, M., Shalev, R. & Vardy, D. A. Prevalence and risk factors for tinea pedis in Israeli soldiers. *Int. J. Dermatol.* 44, 1002–1005 (2005).

Costello, E. K. *et al.* Bacterial community variation in human body habitats across space and time. *Science* 326, 1694–1697 (2009).

Dollive, S. *et al.* A tool kit for quantifying eukaryotic rRNA gene sequences from human microbiome samples. *Genome Biol.* 13, R60 (2012).

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200 (2011).

Gaitanis, G., Magiatis, P., Hantschke, M., Bassukas, I. D. & Velegraki, A. The *Malassezia*genus in skin and systemic diseases. *Clin. Microbiol. Rev.* 25, 106–141 (2012).

Ghannoum, M. A. *et al.* Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* 6, e1000713 (2010).

Gioti, A. *et al.* Genomic insights into the atopic eczema-associated skin commensal yeast*Malassezia sympodialis*. *MBio* 4, e00572–12 (2013).

Grice, E. A. & Segre, J. A. The human microbiome: our second genome. *Annu. Rev. Genomics Hum. Genet.* 13, 151–170 (2012).

Grice, E. A. *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* 324, 1190–1192 (2009).

Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214 (2012).

Iliev, I. D. *et al.* Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 336, 1314–1317 (2012).

James, T. Y. *et al.* Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443, 818–822 (2006).

Larone, D. H. *Medically Important Fungi: A guide to identification* (ASM Press, 2002).

Marples, M., ed. *The Ecology of the Human Skin* (Bannerstone House, 1965).

Matsen, F. A., Kodner, R. B. & Armbrust, E. V. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* 11, 538 (2010).

Paulino, L. C., Tseng, C. H., Strober, B. E. & Blaser, M. J. Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. *J. Clin. Microbiol.* 44,2933–2941 (2006).

Peleg, A. Y., Hogan, D. A. & Mylonakis, E. Medically important bacterial–fungal interactions. *Nature Rev. Microbiol.* 8, 340–349 (2010).

Perea, S. *et al.* Prevalence and risk factors of tinea unguium and tinea pedis in the general population in Spain. *J. Clin. Microbiol.* 38, 3226–3230 (2000).

Perfect, J. R., Lindsay, M. H. & Drew, R. H. Adverse drug reactions to systemic antifungals. Prevention and management. *Drug Saf.* 7, 323–363 (1992).

Pflughoeft, K. J. & Versalovic, J. Human microbiome in health and disease. *Annu. Rev. Pathol.* 7, 99–122 (2012).

Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596 (2013).

Roth, R. R. & James, W. D. Microbial ecology of the skin. *Annu. Rev. Microbiol.* 42,441–464 (1988).

Saunders, C. W., Scheynius, A. & Heitman, J. *Malassezia* fungi are specialized to live on skin and associated with dandruff, eczema, and other skin diseases. *PLoS Pathog.* 8,e1002701 (2012).

Schloss, P. D. *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541 (2009).

Schoch, C. L. *et al.* Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl Acad. Sci. USA* 109, 6241–6246 (2012).

St-Germain, G. & Summerbell, R. *Identifying Fungi: A Clinical Laboratory Handbook* (Star Publishing Company, 2011).

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.*73, 5261–5267 (2007).

A17

DISTINCT MICROBIAL COMMUNITIES WITHIN THE ENDOSPHERE AND RHIZOSPHERE OF *POPULUS DELTOIDES* ROOTS ACROSS CONTRASTING SOIL TYPES[72]

Neil R. Gottel,[73] *Hector F. Castro*,[73,74] *Marilyn Kerley*,[73] *Zamin Yang*,[73] *Dale A. Pelletier*,[73] *Mircea Podar*,[73,75] *Tatiana Karpinets*,[73,76] *Ed Uberbacher*,[73] *Gerald A.* ***Tuskan***,[73,76] *Rytas Vilgalys*,[77] *Mitchel J. Doktycz*,[73] *and Christopher W. Schadt*[73,75,*]

Abstract

The root-rhizosphere interface of *Populus* is the nexus of a variety of associations between bacteria, fungi, and the host plant and an ideal model

[72] Reprinted with permission from Gerald A. Tuskan. Originally printed as Gottel, N. R. et al. 2011. Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* 77(17):5934-5944.

[73] Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831.

[74] Department of Ecology and Evolutionary Biology.

[75] Department of Microbiology.

[76] Department of Plant Sciences, University of Tennessee, Knoxville, Tennessee 37796.

[77] Department of Biology, Duke University, Durham, North Carolina 27708.

* Corresponding author. Mailing address: Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6038. Phone: (865) 576-3982. Fax: (865) 576-8646. E-mail: schadtcw@ornl.gov.

for studying interactions between plants and microorganisms. However, such studies have generally been confined to greenhouse and plantation systems. Here we analyze microbial communities from the root endophytic and rhizospheric habitats of *Populus deltoides* in mature natural trees from both upland and bottomland sites in central Tennessee. Community profiling utilized 454 pyrosequencing with separate primers targeting the V4 region for bacterial 16S rRNA and the D1/D2 region for fungal28S rRNA genes. Rhizosphere bacteria were dominated by *Acidobacteria* (31%) and *Alphaproteobacteria* (30%), whereas most endophytes were from the *Gammaproteobacteria* (54%) as well as *Alphaproteobacteria* (23%). A single *Pseudomonas*-like operational taxonomic unit (OTU) accounted for 34% of endophytic bacterial sequences. Endophytic bacterial richness was also highly variable and 10-fold lower than in rhizosphere samples originating from the same roots. Fungal rhizosphere and endophyte samples had approximately equal amounts of the *Pezizomycotina* (40%), while the *Agaricomycotina* were more abundant in the rhizosphere (34%) than endosphere (17%). Both fungal and bacterial rhizosphere samples were highly clustered compared to the more variable endophyte samples in a UniFrac principal coordinates analysis, regardless of upland or bottomland site origin. Hierarchical clustering of OTU relative abundance patterns also showed that the most abundant bacterial and fungal OTUs tended to be dominant in either the endophyte or rhizosphere samples but not both. Together, these findings demonstrate that root endophytic communities are distinct assemblages rather than opportunistic subsets of the rhizosphere.

Introduction

Populus is considered the model organism for the study of woody perennials (Tuskan et al., 2004) and represents the first tree genome to be fully sequenced (Tuskan et al., 2006). *Populus* has also received attention in bioenergy research for the production of cellulose-derived biofuels (Tuskan et al., 2006). *Populus* can be grown on land not suitable for food production and increase carbon sequestration, thus minimizing the competition between food and fuel production and reducing the carbon debt incurred through land use changes (Sannigrahi et al., 2010). *Populus* may also provide an ideal model for understanding a variety of plant-microbe interactions (Taghavi et al., 2009). *Populus* and other members of the *Salicaceae* are capable of establishing associations with both arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi (Gehring et al., 2006) that may result in unique interactions between these fungi, as well as other endophytic and rhizospheric organisms and the host. *Populus* bacterial rhizosphere and endophytic constituents have received some attention due to their potential role in phytoremediation of industrial chemicals (Moore et al., 2006) and heavy metals (Biro and Takacs, 2007), as well as plant growth-promoting bacteria (PGPB), which benefit plants by providing fixed nitrogen and/or aiding resistance

to infection by pathogens (Jones and Dangl, 2006). However, most studies of such relationships have been greenhouse or plantation based, and the rhizosphere and endophyte communities of *Populus* from natural systems have not been studied comprehensively by molecular ecology approaches. Newly developed, high-throughput sequencing approaches of bacterial and fungal rRNA gene markers should enable an expanded understanding of such plant-microbe relationships and comprehensive descriptions of the full diversity of associations within the *Populus* microbiome.

Roots are the primary sites of plant nutrient import and organic molecule export, which provide carbon and energy sources to nearby microorganisms and result in a "rhizosphere" that supports higher bacterial numbers than do bulk soils (Hinsinger et al., 2009). In many cases these relationships seem to have evolved to the point where certain microorganisms appear to live nonpathogenically as endophytes within the roots or, in the case of some fungi, establish mutually beneficial mycorrhizal relationships (Gehring et al., 2006). In the case of bacterial root endophytes, it is unclear whether these microorganisms represent a specialized community or merely opportunistic rhizosphere microorganisms (Hardoim et al., 2008). Due to the complexity of the plant root habitat and the nearby rhizosphere, as in other terrestrial ecosystems, a great fraction of the microorganisms present likely remain unknown and uncultured (Amann et al., 1995).

This study focuses on the root endophyte and the directly associated rhizosphere communities of two populations of *Populus deltoides* located in upland and bottomland sites near the Caney Fork River in central Tennessee. Both fungi

TABLE A17-1 Selected Tree Dendrometric Measurements and Soil Physicochemical Characteristics for Bottomland and Upland Sites[a]

Tree sample ID no.	DBH* (cm)[b]	Crown ht* (m)	Crown width (radius, m)	Soil texture	Moisture* (%)	pH*
B1	58.4	25.8	7.6 (1.5)[d]	Sandy loam	22.2 (0.1)[c]	6.6
B2	89.6	38.4	8.2 (2.2)	Sandy loam	25.3 (0.6)	6.7
B3	91.1	32.7	7.6 (1.5)	Sandy loam	29.6 (0.3)	6.8
B4	77.9	31.0	7.8 (0.4)	Sandy loam	28.4 (0.2)	6.6
U1	37.0	21.5	4.3 (0.2)	Clay loam	40.0 (1.7)	7.9
U2	38.1	19.9	7.2 (3.1)	Clay	39.8 (0.4)	7.7
U3	38.4	18.9	5.1 (1.3)	Clay	35.0 (0.7)	7.8

[a] A characteristic is followed by an asterisk when the means were significantly different ($P \leq 0.01$) for bottomland (B) versus upland (U) sites.

[b] DBH, diameter at breast height.

[c] OM, organic matter.

[d] Values in parentheses represent standard deviations of 3 measurement replicates.

and bacteria were characterized from the same samples by using bar-coded pyrosequencing of rRNA genes, enabling direct comparisons between these cooccurring communities. By utilizing a nested sampling design, we focused on three main questions: (i) Do endophytic communities reflect a specialized group of organisms or merely an opportunistic subset of the associated rhizosphere community? (ii) To what extent do site and soil conditions modulate endophyte and rhizosphere community composition? (iii) How variable are microbial communities among trees, sites, and the ecological niches of the root endosphere and rhizosphere?

Materials and Methods

Sites Description and Sample Collection

Native *P. deltoides* samples were collected in the basin of the Caney Fork River in the Buffalo Valley Recreation Area, downstream of the Center Hill Dam (bottomland site) and within Edgar Evans State Park (upland site), both in DeKalb County, TN, during the first week of October 2009. Three and four mature *Populus* trees were selected from the upland (36°4′N, 85°50′W) and bottomland (36°6′N, 85°50′W) sites, respectively. The soil characteristics of each tree and surrounding soil are presented in Table A17-1. Three soil cores were taken adjacent to each tree and kept on ice and refrigerated until soil characterizations. Soil moisture was determined gravimetrically after sieving to 4 mm. Other soil characterizations were performed by the Agricultural and Environmental Services

Total C* (%)	Total N* (%)	OM* (%)[c]	K* (ppm)	NH_4-N (ppm)	NO_3-N (ppm)	P* (ppm)
1.7	0.14	2.5	38.6	1.7	5.0	47.1
1.9	0.17	2.7	55.2	2.2	10.1	74.2
2.5	0.20	3.7	56.9	2.5	16.0	68.6
2.2	0.17	3.1	52.3	2.6	11.7	78.4
6.2	0.38	8.7	123.2	2.8	9.6	202.1
5.6	0.41	8.2	134.6	2.9	16.9	249.5
5.1	0.35	7.6	121.3	2.7	5.16	233.7

Laboratory (AESL) at the University of Georgia (http://aesl.ces.uga.edu/) on these same sieved, composited samples. Statistical comparisons of upland and bottomland site and soil characteristics were conducted using Fisher's *t* test as implemented in StatPlus:mac LE (AnalystSoft, Inc.). Three primary lateral roots near each tree were carefully excavated and traced from the originating tree to ensure identity. Tertiary fine roots were removed, shaken over a sieve to remove loose soil, and washed with 100 ml of 10 mM NaCl solution to remove the adhering rhizosphere soil. This wash solution was collected into 50-ml tubes and constituted the rhizosphere samples. Multiple root samples were collected from different lateral roots of each tree, with nine total samples at the upland site (labeled the U site) and 11 root samples at the bottomland site (labeled B site). For a detailed breakdown of the nested sampling schema, refer to Table S1 in the supplemental material. Washed root and rhizosphere samples were transported on ice and stored at 4°C for up to 2 days prior to surface sterilization of the roots and then stored at −80°C thereafter.

Root surface sterilization was performed 2 days after collection by rinsing roots 5 times with sterile distilled water (dH2O) and then transferring roots with a diameter of 2 mm or less to 50-ml centrifuge tubes. The surface sterilization protocol used 3 washes with sterile dH2O before and after the following sterilization steps: 3% H2O2 for 30 s, 100% ethanol for 30 s, 6.15% NaOCl with 2 to 3 drops of Tween 20 per 100 ml for 3 min, and finally again with 3% H2O2 for 30 s. Surface sterility was confirmed for all samples by touching a subsampled root from each collection onto LB plates and incubating overnight at 30°C.

DNA Extraction

Surface-sterilized roots were chopped into 1 mm sections by using a sterilized razor blade in a petri dish. Each root sample was split into five 50 mg subsamples, and total DNA was extracted using the PowerPlant DNA isolation kit (MoBio, Carlsbad, CA), with the following modification to the manufacturer's instructions: 50 µl of 10% cetyltrimethylammonium bromide was added to each lysis tube containing the lysis solution and roots to enhance plant cell lysis, followed by three freeze-thaw cycles (−80°C/65°C; 10 min each) and homogenization in a mixer mill for 20 min at 30 Hz (model MM400; Retsch Inc., Newtown, PA). Each set of 5 subsampled extractions was then concentrated into a single 50-µl DNA sample. DNA was quantified using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). For rhizosphere samples, 2.0 ml of soil slurry and associated cells was pelleted via low-speed centrifugation, and the extractions were carried out using the standard MoBio protocol and PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) and the Retsch mixer mill as described above.

Bacterial and Fungal Ribosomal PCR Amplification and Sequencing

Fifty microliters of 454 sequencing template per sample was produced using the following PCR reagent concentrations: 1× High Fidelity PCR buffer (Invitrogen, Carlsbad, CA), 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 2 mM MgSO4, 0.6 µM forward and reverse primers, 1.0 mg/ml bovine serum albumin (BSA), and 2 units of Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA). To each 49-µl reaction mixture, 1 µl of template DNA (diluted 1:10 in 1× Tris) was added. Thermocycler settings for rhizosphere samples were 2 min at 95°C, then 30 cycles of 95°C for 15 s, 55°C for 45 s, and 68°C for 45 s, with a final extension for 7 min at 68°C. For endophyte samples, 35 cycles were used instead of 30. Bacterial primers targeted the V4 region of the 16S rRNA gene, as described by Vishnivetskaya et al. (2011), and fungal primers targeted the first ~700 bp of the 28S LSU rRNA gene by using the primers LROR and LR3 (Castro et al., 2010), which had been modified to contain the 454 A and B primer, and the A primer was further modified to contain one of 20 8-bp DNA bar codes downloaded from the Ribosomal Database Project (RDP) (Cole et al., 2009). Unincorporated primers, primer dimers, and dNTPs were removed by using the Agencourt AMPure purification system (Beckman Coulter, Danvers, MA). Product purity and concentration were checked with an Agilent 2100 Bioanalyzer (Santa Clara, CA). Emulsion reactions were performed in paired samples containing 2 sample PCR amplicons that were matched for template quantity and quality (Vishnivetskaya et al., 2011). Sequencing was performed on a Roche genome sequencer FLX system (Indianapolis, IN) using the manufacturer's recommended conditions. Twenty samples originating from the rhizosphere and 20 samples originating from the endosphere were loaded onto the A and B regions of the sequencing plates, and separate pyrosequencing runs were performed for bacterial and fungal amplicons.

Sequence Analysis

Raw bacterial sequence outputs from the FLX system were uploaded to the Ribosomal Database Project Pyrosequencing Pipeline (http://pyro.cme.msu.edu/index.jsp) (Cole et al., 2009) for primer removal and quality control and then restricted to the desired length (see Results for further details). *Populus* plastid and mitochondrial 16S rRNA sequences were identified using BLAST search similarity against the entire data set, and those with >95% similarity to the plant sequence were removed from the data set. Bacterial taxonomy was assigned using the RDP Classifier (Cole et al., 2009). Raw fungal sequences were trimmed and quality checked using mothur (Schloss et al., 2009). Fungal sequences were classified by their top BLAST hit compared against the SILVA LSU database in MG-RAST (Meyer et al., 2008) for representative operational taxonomic units (OTUs). The mothur program was further used for alignment of bacterial and fungal sequences based on the full alignments of the rRNA SSU and LSU,

respectively, of the SILVA database, release 104, as a template (Pruesse et al., 2007). The mothur program was also used for preclustering at 2% (20), rarefaction curves, distance calculations, clustering, and further analysis based on OTUs.

Bacterial and fungal data were analyzed separately using the Fast UniFrac program (Meyer et al., 2008), which provides a suite of tools to compare microbial communities under a phylogenetic framework. The bacterial sequences were mapped to relatives contained in the Greengenes database core set. The fungal data set was mapped to a version of the eukaryotic SILVA LSU reference database that was parsed locally to include 565 representative sequences. Phylogenetic trees were built using RAxML v7.0.4 (Vishnivetskaya et al., 2011) using maximum likelihood and an optimized GAMMA model of rate heterogeneity and alpha shape parameter. Trees were analyzed using the unweighted options within Fast UniFrac, and samples were categorized according to sample source (endophyte or rhizosphere) and soil type (bottomland or upland). Both UniFrac and P-test significances were used to compare the microbial communities, as these two methods test different hypotheses and can result in different P values (Meyer et al., 2008). The UniFrac metric tests for differences among treatments by using the branch length, which is unique to each treatment. A P-test uses a phylogenetic tree to test whether two environments are significantly different by using parsimony-based scoring (Lozupone et al., 2006). UniFrac tests were performed using 1,000 permutations and calculated with the Fast UniFrac web application (http://bmf2.colorado.edu/fastunifrac/) (Hamady et al., 2010). UniFrac and P-test significance values were corrected using the Bonferroni correction for multiple comparisons. Principal coordinate analysis (PCoA) was further performed using the Fast UniFrac metric and visualized by origin from either endophyte or rhizosphere in both upland and bottomland sites by using the 3D Java KiNG image program (http://kinemage.biochem.duke.edu/software/).

Hierarchical Clustering

Before clustering, low-abundance and rare OTUs were filtered out by using a two-step procedure. OTUs were first sorted by the sums of their relative abundances across all samples in the data set, and those OTUs accounting for 50% of the total relative abundance in the data set were retained (85 of 8,686 bacterial and 78 of 12,532 fungal OTUs). Second, we removed rare OTUs by retaining only those that occurred in more than 5 samples. The final data set thus included 76 bacterial and 72 fungal/eukaryote OTUs, representing the most abundant and common taxa in our data set. The hierarchical clustering of the percent abundance of the selected OTUs in each sample was implemented using Cluster 3.0 (Eisen et al., 1998) and visualized in TreeView (Schloss et al., 2009) with the Spearman rank correlation coefficients as the similarity metric and a complete linkage clustering criteria.

Results

Tree and Soil Characteristics

A total of 9 upland site samples (3 each from 3 trees) were collected for endophyte, rhizosphere, and bulk soil analysis. The bulk soil samples were pooled from around each tree for soil physical and biochemical characterization. Eleven bottomland site samples, 3 each from 3 trees, and a set of 2 from a fourth tree, were similarly analyzed. Upon examination, the tree ring core sample from the bottomland tree (B3) was found to have significant brown rot in the center of the core. However, other plant phenotypic, soil, and microbial community characteristics from the two samples originating from this tree were not found to substantially differ from the remainder of the samples, and data associated with these samples were thus retained. The upland and bottomland sites differed significantly in both the characteristics of the *Populus* trees sampled (diameter, height, etc.) and most soil characteristics (texture, percent C, percent N, etc.) with the exception of the concentrations of ammonium and nitrate ions, which were highly variable within each site (Table A17-1).

Sequencing Quality Control and Results

Multiple levels of quality control during sequence processing and data analysis were employed. Sequences with a Phred score of <25 were removed, ensuring that the lowest-quality sequences had only ~0.3% probability of an incorrectly called base (Schloss et al., 2009). Sequences with ambiguous bases and homopolymers (>10 bases) and sequences of <150 bp or >300 bp were removed during the initial tag and primer checks through the RDP. Aligned sequences were then trimmed to a common region. Further quality control was achieved by then clustering the entire data set at 97% similarity (bacteria) or 95% similarity (fungi) and eliminating all singletons as, globally, single-sequence clusters are likely sequencing errors (Kunin et al., 2010). However, this may eliminate some extremely rare actual community members.

After initial processing, a total of 177,291 reads for bacterial endophytic samples and 100,311 reads for bacterial rhizosphere samples were generated in our pyrosequencing survey, with an average length read of 205 bp. In the fungal data set, after trimming and processing using mothur, 169,771 endophytic reads and 152,329 rhizosphere reads remained, with an average read length of 244 bp. After singleton removal, there were 116,685 bacterial sequences and 316,360 fungal sequences remaining, out of the original 120,052 bacterial sequences and 322,100 fungal sequences. The number of bacterial sequences was much lower than the fungal sequences, owing to the removal of 67,000 mitochondrial and 65,266 plastid sequences from the endophytic bacterial sample processing that were identified via BLAST similarity against the genomes of each organelle. A detailed breakdown of recovered sequence numbers sample by sample is

presented in Table S1 of the supplemental material. Representative sequences of all OTUs and the entire quality control data set are available from our project website (http://pmi.ornl.gov) or by contacting the corresponding author.

Bacterial endophytic read counts were distributed unevenly among samples, with an average number of 1,117 per sample and a standard deviation of 1,204. Bacterial rhizosphere sequences were more evenly distributed, with an average of 5,015 sequences per sample and a standard deviation of 617. Fungal libraries had a fairly small number of nontarget sequences. On average our LSU rRNA gene fungal endophyte libraries contained ~78% sequences that were classifiable as fungi, ~11% as *Viridiplantae*, and ~10% as metazoan, with the small remainder coming from *Alveolates*, *Cercozoa*, and other eukaryotes. Rhizosphere fungal libraries were on average ~87% fungi, 3.1% *Viridiplantae*, 9.7% metazoans, and a small number from other eukaryotes. The fungal primers used in the study were designed for fungal specificity as well as breadth, so the overall abundance of these nonfungal eukaryotic groups should not be considered to have ecological meaning in these studies. Thus, we excluded nonfungal sequences from the overall phylogenetic classifications (see Figure A17-4, below). However, nonfungal sequences were not excluded from OTU-level comparisons, as individual OTUs that amplified are unlikely to be affected by primer biases; thus, Figures A17-2, A17-5, and A17-6, below, contain nonfungal, eukaryotic OTUs.

Characterization of Endophyte and Rhizosphere Bacterial and Fungal Communities

Rarefaction curves were generated via mothur using a 97% identity cutoff for bacterial samples (Figure A17-1) and a 95% identity cutoff for fungal samples (Figure A17-2). As expected, endophytic microbial communities were less diverse than the rhizosphere community for both bacteria and fungi. However, bacterial endophytes exhibited a high degree of variation in the shape of their rarefaction curves compared to the other sample types. The majority of the bacterial endophyte samples saturated below 50 OTUs, although several samples continued to increase past 200 OTUs. No differences were apparent for rarefaction curves for microbial communities originating from upland versus bottomland sites. The fungal rarefaction curves displayed similar trends as the bacterial curves, with greater richness in the rhizosphere; however, endophytic samples were not as variable as the bacterial communities and on average contained more OTUs at equal sequencing depths (Figure A17-2).

Rhizospheric and endophytic bacterial communities exhibited different overall patterns of relative abundance of the major groups at the phylum level (Figure A17-3). No major differences in the phyla relative abundance patterns were observed between the upland and bottomland environments, and sample-to-sample variation was also low (data not shown). Rhizosphere bacterial communities were similar to previously reported soil communities (Kend and Triplett,

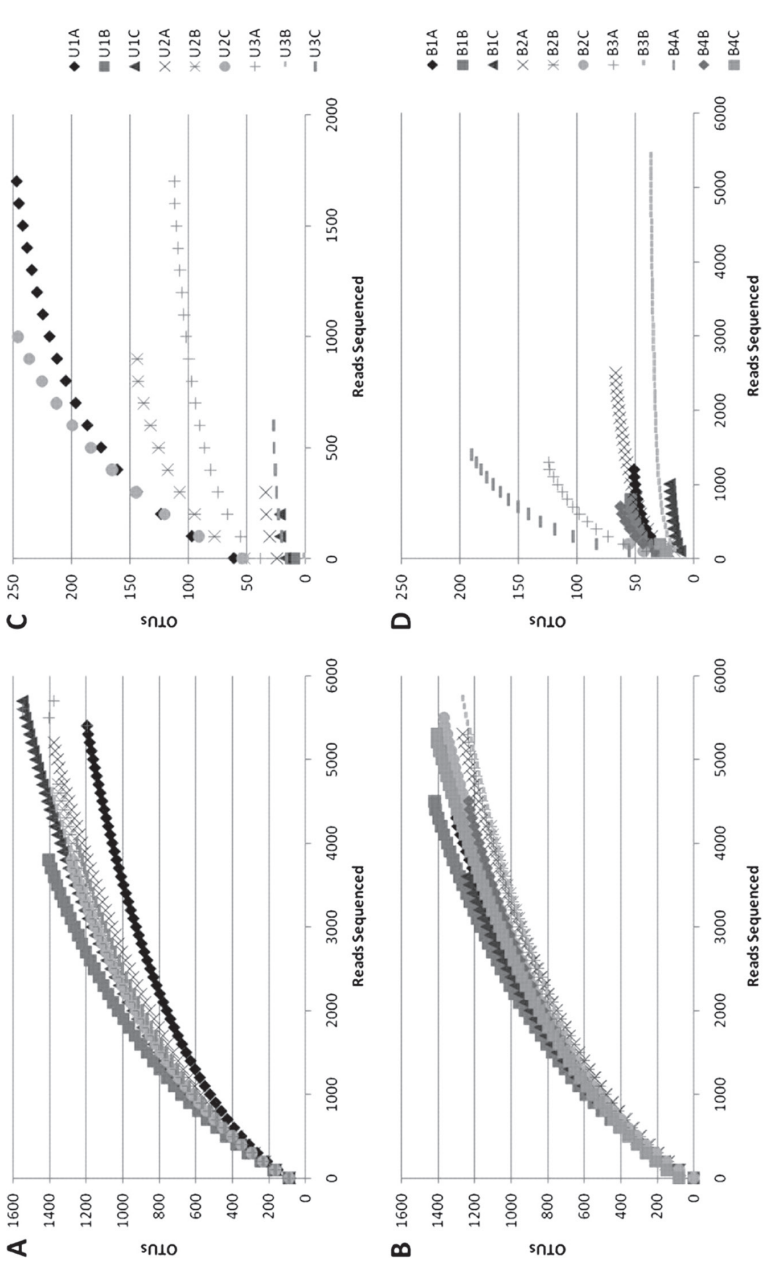


FIGURE A17-1 Rarefaction curves for bacterial OTUs, clustering at 97% rRNA sequence similarity. Curves represent sequences for multiple samples of rhizospheric (A and B) or endophytic (C and D) communities originating from samples of either upland (A and C) or bottomland (B and D) trees. See Table S1 in the supplemental material for a more detailed breakdown of properties on a sample-by-sample basis.

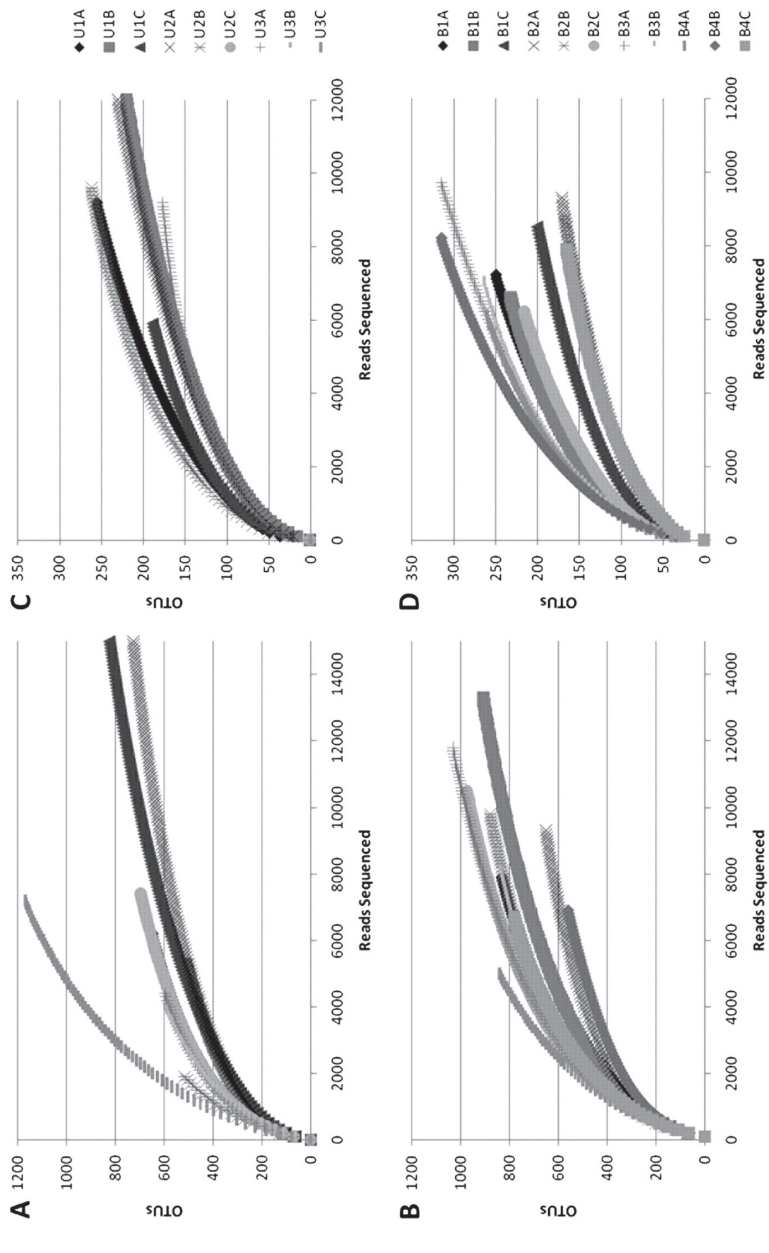


FIGURE A17-2 Rarefaction curves for fungal OTUs, clustering at 95% rRNA sequence similarity. Curves represent sequences for multiple samples of rhizospheric (A and B) and endophytic (C and D) communities originating from samples of either upland (A and C) or bottomland (B and D) trees. See Table S1 in the supplemental material for a more detailed breakdown of properties on a sample-by-sample basis.

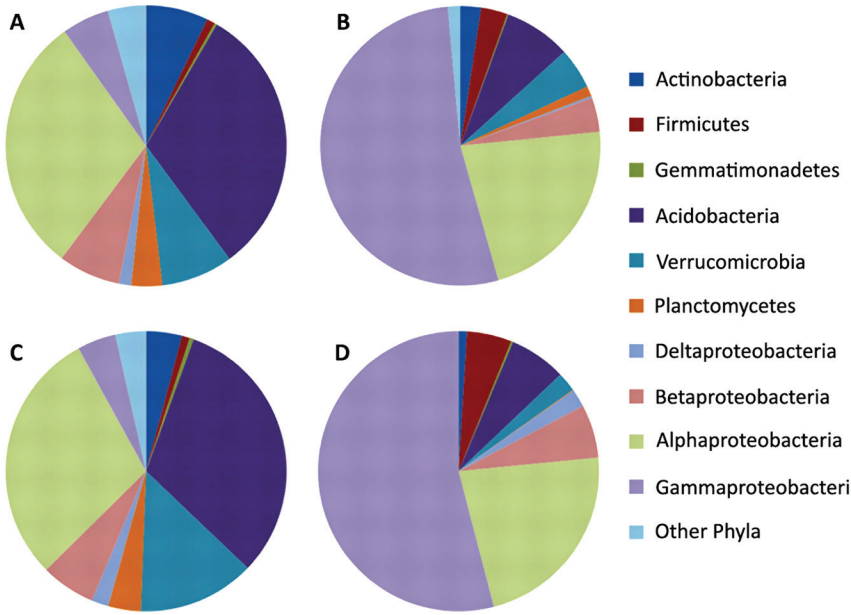


FIGURE A17-3 Bacterial classifications using the RDP classifier at 80% identity as implemented in mothur, shown at the phylum level except for *Proteobacteria*, which are classified by class. The charts represent average results for rhizospheric (A and B) and endophytic (C and D) or communities originating from samples of either bottomland (B and D) or upland (A and C) trees. To aid in distinguishing the colors, phylogenetic groups are presented in the same order in the pie charts (clockwise) as in the legend (top to bottom) in each subchart.

2002), where *Proteobacteria* were dominant (~40%), followed by *Acidobacteria* (33%), and *Verrucomicrobia* (~10%) in the samples. The Proteobacteria within the rhizosphere were primarily composed of the *Alphaproteobacteria* subclass (60%), with lower levels of *Betaproteobacteria* (15%) and *Gammaproteobacteria* (10%). The majority of *Acidobacteria* were group 6 (60%), with 15% each from groups 17 and 4, a typical distribution for soil communities (Barns et al., 2007). Endophytic bacterial communities were heavily dominated by *Proteobacteria* at the phylum level, at >80% of the sequences in each sample, and *Acidobacteria* comprised only 6%. Within the *Proteobacteria*, endophyte communities were heavily dominated by *Gammaproteobacteria*, followed by *Alphaproteobacteria*.

Fungal classification results indicated that fungal communities within both the rhizosphere and endosphere were dominated by the *Ascomycetes* within the *Pezizomycotina* (~40%) and *Basidiomycetes* within the *Agaricomycotina* (~25%). Similar to the bacterial patterns, relative abundance patterns of these broad fungal phylogenetic groups showed little difference between upland and

bottomland but major changes between rhizosphere and endosphere habitat (Figure A17-4). Approximately one-quarter of the LSU sequences were unidentified even to a phylum level with the BLAST-based classifications employed. An additional difference was in the bottomland endophytic community, of which 17% of sequences were attributable to *Pucciniomycotina*, which were at very low abundance levels in upland endophyte samples and both rhizospheres. The majority of these sequences originated from 9 of 11 endophyte samples within the bottomland data set, but *Pucciniomycotina* were also present in low abundance in rhizosphere samples.

Fast UniFrac and OTU-Based Hierarchical Clustering Analyses

Principal coordinate analysis generated by Fast UniFrac showed that the rhizosphere and endophyte bacterial and fungal communities form distinct clusters; within each of these clusters, the bottomland and upland communities exhibit considerable overlap (Figure A17-5). These results are similar to the trends in

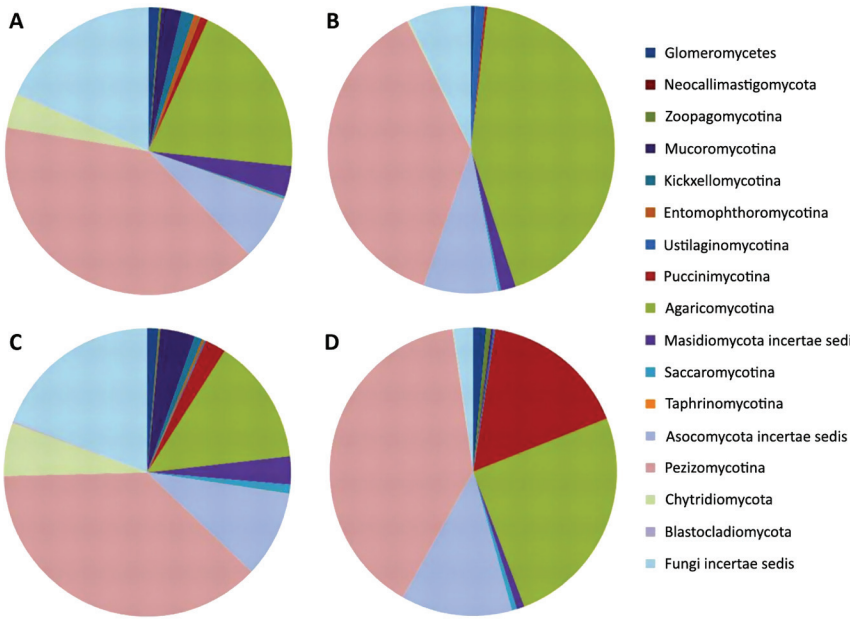


FIGURE A17-4 Fungal sequence classifications as identified from a consensus among the top BLAST scores against the SILVA LSU database. The charts represent average results for rhizospheric (A and B) or endophytic (C and D) or communities originating from samples of either bottomland (B and D) or upland (A and C) trees. To aid in distinguishing the colors, phylogenetic groups are presented in the same order in the pie charts (clockwise) as in the legend (top to bottom) in each subchart.

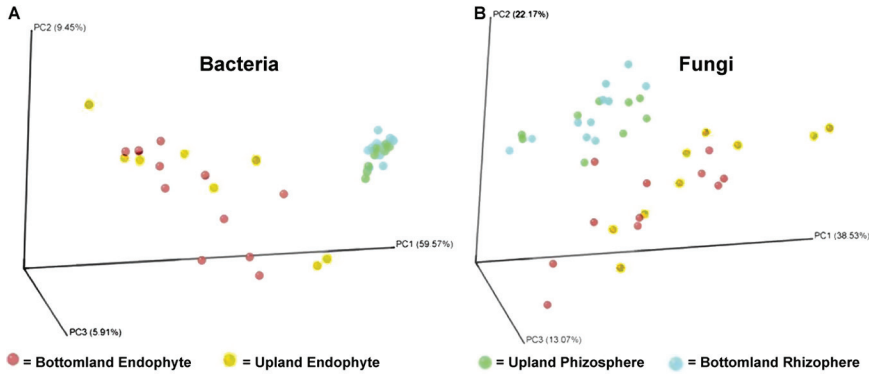


FIGURE A17-5 Principle coordinate analysis of bacterial (A) and fungal (B) communities, based on Fast UniFrac analysis. Circles are color coded by sample type: rhizosphere bottomland, green; rhizosphere upland, blue; endophyte bottomland, red; endophyte upland, yellow.

the phylogenetic classification and rarefaction results, where there were marked differences between the rhizosphere and endophytes but no consistent changes between the upland and bottomland sites. Correcting for multiple comparisons, UniFrac significance values for the bacterial data set significantly differed for the major treatments: endophyte versus rhizosphere and bottomland versus upland sites (see Table S2 in the supplemental material). Using the P-test, there was a highly significant difference between the bottomland endophyte sample and both of the rhizosphere data sets ($P < 0.001$), but none of the other comparisons resulted in significant differences. For the fungal data set and using the UniFrac test, significant differences were detected according to sample source (endophyte versus rhizosphere) but not according to site (bottomland versus upland) (see Table S2). When corrected for multiple comparisons, the P-test did not reveal any significant differences among fungal samples. We also used the UniFrac and P-test significance tests to check for differences between roots from the same trees and between trees from the same sites. Results from these tests were highly variable (see Table S3 in the supplemental material). While the majority of P-tests showed nonsignificant differences from samples within trees and between trees, many of the UniFrac tests did show significant differences, especially in diverse rhizosphere samples. These differences between the two unique tests (UniFrac and P-test) were likely due to the fact that the P-test takes into account only the tree topology while the UniFrac test takes into account both the tree topology and the branch length (evolutionary distance) between members (Lozupone et al., 2006). Additionally, UniFrac significance tests also have been noted by its developers to overestimate significance from diverse samples using next-generation sequencing, and it has been recommended that these tests be replaced

by PCoA and hierarchical clustering (Hinsinger et al., 2003), as presented in Figure A17-5 and Figure A17-6.

Hierarchical cluster analysis of the relative abundance of the most abundant OTUs in the fungal and bacterial data sets with high occurrence rates revealed two major linkage groups that separated completely by rhizosphere or endosphere origin in both bacterial (Figure A17-6A) and fungal (Figure A17-6B) samples. Soil type also often separated within subgroups, with numerous exceptions. Information on the identity of the major OTUs identified that are numbered and shown in Figure A17-6 can be also be found in Table S4 of the supplemental material.

Discussion

Very little is known about *Populus* interactions with the microbial community in mature, natural ecosystems, as most studies have originated from either

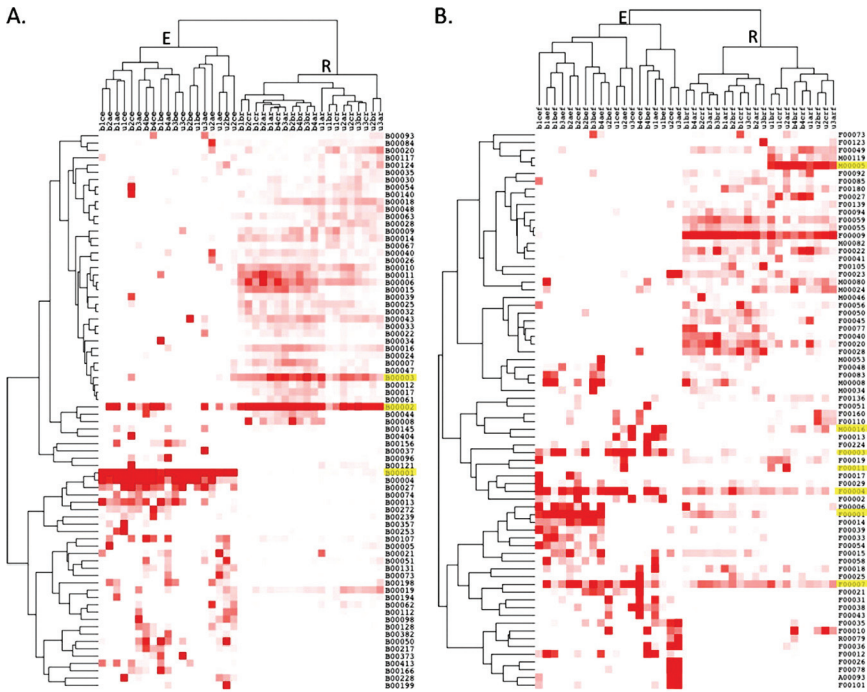


FIGURE A17-6 Heat map and hierarchical cluster analysis based on the relative abundances of the top OTUs identified in >5 samples in the bacterial (A) and fungal (B) data sets. Cluster analysis completely separated OTU abundance by endophyte or rhizosphere origin. OTUs highlighted in yellow are discussed in the text. For classification details of the OTUs depicted here, see Table S4 in the supplemental material.

greenhouse cuttings (Moore et al., 2006; van der Lelie et al., 2009) or short-rotation, plantation-grown trees (Stefani et al., 2009; van der Lelie et al., 2009). To our knowledge no studies have comprehensively examined the bacterial and fungal communities simultaneously in the same sample sets for any part of the *Populus* microbiome, and few have employed culture-independent methods. In an attempt to disentangle the root endophytic and rhizospheric *Populus*-associated microbial communities, we used 454 pyrosequencing surveys to characterize the bacterial and fungal communities of *P. deltoides* trees in upland and bottomland sites along the Caney Fork River in Tennessee. These sites differed significantly in both soil and stand characteristics (Table A17-1). In this study, both the fungal and bacterial communities were described from the same DNA extractions: the paired assessments of rhizospheric (consisting of adhering soils washed from the roots) and endophytic populations (extracted from surface-sterilized roots) were obtained from the same root systems and samples. Our results suggest that the diversity and composition of the associated microbial communities are largely consistent regardless of the differences in host trees and soil physicochemical characteristics associated with the two upland and bottomland sites studied and that the rhizosphere and endophyte communities are largely independent with little overlap in the dominant phyla or OTUs. These findings contradict previous reports that indicated soil properties are a major driver of differences in the distributions and compositions of microbial communities (Buyer et al., 2002; Jesus et al., 2010).

Variability in Rhizosphere and Endosphere Populations of P. deltoides

The number of OTUs in the bacterial endophytic samples (83 ± 78) was much lower than in the rhizosphere (1,319 ± 99) and more variable from sample to sample. The bacterial endophytic samples with the most diversity were an order of magnitude less diverse than the most diverse rhizosphere samples at similar sequencing depths (Figure A17-1). It is possible that this was caused by sporadic and nonuniform colonization of *Populus* roots by rhizosphere bacteria, which could contribute to the observed high variability. However, this variation may also have arisen partially from our failure to have sequenced the bacterial endophyte community deeply and uniformly. As seen in the rarefaction curves, many endophytic samples had an order of magnitude fewer reads than their counterpart rhizosphere samples. This was due to the high levels of plastid and mitochondrial 16S sequences within the amplified community DNA that were removed from the analysis, including ~67,000 mitochondrial and ~65,266 plastid rRNA gene sequences. The fungal data set was not impacted by this problem (Figure A17-2), as we only found low numbers of fungal reads that were identified as originating from the *Populus* host tree, or of other soil eukaryotes, such as nematodes. In contrast to the bacterial endophyte rarefaction curves, the fungal endophyte curves exhibited many more OTUs at comparable levels

of sampling effort/sequencing depth and much less variability from sample to sample. However, high-throughput sequencing of fungal rRNA genes currently has far less support than bacterial and archaeal rRNA pyrosequencing based on previous studies, databases, and robust alignments that account for secondary structure. Previous fungal studies typically sequenced the ITS regions (Buee et al., 2009; Jumpponen et al., 2010) or the 18S region (Rousk et al., 2010) of the rRNA genes. The ITS regions are hypervariable, which prevents sequence alignment across the breadth of the fungi impossible, and therefore the identity of many uncultured and underdocumented fungi cannot be determined accurately. Additionally, many community comparison methods based on alignments and phylogenetic methods (e.g., UniFrac) are not possible without alignments. Conversely, 18S regions are often highly conserved and prevent identification past the family level. The D1/D2 region of the 28S LSU rRNA gene was targeted in this study in order to identify known and unknown fungal organisms, even at deep phylogenic branch points (Porter et al., 2008; Schadt et al., 2003), while also enabling alignment and phylogeny-based community analysis methods.

Higher-Order Phylogenetic Composition of Endophyte Versus Rhizosphere Populations of P. deltoides *in Contrasting Soils*

Rhizosphere bacterial samples were dominated by *Acidobacteria* and *Proteobacteria* in both soil types, both of which are common phyla recovered from soil sequencing surveys (Castro et al., 2010; Janssen, 2006; Jesus et al., 2010) (Figure A17-3). The ratio of *Proteobacteria* to *Acidobacteria* has been suggested to be an indicator of the trophic level of soils (Castro et al., 2010; Smit et al., 2001), favoring *Proteobacteria* in nutrient-rich soils and *Acidobacteria* in nutrient-poor soils. Selection for *Proteobacteria* in the rhizosphere of *Trifolium repens* and *Lolium perenne* has been attributed to the nutrient-rich conditions of the rhizosphere (Marilley and Aragno, 1999), and similar results have been obtained in other plant systems, such as maize (Sanguin et al., 2006), soybean (Xu et al., 2009), and grasslands (Singh et al., 2007). However, in other woody species, such as chestnut trees (Lee et al., 2008) and black spruce (Filion et al., 2004), *Acidobacteria* have been shown to dominate rhizosphere systems. In our study, *Proteobacteria* were only slightly more prevalent (43% of sequences) than the *Acidobacteria* (38%), which may indicate a level intermediate between the copiotrophic and oligotrophic conditions thought to characterize the preferred habitats of these groups. However, numerous other factors may be driving these ratios. Acidobacterial relative abundance in soils has also been shown to correlate with soil pH (Lauber et al., 2009) and relative moisture (Castro et al., 2010). The pH of the bulk soils in the upland site was a full unit higher than the bottomland (Table A17-1), yet on average the relative abundance of *Acidobacteria* was slightly greater in the upland samples (41%) than in the bottomland (35%). However, we did not measure the pH of the rhizosphere directly to determine

whether it was different than that of the bulk soil, and it is well-established that some plants modify the rhizosphere pH (Hinsinger et al., 2003).

Populus is unusual among most higher plants because it can associate with both endomycorrhizal *Glomeromycete* fungi as well as ectomycorrhizal fungi in the *Ascomycotina* and *Basidiomycotina* (Luckac et al., 2003; Vozzo and Hackayl, 1974). The higher-level fungal communities observed in our study clearly differ between the rhizosphere and endosphere, even at broad taxonomic levels, and in some cases between the upland and bottomland locations (Figure A17-4). The differences between upland and bottomland communities are primarily due to large numbers of an individual OTU (F00001), similar to basidiomycetous yeasts from the *Pucciniomycotina*, which dominated most bottomland endophyte samples, comprising on average 10% (Figure A17-6). Using traditional ITS cloning techniques targeting the ECM fungi in transgenic plantation-grown *Populus*, Stefani et al. (Stefani et al., 2009) did not find any differences among the fungal communities of transformed and untransformed *Populus deltoides* × *P. trichocarpa* hybrids. They found a high frequency of ECM fungi in the root tips but only recovered about 42 OTUs from the root tips and 58 OTUs from soil cloning (at a sequence similarity of 98%), of which 39 and 26, respectively, were ECM fungi (Stefani et al., 2009). These OTU values are surprisingly low compared to other fungal surveys and to our study of mature natural stands. However, it is likely that the Stefani et al. study also undersampled OTUs, as they used a much lower sequencing depth, which is inherent in clone-based analyses. Jumpponen et al., using ITS pyrosequencing, recovered 1,077 OTUs (at 95% sequence similarity) from *Quercus* spp. roots (Jumpponen et al., 2010). The number of OTUs recovered from *Populus* roots in our study was 298 ± 90 (95% sequence similarity). Buée et al. observed a comparable number of fungal OTUs (600 to 1,000) in a survey of forest soils (Buee et al., 2009) as we found in our survey of rhizosphere soils (1,036 ± 278), and they observed some differences in OTU number and composition based on soil type. Our OTU numbers were also lower than estimates from the clone-based study of Fierer et al. of rainforest soils (1,000 to 2,000 OTUs) (Fierer et al., 2007). Many of the difficulties in comparing such OTU levels stem not only from different sampling/sequencing depths but also the inherent differences in variability between the ITS, SSU, and LSU rRNA gene regions employed and differing opinions on OTU cutoff similarities. Our analysis used a conservative 95% similarity cutoff for the D1 region of the LSU, which may have led to lower OTU numbers.

Several authors have proposed that soil type is one of the major drivers of rhizosphere microbial communities (Bachmann and Kinzel 1992; Hinsinger et al., 2009), while other authors have suggested that the plant growth stage can shape the rhizosphere microbial communities (Chiarini et al., 1998; Hinsinger et al., 2009). Our limited study of two contrasting soil types showed that both bacterial and fungal communities did not differ significantly in higher-order composition in either the rhizosphere or endosphere environment. In addition to

divergent soil properties, the upland and bottomland sites had statistically different tree sizes and age classes (Table A17-1), but we did not see major differences in community composition between these sites. Taken together our results suggest that the presence of *Populus* trees has a dominant effect over other factors in determining overall microbial community patterns in the rhizosphere as well as the endosphere. However, additional studies that incorporate diverse *Populus* genotypes and development stages as well as broader sets of soil conditions and cooccurring tree species would be required to fully understand and enumerate the effects of such factors.

Is the Root Endosphere Community a Subset of the Rhizosphere Community?

It has been speculated that endophytic root bacterial communities comprise a subset of colonists originating from the surrounding rhizosphere soil (Cocking 2003; Hallmann et al., 1997), and the resulting community composition is affected by the surrounding soil and environmental properties. Therefore, if endophytes are mostly facultative rhizosphere organisms and/or accidental passengers within the root, then the rhizosphere and endosphere will have similar overall patterns of dominant phylogenetic groups and OTU abundance patterns. However, this pattern was not observed in our data. The higher-order classifications of both communities differed in the abundance of major phyla (Figure A17-3 and Biro and Takacs, 2007), and perhaps more tellingly, the phylogeny-based UniFrac PCoA (Figure A17-5) and the OTU-based cluster analysis (Figure A17-6) differed dramatically between these two plant-associated environments. Considering that each of the endosphere and rhizosphere sample pairs were derived from the same root collections, this pattern is especially striking.

Previous reports have shown that endophytic communities are dominated by *Gammaproteobacteria*, followed by *Betaproteobacteria* and *Alphaproteobacteria* (van der Lelie et al., 2009). These studies were based primarily on culture-dependent isolation techniques (Moore et al., 2006; Taghavi et al., 2009). Our pyrosequencing surveys had a similarly high abundance of *Gammaproteobacteria*, but with a greater number of *Alphaproteobacteria* than *Betaproteobacteria* (Figure A17-3). These discrepancies could be due to differences between culture-dependent and -independent methods or because previous knowledge of endosphere and rhizosphere communities has often originated from entirely separate samples or studies. Perhaps we were able to clearly observe differences between these communities because the endosphere and rhizosphere populations originated from the same root samples or because of the more comprehensive analyses enabled by pyrosequencing. With regard to the dominant endophytes of *Populus*, many studies have shown that the *Gammaproteobacteria* dominates (49). In our study of natural populations and previous culture-dependent studies, dominant bacterial endophytes were often *Pseudomonas* spp. One OTU (B00001) attributable to a *P. fluorescens*-like organism dominated both upland (33.8%) and

bottomland (34.6%) endophyte samples but accounted for small fractions in the rhizosphere (0.2 and 0.08% of upland and bottomland, respectively). A similar disparity between endosphere and rhizosphere abundances can be seen across many of the OTUs in Figure A17-6. Another study in the presence of trichloroethylene (TCE) showed the dominant *Gammaproteobacteria* to be *Serratia* spp. in culture-based assessments (Xu et al., 2009). *Serratia*-like OTUs were exceedingly uncommon in our study and comprised <0.001% of the data set. Ulrich et al. reported differing endophytic phyllosphere communities across various genotypes of plantation-grown *Populus* in Europe (Ulrich et al., 2008) but did not examine root or rhizosphere communities.

The number of OTUs attributable to ECM and other mycorrhizal fungi was lower than expected in our samples, and some ongoing studies suggest *P. deltoides* is only weakly ectomycorrhizal compared to other *Populus* species and hybrids or compared to other tree species, such as oak and pine (F. Martin and R. Vilgalys, unpublished data). A study by Stefani et al. (2009) of plantation-grown *Populus* hybrids found differences in the rank abundance of different OTUs identified within roots and in bulk soils but also observed that over half of the ECM OTUs cooccurred in both habitats, even at the low sequencing depths achievable with clone library examinations. However, it is unclear how the Stephani et al. sampling strategy would compare with the surface-sterilized endophyte and rhizosphere sampling methods employed in our study. ECM OTUs were also observed in both the rhizosphere and endophyte samples in our study. For example, OTU F00003 is attributable to a *Cortinarius*-like organism (*Basidiomycotina*) and occurred at an average frequency of 4.7% in endosphere samples but only at 0.2% in the rhizosphere (Figure A17-6). Conversely, OTU F00011 was dominant in the rhizosphere and endosphere of several samples and attributable to a *Tuber*-like ECM organism (*Ascomycotina*).

The uniqueness of rhizosphere root endophyte communities is illustrated in the hierarchical clustering-based analysis of the dominant OTUs from each environment (Figure A17-6). With the exception of a few OTUs, most are abundant in either the endophyte or rhizosphere samples, but not both. Of the OTUs that are prominent in both environments, most are fungal. For example, OTUs F00007 and F00004 were attributable to a *Neonectria*-like species and a *Veronia*-like species, present in high abundance in both the endophyte and rhizosphere samples. These *Ascomycete* genera are known as plant-associated pathogens; however, many species have unknown effects on plant growth (Rossman et al., 1999). The most dominant bacterial rhizosphere OTU (B00002) was a *Bradyrhizobium*-like organism that was also sporadically present in the endosphere. OTU B0003 is the second most dominant across rhizospheric environments and classified as *Chloroflexi*-like. *Chloroflexi* are common soil organisms but have not been reported as plant endophytes. Some nonfungal metazoan taxa (e.g., nematodes) present in the fungal data set have distinct OTU distribution patterns. For example, OTU M00005 represents a *Steinernema*-like nematode that occurs in the

rhizosphere, whereas M00016 represents a *Hoploimus*-like nematode prominent in the endosphere. While such patterns of species partitioning in rhizosphere and endosphere habitats are preliminary, considering the small number of environments sampled, they begin to suggest that segregating of the myriad of soil niches may be possible with further dissection and application of deep pyrosequencing techniques.

Conclusions

At broad taxonomic levels as well as the individual OTU level, rhizosphere and endophyte communities of both bacteria and fungi associated with native *P. deltoides* are clearly distinct, suggesting that the tissues within naturally occurring *Populus* roots represent a unique niche for microbial communities. There appears to be little variation in dominant phyla within rhizosphere and endophyte habitats between the two soils and ecotypes of *Populus* examined thus far. Future work that includes more diverse soil types and the analysis of the specific effects of host genotype and chemical phenotypes should further elucidate the relative effects of environment and host factors in microbial associations with *Populus*.

Acknowledgments

We thank S. Retterer, A. Gorin, D. Weston, C. Pan, and L. Gunter for their assistance in conducting field and lab work. We also thank the Army Corps of Engineers and the Tennessee Parks Department for access to sampling sites.

This research was sponsored by the Genomic Science Program, U.S. Department of Energy, Office of Science, Biological and Environmental Research, as part of the Plant Microbe Interfaces Scientific Focus Area (http://pmi.ornl.gov). Oak Ridge National Laboratory is managed by UT-Battelle LLC, for the U.S. Department of Energy under contract DE-AC05-00OR22725.

The submitted manuscript has been authored by a contractor of the U.S. Government under contract DE-AC05-00OR22725.

References

Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.

Bachmann, G., and H. Kinzel. 1992. Physiological and ecological aspects of the interactions between plant-roots and rhizosphere soil. Soil Biol. Biochem. 24:543–552.

Barns, S. M., E. C. Cain, L. Sommerville, and C. R. Kuske. 2007. Acidobactetia phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Appl. Environ. Microbiol. 73:3113–3116.

Biro, I., and T. Takacs. 2007. Study of heavy metal uptake of *Populus nigra* in relation to phytoremediation. Cereal Res. Commun. 35:265–268.

Buee, M., et al. 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol. 184:449–456.

Buyer, J. S., D. P. Roberts, and E. Russek-Cohen. 2002. Soil and plant effects on microbial community structure. Can. J. Microbiol. 48:955–964.

Castro, H. F., A. T. Classen, E. E. Austin, R. J. Norby, and C. W. Schadt. 2010. Soil microbial community responses to multiple experimental climate change drivers. Appl. Environ. Microbiol. 76:999–1007.

Chiarini, L., A. Bevivino, C. Dalmastri, C. Nacamulli, and S. Tabacchioni. 1998. Influence of plant development, cultivar and soil type on microbial colonization of maize roots. Appl. Soil Ecol. 8:11–18.

Cocking, E. C. 2003. Endophytic colonization of plant roots by nitrogenfixing bacteria. Plant Soil 252:169–175.

Cole, J. R., et al. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37:D141–D145.

Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A. 95:14863–14868.

Fierer, N., et al. 2007. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Appl. Environ. Microbiol. 73:7059–7066.

Filion, M., R. C. Hamelin, L. Bernier, and M. St. Arnaud. 2004. Molecular profiling of rhizosphere microbial communities associated with healthy and diseased black spruce (*Picea mariana*) seedlings grown in a nursery. Appl. Environ. Microbiol. 70:3541–3551.

Gehring, C. A., R. C. Mueller, and T. G. Whitham. 2006. Environmental and genetic effects on the formation of ectomycorrhizal and arbuscular mycorrhizal associations in cottonwoods. Oecologia 149:158–164.

Hallmann, J., A. Quadt-Hallmann, W. F. Mahaffee, and J. W. Kloepper. 1997. Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43:895–914.

Hamady, M., C. Lozupone, and R. Knight. 2010. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J. 4:17–27.

Hardoim, P. R., L. S. van Overbeek, and J. D. van Elsas. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16:463–471.

Hinsinger, P., A. G. Bengough, D. Vetterlein, and I. M. Young. 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant Soil. 321:117–152.

Hinsinger, P., C. Plassard, C. X. Tang, and B. Jaillard. 2003. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: a review. Plant Soil 248:43–59.

Huse, S. M., D. M. Welch, H. G. Morrison, and M. L. Sogin. 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ. Microbiol. 12:1889–1898.

Janssen, P. H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728.

Jesus, E. D., et al. 2010. Bacterial communities in the rhizosphere of biofuel crops grown on marginal lands as evaluated by 16S rRNA gene pyrosequences. Bioenerg. Res. 3:20–27.

Jones, J. D. G., and J. L. Dangl. 2006. The plant immune system. Nature 444:323–329.

Jumpponen, A., K. L. Jones, D. Mattox, and C. Yaege. 2010. Massively parallel 454-sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. Mol. Ecol. 19: 41–53.

Kent, A. D., and E. W. Triplett. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. 56: 211–236.

Kunin, V., A. Engelbrektson, H. Ochman, and P. Hugenholtz. 2010. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environ. Microbiol. 12:118–123.

Lauber, C. L., M. Hamady, R. Knight, and N. Fierer. 2009. Pyrosequencingbased assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl. Environ. Microbiol. 75:5111–5120.

Lee, S. H., J. O. Ka, and J. C. Cho. 2008. Members of the phylum Acidobacteria are dominant and metabolically active in rhizosphere soil. FEMS Microbiol. Lett. 285:263–269.

Lozupone, C., M. Hamady, and R. Knight. 2006. UniFrac: an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 7:371.

Lukac, M., C. Calfapietra, and D. L. Godbold. 2003. Production, turnover and mycorrhizal colonization of root systems of three Populus species grown under elevated CO2 (POPFACE). Global Change Biol. 9:838–848.

Marilley, L., and M. Aragno. 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl. Soil Ecol. 13:127–136.

Meyer, F., et al. 2008. The metagenomics RAST server: a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386.

Moore, F. P., et al. 2006. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: the characterisation of isolates with potential to enhance phytoreniediation. Syst. Appl. Microbiol. 29:539–556.

Porter, T. M., et al. 2008. Widespread occurrence and phylogenetic placement of a soil clone group adds a prominent new branch to the fungal tree of life. Mol. Phylogenet. Evol. 46:635–644.

Pruesse, E., et al. 2007. SILVA: a comprehensive online resource for quality checked and aligned rRNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.

Rossman, A. Y., G. J. Samuels, C. T. Rogerson, and R. Lowen. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud. Mycol. 42:1–248.

Rousk, J., et al. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 4:1340–1351.

Sanguin, H., et al. 2006. Potential of a 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on Agrobacterium spp. and bacterial communities. Appl. Environ. Microbiol. 72:4302–4312.

Sannigrahi, P., A. J. Ragauskas, and G. A. Tuskan. 2010. Poplar as a feedstock for biofuels: a review of compositional characteristics. Biofuels Bioprod. Bior. 4:209–226.

Schadt, C. W., A. P. Martin, D. A. Lipson, and S. K. Schmidt. 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science 301:1359–1361.

Schloss, P. D., et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537–7541.

Singh, B. K., S. Munro, J. M. Potts, and P. Millard. 2007. Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. Appl. Soil Ecol. 36:147–155.

Smit, E., et al. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. 67:2284–2291.

Stefani, F. O. P., J. M. Moncalvo, A. Seguin, J. A. Berube, and R. C. Hamelin. 2009. Impact of an 8-year-old transgenic poplar plantation on the ectomycorrhizal fungal community. Appl. Environ. Microbiol. 75:7527–7536.

Taghavi, S., et al. 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl. Environ. Microbiol. 75:748–757.

Tuskan, G., et al. 2004. The Populus genome: are there discernable differences between the genomes of perennial woody plants and herbaceous annuals? Acta Physiol. Plant 26:292–293.

Tuskan, G. A., et al. 2006. The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science 313:1596–1604.

Ulrich, K., A. Ulrich, and D. Ewald. 2008. Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol. Ecol. 63:169–180.

van der Lelie, D., et al. 2009. Poplar and its bacterial endophytes: coexistence and harmony. Crit. Rev. Plant Sci. 28:346–358.
Vishnivetskaya, T. A., et al. 2011. Mercury and other heavy metals influence bacterial community structure in low-order Tennessee streams. Appl. Environ. Microbiol. 77:302–311.
Vozzo, J. A., and E. Hacskayl. 1974. Endomycorrhizal and ectomycorrhizal associations in 5 Populus species. B Torrey Bot. Club 101:182–186.
Xu, Y. X., et al. 2009. Bacterial communities in soybean rhizosphere in response to soil type, soybean genotype, and their growth stage. Soil Biol. Biochem. 41:919–925.

A18

INTERACTIONS BETWEEN COMMENSAL FUNGI AND THE C-TYPE LECTIN RECEPTOR DECTIN-1 INFLUENCE COLITIS[78]

Iliyan D. Iliev,[79] *Vincent A. Funari*,[80,81] *Kent D. Taylor*,[80] *Quoclinh Nguyen*,[80] *Christopher N. Reyes*,[79] *Samuel P. Strom*,[80] *Jordan Brown*,[80] *Courtney A. Becker*,[79] *Phillip R. Fleshner*,[82] *Marla Dubinsky*,[79,83] *Jerome I. Rotter*,[80] *Hanlin L. Wang*,[84] *Dermot P. B. McGovern*,[79,80] *Gordon D. Brown*,[85] *and David M.* ***Underhill*** [79,84,86,*]

Abstract

The intestinal microflora, typically equated with bacteria, influences diseases such as obesity and inflammatory bowel disease (IBD). Here we show that the mammalian gut contains a rich fungal community that interacts with the immune system through the innate immune receptor Dectin-1. Mice lacking

[78] From Iliev, I. D., et al. 2012. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 336(6086):1314-1317. Reprinted with permission from AAAS.

[79] Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
[80] Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
[81] Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.
[82] Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
[83] Department of Pediatrics, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.
[84] Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.
[85] Section of Immunology and Infection, Division of Applied Medicine, Institute of Medical Sciences & The Aberdeen Fungal Group, University of Aberdeen, Aberdeen, UK.
[86] Research Division of Immunology, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA.

* Correspondence to: David M. Underhill, Inflammatory Bowel & Immunobiology Research Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Los Angeles, CA 90048, Email: David.Underhill@csmc.edu.

Dectin-1 exhibited increased susceptibility to chemically-induced colitis, which was the result of altered responses to indigenous fungi. In humans we identified a polymorphism in the gene for Dectin-1 (*CLEC7A*) that is strongly linked to a severe form of ulcerative colitis. Together our findings reveal a novel eukaryotic fungal community in the gut (the "mycobiome") that coexists with bacteria and substantially expands the repertoire of organisms interacting with the intestinal immune system to influence health and disease.

Interactions between the commensal microflora and the gut immune system are critical for establishing a balance between immunity and tissue health. Changes in gut bacteria described as "dysbiosis" have been associated with intestinal inflammation (Elinav et al., 2011; Lupp et al., 2007; Willing et al., 2010) and metabolic syndrome (Arumugam et al., 2011; Henao-Mejia et al., 2012; Vijay-Kumar, et al., 2010). The vast majority of studies on commensal microbiota have focused on gut bacteria, and the terms "intestinal microbiota" and "intestinal bacteria" are often used interchangeably. Recent studies have begun to note, however, that a fraction of gut microorganisms are not bacterial (Qin et al., 2010). Although a few studies have suggested the presence of commensal fungi in the gut (Ott et al., 2008; Qin et al., 2010), whether they interact with the mucosal immune system or influence diseases is unknown.

Fungi are recognized by a number of immune receptors among which Dectin-1 has emerged as key for phagocytosis and killing by myeloid phagocytes. Dectin-1 is a C-type lectin receptor that recognizes β-1,3-glucans found in the cell walls of nearly all fungi. Dectin-1 activates intracellular signals through caspase recruitment domain–containing protein 9 (CARD9), which leads to inflammatory cytokine production and induction of T helper 17 (T_H17) immune responses (Cheng et al., 2011; Conti et al., 2009; Gringhuis et al., 2012; LeibundGut-Ladmann et al., 2007). Deficiencies in either Dectin-1 or CARD9 result in enhanced susceptibility to pathogenic fungal infections in humans and mice (Ferwada, et al., 2009; Glocker et al., 2009; Taylor et al, 2007). Polymorphic variants in the gene for CARD9 are strongly associated with Crohn's disease and ulcerative colitis in humans (Franke et al., 2010; McGovern et al., 2010). Furthermore, anti–*Saccharomyces cerevisiae* antibodies (ASCA) against yeast mannan have been strongly associated with Crohn's disease (Joossens et al., 2002; Seow et al., 2009). Together, these last-named findings suggest a possible link between immune responses to commensal fungi and intestinal disease.

We examined fungal distribution and detected fungal ribosomal DNA (rDNA) throughout the murine gastrointestinal tract with highest densities in the terminal colon of C57BL/6 (Figure A18-1A) and 129S2/Sv (fig. S1A) mice. We stained colonic tissue sections and observed that fungi are abundant and in close proximity with commensal bacteria (Figure A18-1B and figs. S1B and S2 to S4). Furthermore, we found that a soluble Dectin-1 probe (*Ganter* et al., 2005) binds to 5 to 7% of the fecal material, consisting of fungal cells with various

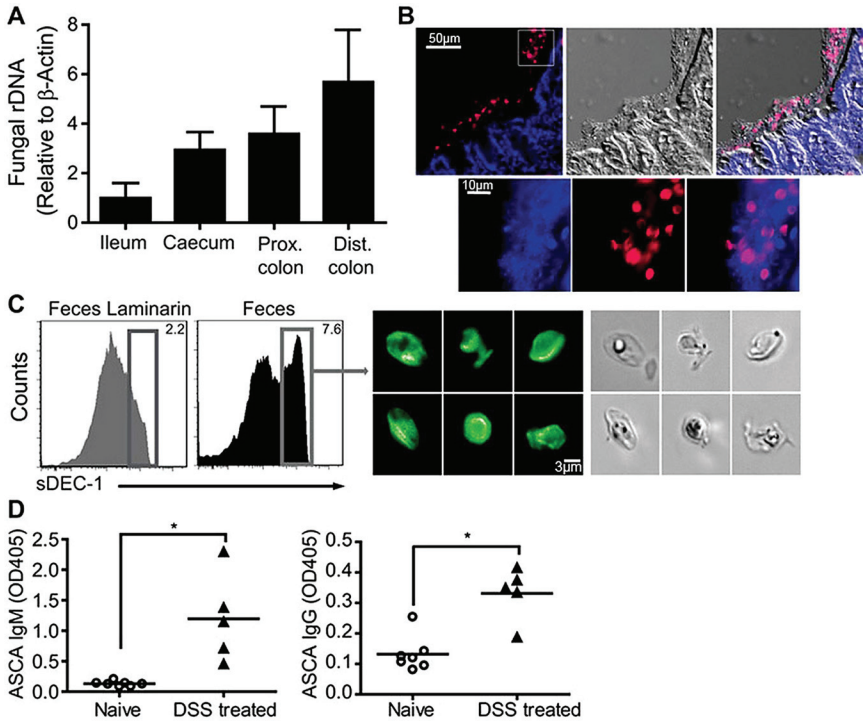


FIGURE A18-1 Commensal fungi are present in the intestine and are recognized by Dectin-1. (A) Prevalence of fungi in mucosa isolated from ileum, cecum, proximal (prox) and distal (dist) colon of C57BL/6J mice. ITS1-2 rDNA level was analyzed by quantitative polymerase chain reaction and normalized to β-actin DNA. (B) Visualization of commensal fungi in the intestine. Colon sections were stained with a soluble Dectin-1 probe (sDEC-1) and counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI). The DAPI signal has been amplified in (B, bottom) to show that DAPI-stained bacteria and fungi are in close proximity to each other. (C) Intestinal fungi are recognized by Dectin-1. Fecal pellets were homogenized and labeled with sDEC-1 in the presence (gray histogram) or absence (black histogram) of laminarin (a soluble β-glucan) to block specific binding. Binding was assessed by flow cytometry (left panels). Dectin-1–binding fungi were sorted (right) and visualized by confocal microscopy. (D) ASCA generation after DSS colitis. Mice were exposed twice to 2.5% DSS-supplemented water for 7 days each separated by 2 weeks of recovery. Serum samples were collected before DSS treatment (day 0) and 2 weeks after the last DSS cycle (42 days total), and ASCA IgM and IgG were measured by enzyme-linked immunosorbent assay (ELISA). Each symbol represents a mouse, all error bars indicate the SD; unpaired *t* test, $*P < 0.05$. All data are representative of at least two independent experiments with similar results.

morphologies (Figure A18-1C and fig. S5). Fungi were also present in rat, guinea pig, rabbit, pig, dog, and human feces (fig. S1C). Together, the data demonstrate that commensal fungi contribute to the intestinal microbial community in many species.

We next examined whether gut fungi can be detected by the immune system upon intestinal insult. We utilized a mouse model of dextran sodium sulfate (DSS)–induced colitis extended to allow antibody responses to develop. We found that DSS-induced intestinal inflammation led to the development of circulating immunoglobulin G (IgG) and IgM antibodies against fungi (ASCA) (Figure A18-1D), which suggested that fungal antigens indigenous to the gut might be responsible for the induction of ASCA during colitis.

Because gut commensal fungi are recognized by Dectin-1, we tested whether Dectin-1–deficient mice (*Clec7a*$^{-/-}$) are susceptible to DSS-induced colitis. *Clec7a*$^{-/-}$ mice experienced increased weight loss (Figure A18-2A) and displayed altered histology characterized by increased mucosal erosion, crypt destruction, inflammatory cell infiltration, and tumor necrosis factor–α (TNF-α) production in the colon (Figure A18-2, B to D) as compared with their wild-type (WT) littermate controls. We further detected augmented production of interferon-γ (IFN-γ) and interleukin-17 (IL-17) in intestines from *Clec7a*$^{-/-}$ mice (fig. S6). Similar results were obtained comparing cohoused animals (fig. S7). These results indicate that Dectin-1 deficiency leads to increased susceptibility to colitis.

Many studies have documented the importance of bacteria in intestinal inflammation, so we examined whether bacteria could contribute to the susceptible phenotype. We observed no significant differences in major phyla of commensal bacteria between WT and *Clec7a*$^{-/-}$ mice (fig. S8). To directly determine whether microflora can transfer disease, we depleted intestinal bacteria and fungi with antibiotics, transplanted fecal microflora from WT or *Clec7a*$^{-/-}$ mice, and exposed mice to DSS. Microflora from *Clec7a*$^{-/-}$ mice did not transfer susceptibility to disease (Figure A18-2, E and F, and figs. S9 and S10). The data demonstrate that the disease phenotype in the *Clec7a*$^{-/-}$ mice is affected by the genotype of the mouse, not by initial differences in microflora.

We know very little about what commensal fungi populate the murine gut or how they contribute to colitis in Dectin-1 deficiency. To define the mouse intestinal fungal microbiome, we isolated DNA from murine feces, amplified the internal transcribed spacer region (ITS1-2) of fungal rDNA, and performed high-throughput sequencing (Ghannoum et al., 2010). Combining data from 23 mice, we obtained >30 Mb of raw data from 454 pyrosequencing and >2.2 Gb of raw data from Illumina genome analyzer (GA) sequencing, together containing >1.3 million individual sequences that passed quality control. Detailed analysis identified >100 different well-annotated fungal species representing at least 50 genera, which illustrated the fungal diversity. In addition, we identified >100 novel and/or unannotated fungi representing the large uncharacterized nature of the mycobiome in the gut (figs. S11 and S12). Note that 97.3% of all the fungal

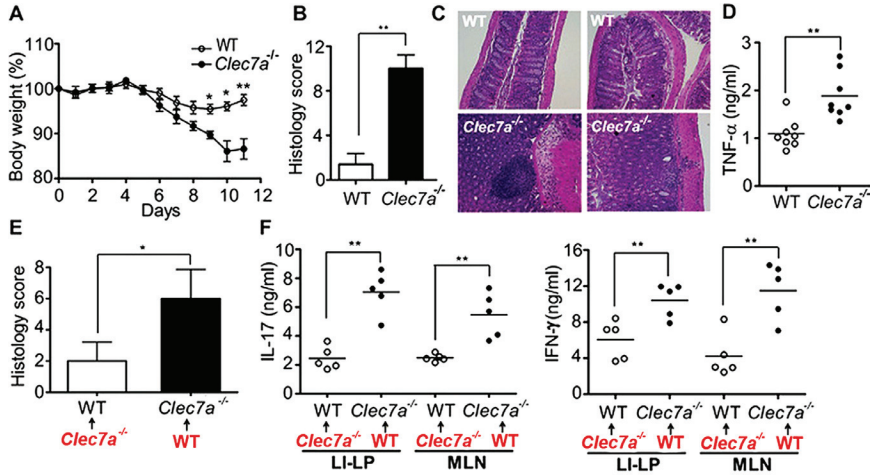


FIGURE A18-2 Dectin-1 regulates the severity of colitis. WT and *Clec7a*$^{-/-}$ littermates were treated with 2.5% DSS for 7 days and kept on water for 4 additional days. Colitis progress and severity were assessed by measuring body weight during treatment (A), histology (B and C), and TNF-α production in the colon (D) on day 11. (E and F) WT and *Clec7a*$^{-/-}$ mice were given an antibiotic cocktail including fluconazole for 3 weeks, given a transplant as indicated (red) with fecal microflora from WT or *Clec7a*$^{-/-}$ mice, and treated with DSS as in (A). Disease severity was accessed by histology score (E) and by cytokine production stimulated by antibodies against CD3 and CD28 in large-intestine lamina propria (LI-LP) and MLN T cells (F). Each symbol represents a different mouse. One of four independent experiments is shown. Error bars, SD; $*P < 0.05$, $**P < 0.01$.

sequences identified belonged to 10 fungal species, with 65.2% of the sequences belonging to a single fungus: *Candida tropicalis* (Figure A18-3A and fig. S13). We found 7 of the 20 most common gut fungi also in mouse food (fig. S13 and S14). These accounted, however, for only 1.5% of total fungi in the intestines, which suggests that highly represented fungal species are indigenous to the gut.

Many studies have shown that intestinal inflammation can lead to changes in commensal bacteria that affect the host (*Garret* et al., 2010; Lupp et al., 2007; Willing et al., 2010). Whether colitis affects the makeup of the commensal mycobiome is unknown. One study has reported increased fungal burden in intestines of Crohn's disease patients (Ott et al., 2008), and another has shown increased colonization with exogenously added *C. albicans* during DSS colitis in mice (Jawhara et al., 2008). Notably, we found that, during colitis in *Clec7a*$^{-/-}$ mice the proportion of opportunistic pathogenic fungi including *Candida* and *Trichosporon* increases, whereas nonpathogenic *Saccharomyces* decreases (Figure A18-3B and fig. S15). Examination of colons revealed that fungi invade inflamed tissues in DSS-treated *Clec7a*$^{-/-}$ mice but remain in the lumen of DSS-treated WT mice

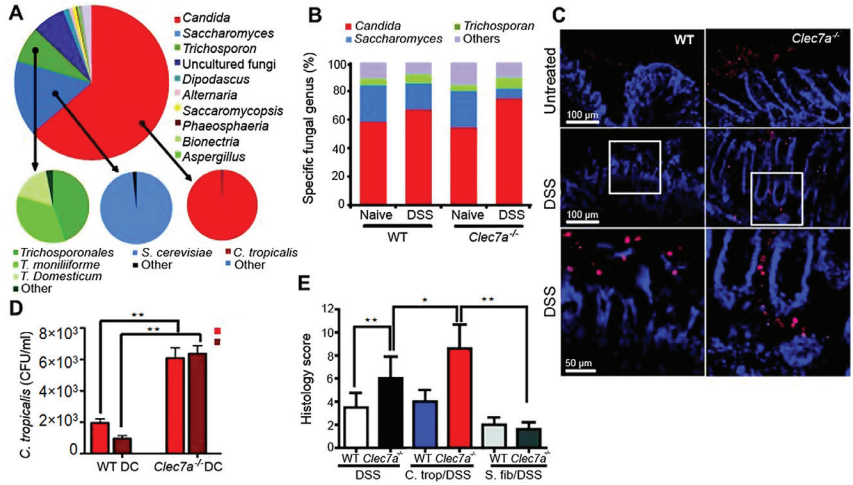


FIGURE A18-3 Defining the fungal microbiome and characterizing the specific role of Dectin-1–mediated host defense during colitis. (A) DNA was isolated from murine feces, and mycobiome analysis was performed using Roche 454 and Illumina GA sequencing of ITS1-2 rDNA. The taxonomic distribution of the most abundant fungal genera is shown (large pie chart), and the species breakdowns for major groups are provided (small pie charts). (B) Quantitative analysis of the major intestinal fungal genera in WT and *Clec7a*$^{-/-}$ mice before and after treatment with DSS. Illumina GA data were analyzed and presented as relative percentage of dominant fungal genera (n =16 mice). (C) Fungal invasion of colonic tissue in *Clec*7a$^{-/-}$ mice during colitis. Colon sections from WT and *Clec7a*$^{-/-}$ mice before and after colitis were stained with the sDEC-1 probe and counterstained with DAPI. (D) Intestinally conditioned dendritic cells were incubated with live C. tropicalis, and killing was assessed after 6 and 18 hours. (E) Histology score of WT and *Clec7a*$^{-/-}$ mice supplemented or not with four doses of *C. tropicalis* or *S. fibuligera* every other day, and then treated with 2.5% DSS for 7 days and kept on water for 4 additional days. Data are representative of at least two independent experiments with similar results. Error bars, SD; $*P < 0.05$, $**P < 0.01$.

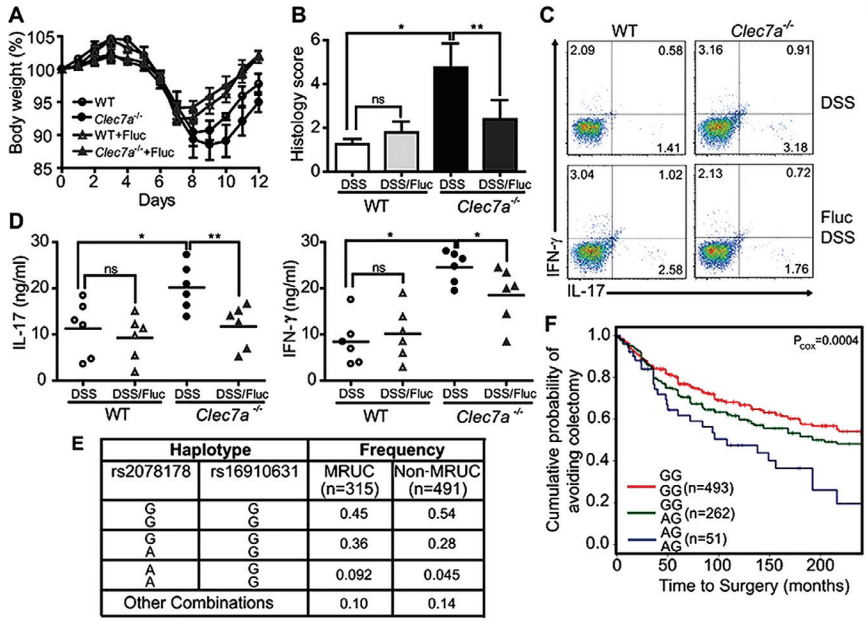
(Figure A18-3C and fig. S16). These data are consistent with the observation that intestinally conditioned *Clec7a*$^{-/-}$ dendritic cells are less capable of killing *C. tropicalis* in vitro (Figure A18-3D). Together, the data suggest that Dectin-1 deficiency leads to altered immunity to commensal fungi in the gut.

Given that *C. tropicalis* is an opportunistic pathogen, we further analyzed its role during colitis. We supplemented mice with *C. tropicalis* and subjected them to DSS. (See fig. S17A for dosing schedule.) For comparison, another group of mice was supplemented with *S. fibuligera*, a nonpathogenic fungus that, like *C. tropicalis*, grows in yeast and filamentous forms and is recognized by Dectin-1 (fig. S18). Colitis symptoms including weight loss, crypt loss, and

inflammatory cell infiltration were more severe in *Clec7a*$^{-/-}$ mice supplemented with *C. tropicalis* compared with the *Clec7a*$^{-/-}$ controls (Figure A18-3E and fig. S17, B and C). In contrast, *C. tropicalis* supplementation did not aggravate colitis in WT mice. Consistent with the pathology, we detected increased IL-17 and IFN-γ production by T cells from the mesenteric lymph nodes (MLNs) and colons of *Clec7a*$^{-/-}$ mice supplemented with *C. tropicalis* (fig. S17, D and E), as well as increased message for TNF-α, IL-23p19, IL-17a, Cxcl2, and defensins in colons (fig. S19). This correlated with higher loads of *C. tropicalis* in the intestines of DSS-treated *Clec7a*$^{-/-}$ mice (fig. S20B). In contrast, *S. fibuligera* supplementation did not contribute to colitis pathology (Figure A18-3E and figs. S17 and S19), and fungal loads were unchanged (fig. S20C). The data suggest that an inability of *Clec7a*$^{-/-}$ mice to mount effective immune responses to specific intestinal fungi creates conditions that promote inflammation.

To determine whether the altered fungal burden during colitis contributes to disease severity in the absence of Dectin-1, we suppressed fungal growth with fluconazole, a specific antifungal drug (fig. S3). Fluconazole treatment during colitis led to reduced weight loss (Figure A18-4A), and milder histological disease characteristics (Figure A18-4B), specifically in *Clec7a*$^{-/-}$ mice. We similarly observed decreased T_H1 and T_H17 responses (Figure A18-4, C and D, and fig. S21, A and B) and decreased production of inflammatory cytokines (fig. S21, C and D). Taken together, these results further support the conclusion that an inability to control fungi in the gut leads to more severe colitis in Dectin-1 knockout mice.

Having established a role for Dectin-1 in fungal control during colitis in mice, we next explored whether there is an association between inflammatory bowel disease (IBD) and genetic variation of the human Dectin-1 gene (*CLEC7A*). Because the mouse model suggested that Dectin-1 is involved in the severity of colonic disease, we focused our human studies on ulcerative colitis (UC), a disease of the colon, and in particular on severe UC. Up to 30% of patients with UC require colectomy, usually for severe disease that will not respond to medical therapy, including systemic corticosteroids, cyclosporine, and biological therapies [that is, medically refractory UC (MRUC)]. We compared *CLEC7A* alleles in an MRUC group with those from a group of patients with UC who had not required colectomy (non-MRUC) (Haritunians et al., 2010). We identified an association of *CLEC7A* single-nucleotide polymorphism rs2078178 in patients with MRUC (logistic regression, $P = 0.007$). Notably, a two-marker haplotype, rs2078178 to rs16910631, was more strongly associated with MRUC (AG haplotype; logistic regression, $P = 0.00013$; and Fisher's test, $P = 0.0005$) (Figure A18-4E and fig. S22 and table S1), a shorter time to surgery, and thus with a more severe UC (Figure A18-4F). Compared with healthy controls, the haplotype is strongly associated with MRUC and not with non-MRUC, further consistent with the idea that the haplotype is associated with severe disease (table S2). *CLEC7A* has not been identified in any genome-wide association study yet as an IBD susceptibility



Haplotype		Frequency	
rs2078178	rs16910631	MRUC (n=315)	Non-MRUC (n=491)
G G	G G	0.45	0.54
G A	G G	0.36	0.28
A A	G G	0.092	0.045
Other Combinations		0.10	0.14

FIGURE A18-4 Anti-fungal therapy ameliorates colitis in *Clec7a*$^{-/-}$ mice and *CLEC7A* associates with ulcerative colitis severity in humans. (A) WT and *Clec7a*$^{-/-}$ mice were given fluconazole in their drinking water for total of 14 days (starting 2 days prior the induction of DSS colitis), and body weight was measured. Weight loss is shown in (A) ($p<0.05$). Histology score (B), the percentage of IL-17 and IFN-γ producing CD4^{+} T cells in LI-LP (C), and IL-17 and IFN-γ production in MLNs (D) were determined 4 days after the 7 days of DSS treatment. Each symbol represents a different mouse. One of three independent experiments with similar results is shown. Error bars, s.d., * $P < 0.05$, ** $P < 0.01$. (E) Specific *CLEC7A* haplotypes associate with medically refractory ulcerative colitis (MRUC). Haplotypes were formed from rs2078178 and rs16910631 using PHASE v2.3. Haplotypes listed as "Other Combinations" were those that could not be reliably determined (posterior $p<0.95$). (F) The *CLEC7A* "AG/AG" haplotype associates with severity of disease as indicated by earlier progression to colectomy. Haplotypes were tested for association with time to surgery by fitting the MRUC/non-MRUC and time to surgery with a Cox proportional hazards model.

gene. Unlike susceptibility genes that predispose to disease, severity gene variants aggravate disease that is initially established through other mechanisms. The *CLEC7A* risk haplotype we report here fits the latter situation and agrees with our observation that *Clec7a*$^{-/-}$ mice do not develop spontaneous colitis. These findings suggest more in-depth studies on the role of the *CLEC7A* gene and pathway on the natural history of UC and will require further validation in independent cohorts.

A deeper understanding of the mechanisms by which fungi stimulate inflammatory immune responses in the gut may lead to better therapies for IBD and may be especially beneficial to patients with particularly severe forms of UC carrying the risk haplotype of the gene for Dectin-1. Overall, the idea that fungi are present in the gut and that they interact strongly with the immune system will fundamentally alter how we think about the gut microflora and inflammatory bowel diseases.

Acknowledgments

This study was supported in part by the National Institute of Allergy and Infectious Diseases, NIH (AI071116 to D.M.U.) and the Janis and William Wetsman Family Chair in Inflammatory Bowel Disease Research (D.M.U.). I.D.I. held a Research Fellowship Award (3064) from the Crohn's and Colitis Foundation of America. Further support came from National Institute of Diabetes and Digestive and Kidney Diseases, NIH, grant P01-DK046763; UCLA Clinical and Translational Science Institute grant UL1RR033176; Cedars-Sinai Medical Center Inflammatory Bowel and Immunobiology Research Institute Funds; The Feintech Family Chair in IBD (S. R. Targan); The Cedars-Sinai Board of Governors' Chair in Medical Genetics (J.I.R.); The Abe and Claire Levine Chair in Pediatric IBD (M.D.); and The Joshua L. and Lisa Z. Greer Chair in IBD Genetics (D.M.). G.D.B. is supported by the Wellcome Trust. *Clec7a*$^{-/-}$ mice are available through a Material Transfer Agreement with the University of Aberdeen. The data presented in this paper are tabulated in the main paper and in the supplementary materials. All sequences generated in this study have been deposited in the National Center for Biotechnology Information, NIH, Short Read Archive (www.ncbi.nlm.nih.gov/Traces/sra, accession no. SRA051853.1). G.D.B. is an advisory board member of MiniVax and Fiberbiotics. M.D. is a consultant for Prometheus Labs.

Supplementary Materials

Visit www.sciencemag.org/cgi/content/full/336/6086/1314/DC1.

References

Arumugam M, et al. Enterotypes of the human gut microbiome. Nature. 2011;473:174.

Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335.

Cheng SC, et al. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*. J Leukoc Biol.2011;90:357.

Chenna R, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res.2003;31:3497.

Conti HR, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med. 2009;206:299.

Elinav E, et al. NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. Cell.2011

Ferwerda B, et al. Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med.2009;361:1760.

Franke A, et al. Genome-wide association study for ulcerative colitis identifies risk loci at 7q22 and 22q13 (IL17REL) Nat Genet. 2010;42:292.

Fried LP, et al. The Cardiovascular Health Study: design and rationale. Ann Epidemiol. 1991;1:263.

Gantner BN, Simmons RM, Underhill DM. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J. 2005;24:1277.

Garrett WS, et al. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. Cell Host Microbe. 2010;8:292.

Ghannoum MA, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010;6:e1000713.

Glocker EO, et al. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med. 2009;361:1727.

Gringhuis SI, et al. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol. 2012

Haritunians T, et al. Genetic predictors of medically refractory ulcerative colitis. Inflamm Bowel Dis.2010;16:1830.

Henao-Mejia J, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482:179.

Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol.2009;2:340.

Jawhara S, et al. Colonization of mice by *Candida albicans* is promoted by chemically induced colitis and augments inflammatory responses through galectin-3. J Infect Dis. 2008;197:972.

Joossens S, et al. The value of serologic markers in indeterminate colitis: a prospective follow-up study. Gastroenterology. 2002;122:1242.

Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal Immunol. 2008;1:460.

Kent WJ. BLAT--the BLAST-like alignment tool. Genome Res. 2002;12:656.

Landers CJ, et al. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. Gastroenterology. 2002;123:689.

LeibundGut-Landmann S, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol. 2007;8:630.

Li H, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078.

Liu Z, DeSantis TZ, Andersen GL, Knight R. Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res. 2008;36:e120.

Lupp C, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007;2:204.

Matteoli G, et al. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut. 2010;59:595.

McGovern DP, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. Nat Genet. 2010;42:332.

Nilsson RH, Bok G, Ryberg M, Kristiansson E, Hallenberg N. A software pipeline for processing and identification of fungal ITS sequences. Source Code Biol Med. 2009;4:1.

Ott SJ, et al. Fungi and inflammatory bowel diseases: Alterations of composition and diversity. Scand J Gastroenterol. 2008;43:831.

Psaty BM, et al. Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium: Design of prospective meta-analyses of genome-wide association studies from 5 cohorts. Circ Cardiovasc Genet. 2009;2:73.

Qin J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature.2010;464:59.

R. D. C. Team R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2005

Scupham AJ, et al. Abundant and diverse fungal microbiota in the murine intestine. Appl Environ Microbiol. 2006;72:793.

Seow CH, et al. Novel anti-glycan antibodies related to inflammatory bowel disease diagnosis and phenotype. Am J Gastroenterol. 2009;104:1426.

Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet. 2001;68:978.

Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet. 2003;73:1162.

Taylor PR, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol. 2007;8:31.

Vijay-Kumar M, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science. 2010;328:228.

Willing BP, et al. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. Gastroenterology. 2010;139:1844.

A19

METAGENOMICS AND PERSONALIZED MEDICINE[87]

*Herbert W. **Virgin***[88,*] *and John A. Todd*[89,*]

The microbiome is a complex community of Bacteria, Archaea, Eukarya, and viruses that infect humans and live in our tissues. It contributes the majority of genetic information to our metagenome and, consequently, influences our resistance and susceptibility to diseases, especially common inflammatory diseases, such as type 1 diabetes, ulcerative colitis, and Crohn's disease. Here we discuss how host–gene–microbial interactions are major determinants for the development of these multifactorial chronic disorders

[87] Reprinted from *Cell* 147(1), Virgin, H. V., J.A. Todd. 2011. Metagenomics and personalized medicine, pages 44-56, Copyright 2011, with permission from Elsevier.

[88] Department of Pathology and Immunology, Department of Molecular Microbiology, and Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, Washington University School of Medicine, St. Louis, MO, 63110, USA.

[89] Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0XY, UK.

* Correspondence: virgin@wustl.edu (H.W.V.), john.todd@cimr.cam.ac.uk (J.A.T.).

and, thus, for the relationship between genotype and phenotype. We also explore how genome-wide association studies (GWAS) on autoimmune and inflammatory diseases are uncovering mechanism-based subtypes for these disorders. Applying these emerging concepts will permit a more complete understanding of the etiologies of complex diseases and underpin the development of both next-generation animal models and new therapeutic strategies for targeting personalized disease phenotypes.

Recent advances in diverse areas of science and technology make this a unique time to study the genetics and pathogenesis of complex diseases, such type 1 diabetes (T1D) and inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC). These distinct diseases are now understood to share important common characteristics and aspects of their disease mechanisms. In all three diseases, the immune system damages tissues: T1D is likely an autoimmune disease, whereas CD and UC are likely caused by inappropriate inflammatory responses to components of our microbiome (see Box A19-1 for definition of key terms). Many genetic loci regulate the risk for each disease. Although a threshold dose of these susceptibility alleles provides the foundation for developing the disease, these alleles are not sufficient to cause the disease.

It has been obvious for decades that complex gene–gene and gene–environment interactions govern these diseases, but not surprisingly, untangling this web of interactions has been extremely difficult (Figure A19-1). Despite the failure to identify single causal agents for each disease, there is strong evidence that microbes contribute to pathogenesis. Furthermore, genomewide association studies (GWAS), which use large study populations and careful replication of results, have effectively identified many important loci in the host that increase one's risk for the disease, and these results have fundamentally altered how we conceptualize these diseases (Stappenbeck et al., 2011; Khor et al., 2011; Anderson et al., 2011; Franke et al., 2010; Todd, 2010). Correlation of GWAS data with genome-wide gene expression analyses (eQTLs), in combination with protein–protein interaction data, is greatly assisting the identification of candidate causal genes within these loci (Anderson et al., 2011; Franke et al., 2010; Cotsapas et al., 2011; Rossin et al., 2011; Fehrmann et al., 2011). Recently, numerous approaches have been developed to start defining mechanisms for complex inflammatory diseases by using leads from GWAS and analyses of the microbiome. These promising approaches include the following: the introduction of mutations in GWAS-identified loci into the mouse genome (Cadwell et al., 2010; Bloom et al., 2011); the creation of induced pluripotent stem cells (iPSCs) from patients and their differentiation into relevant cell types (e.g., Rashid et al., 2010); and humanized mouse models in which the murine immune system is replaced by transplantation (e.g., Brehm et al., 2010; Esplugues et al., 2011) or human microbial communities are transplanted into formerly germ-free mice (Goodman et al., 2011). Currently the great challenges in this field are to (1) understand how

BOX A19-1
Definition of Terms

Dysbiosis: Most commonly refers to a disruption in the normal homeostatic and beneficial relationship between microbes and their host, including disruptions in microbial community structure and function. Alterations in microbial community structure, involving Bacteria, Archaea, and/or Eukarya, can occur in any body habitat but have been best described in the gut where they have been associated with a number of disease states including, for example, inflammatory bowel disease.

Familial clustering: If a family member is diagnosed with a disease such as type 1 diabetes, ulcerative colitis, or Crohn's disease, then the risk of other first-degree family members is much greater (perhaps as much as 50-fold for some multiactorial disorders) than that for a person taken at random from the general population. Familial clustering is caused by a combination of inherited genetic variants from the parents to the children and shared environmental factors within the families. Susceptibility variants are being discovered rapidly by GWAS, but the environmental factors remain unknown, although numerous candidates are recognized, most particularly a role for the microbiome and infections.

GWAS: Analysis of common alleles (mostly single-nucleotide polymorphisms, SNPs) in a population that associates genetic loci with disease susceptibility. These loci contain "candidate" disease genes.

Metagenetics: Approaching genetic and genomic studies by considering all of the genes in the metagenome as opposed to considering, in isolation, host genes or genes that confer particular properties (e.g., virulence or commensalism) upon an individual microbe. Importantly, the history of microbial inputs into the metagenomic profile of an individual is important for identifying the causes of complex disease, requiring expensive but essential longitudinal studies, including information from maternal and gestational exposures and phenotypes.

Metagenome: As used here, metagenome is the sum of all genes and genetic elements and their modifications in the somatic and germ cells of a host plus all genes and genetic elements in all microorganisms that live on or in that host at a given time. The metagenome has transient elements (e.g., during infection with a pathogen) and more persistent elements (e.g., infection with latent eukaryotic virus; presence of commensal bacteria).

Microbiome: As used here, the microbiome is the sum of all microbial organisms that live in or on the host at a given time. The microbiome includes members of Bacteria, Archaea, Eukarya, and the viruses of these organisms. In other articles this term may be used to refer to the genes of these organisms.

Virome: The sum of all viruses living in the tissues of the host or infecting organisms in the microbiome. These viruses maybe further divided into viruses that infect members of each of the three domains of life (e.g., bacterial virome or bacterial phages or the eukaryotic virome).

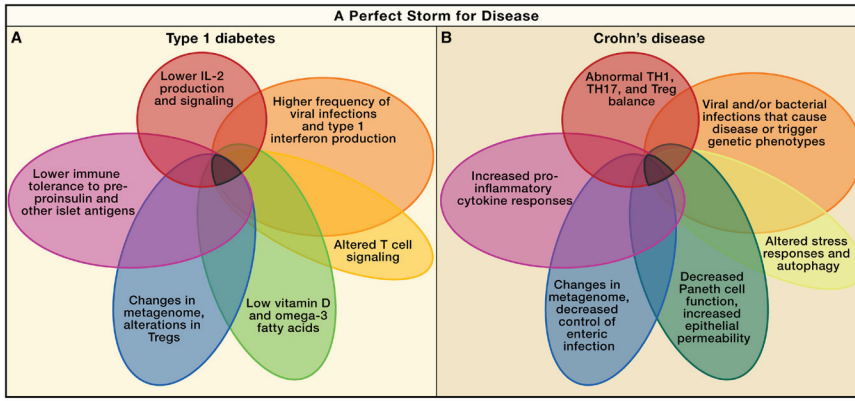


FIGURE A19-1 Perfect storms for developing Crohn's disease and type 1 diabetes. A series of overlapping events and phenotypes driven by metagenetic and environmental processes that, in sum, contribute to the development and pathogenesis of type 1 diabetes (A) and Crohn's disease (B).

both microbiome and GWAS-identified genes contribute to disease; (2) elucidate the molecular mechanisms by which causal genes act during pathogenesis; and (3) validate biomarkers and druggable pathways via genotype- phenotype studies (e.g., Dendrou et al., 2009; Bloom et al., 2011; Cadwell et al., 2010).

By peering through the lens of recent studies on CD, UC, and T1D, this review seeks to delineate emerging concepts in research on complex inflammatory diseases and to comment on the implications of these concepts for the interpretation of genetic and pathogenetic data. Two concepts are emphasized and integrated herein: (1) that single disease diagnoses are unlikely to be single phenotypes and may instead be the sum of multiple mechanism-based disease subsets, and (2) that the interactions of individual microorganisms and their genomes with specific host genes or pathways underpin the relationship between genotype and phenotype in these complex diseases. In this view, disease genetics may be combinatorial with different host–gene–microbial interactions, contributing to the pathogenesis of disease in subsets of patients. These two interrelated concepts, therefore, define T1D, CD, and UC as metagenetic (Box A19-1), rather than simply "genetic," diseases. These concepts will guide the design and interpretation of future experiments that seek to dissect the pathophysiologic mechanisms underlying a number of complex diseases and to identify more effective approaches for their treatment and prevention.

Host Genetic Grist for the Metagenetic Mill

Recently, meta-analyses of GWAS of large cohorts of patients of European descent with UC or CD have been performed (Franke et al., 2010; Anderson et

al., 2011; Khor et al., 2011). These studies identified 98 loci, and candidate genes within these loci, that have a putative role in IBD. Similar studies of T1D identified 53 disease susceptibility loci (Barrett et al., 2009) (http://www.t1dbase.org). Importantly, many disease susceptibility loci are shared among common autoimmune and inflammatory diseases, including T1D, Graves' disease, celiac disease, CD, UC, psoriasis, rheumatoid arthritis, alopecia areata, multiple sclerosis, and systemic lupus erythematosus (Cotsapas et al., 2011; Khor et al., 2011). It is striking that T1D and CD share 13/52 (25%) risk loci outside the human leukocyte antigen (HLA) gene complex despite the fact that these diseases are neither thought to be related diseases nor reported to be shared within families more often than expected by chance (http://www.t1dbase.org). Notably, the candidate causal genes in these 13 susceptibility loci regulate immunity. These include (Khor et al., 2011) *PTPN22*, which is involved in T and B cell signaling; *IL10*, encoding a powerful cytokine that suppresses inflammatory responses (including in specialized T regulatory cells in the gut) (Maloy and Powrie, 2011); *BACH2*, which regulates B cell gene expression and possibly IgA production; *TAGAP*, which is involved in T cell activation; *IKZF1*, which negatively regulates B cells; *IL2RA*, which controls T regulatory lymphocyte development and function; *GSDMB/GSDMA/ORMDL3*, which is involved in stress responses; *FUT2*, which controls microbial susceptibility (Smyth et al., 2011; Franke et al., 2010; McGovern et al., 2010); and *IL27*, which suppresses inflammatory responses and regulates IL-10 signaling (Imielinski et al., 2009; Barrett et al., 2009). This is a remarkable concordance of involved genes for two unrelated diseases, indicating that different diseases can have common mechanistic components and that the immune system is key for both diseases.

However, not withstanding all insights into disease mechanisms that the GWAS approach has already provided, the inheritance and the strong clustering of these multifactorial diseases within families (Box A19-1), which encompass both inherited genetic variants and intrafamilial environmental factors, remain only partially explained. Assuming a simple statistical model of gene interaction (Clayton, 2009), the numerous identified loci account for not more than 25% of the familial clustering of CD and UC (Anderson et al., 2011; Franke et al., 2010). This contrasts with T1D, in which the HLA effect is uniquely large and, together with 52 non-HLA loci, can account for almost all of the familial clustering (Clayton, 2009). For T1D, the massive effect of the HLA region, owing to functional polymorphisms in the HLA class II and class I genes, contributes almost 50% of familial clustering on its own (Clayton, 2009; Todd, 2010). There are, however, probably hundreds of non-HLA loci affecting the risk of CD, UC, and T1D that remain unmapped owing to their very small effect sizes (Barrett et al., 2009; Anderson et al., 2011; Franke et al., 2010). These putative loci will be difficult to map unless they contain rare mutations of higher penetrance, an occurrence that is just beginning to yield informative findings (Nejentsev et al., 2009; Rivas et al., 2011) and holds continued promise with the rapid use of high-throughput next-generation sequencing.

In humans, the HLA locus contains a large number of genes encoding the major histocompatibility complex (MHC) molecules (which are responsible for presenting antigens to cells of the immune system), along with a number of other genes that modulate immune responses. The remarkable contribution of HLA variations (Todd, 2010) to T1D risk is an unusual feature of a common disease. Nevertheless, HLA genotypes that greatly predispose individuals to T1D are not sufficient to cause the disease because only ~5% of high-risk HLA carriers develop T1D. HLAs are expressed by antigen-presenting cells (APCs), such as macrophages, B lymphocytes, and dendritic cells (DCs). DCs are highly potent APCs that reside in the pancreas and its islets (i.e., collections of insulin-producing beta cells and other endocrine cells) and could initiate the autoimmune destruction of beta cells by T cells (Calderon et al., 2011a, 2011b). Interestingly, the pancreatic lymph nodes, where DC priming of T cells for the induction of T1D may occur, also drain parts of the intestine, providing a site where the microbiome might influence the genesis of T1D (Turley et al., 2005; Wen et al., 2008). Because the insulin gene is one of the strongest non-HLA T1D susceptibility loci in the genome (Todd, 2010) (http://www.t1dbase.org), insulin and its precursors are likely primary autoantigens. These very strong associations with both HLA and this autoantigen gene are not a feature of CD or UC, in which no particular antigen is known to be targeted, hence their classification as inflammatory rather than autoimmune diseases. GWAS point to several other immunologic components of T1D etiology, including IL-2 production and receptor signaling (IL-2 gene, IL-2 receptors *IL2RA* [CD25] and *IL2RB* [CD132]; Todd, 2010), immune tolerance and T cell receptor signaling (*PTPN2* [Long et al., 2011], *PTPN22* [Arechiga et al., 2009; Bottini et al., 2006]), and recently, the immune response to viral infections and the type 1 interferon responses (*IFIH1* [encoding MDA5], *GPR183* [*EBI2*] [Heinig et al., 2010], *TLR7* and *TLR8*, and *FUT2* [Smyth et al., 2011]).

Twenty-eight loci (28/71, 39%) of CD risk loci are shared with UC, indicating that a set of core mechanisms participate in these diseases (Figure A19-2) (Khor et al., 2011). These diseases genes implicate numerous processes in both CD and UC, including T cell differentiation and function, autophagy, endoplasmic reticulum stress, oxidative stress, and mucosal immune defenses, among others. There are important gene–gene and pathway–pathway interactions within this core set of processes. For example, the CD risk gene *NOD2* links to autophagy through interactions with the Nod2 protein and with Atg16L1, induction of proinflammatory cytokines, control of bacterial infection, and sensing of pathogen-associated molecular patterns (Levine et al., 2011). Particularly notable are pathways involving the cytokines IL-23 and IL-12, which regulate the development of TH1 and TH17 CD4 T cells, and IL-10, which is essential in the function of certain regulatory T cells (Tregs) via its anti-inflammatory activity. Rare mutations in genes encoding IL-10 receptors confer susceptibility to early-onset IBD (Glocker et al., 2009). These genetic clues point to a key role for regulating

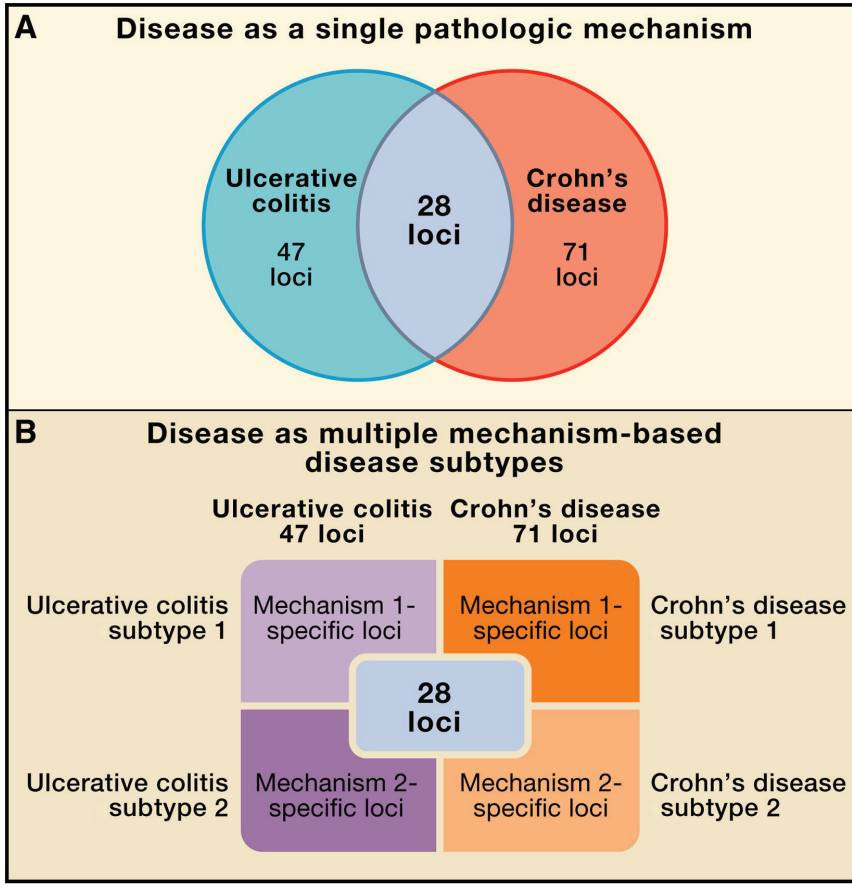


FIGURE A19-2 Refining the relationship between genotype and phenotype in complex inflammatory diseases. (A) Traditionally, a disease is considered as a single phenotype, with genes or loci conferring risk to two diseases shown as overlapping in a Venn diagram. (B) We propose a new view of the genotype–phenotype relationship in which different sets of loci are responsible for mechanistically distinct subtypes of diseases, and the sum of these subtypes constitutes the overall diagnosis. Here two disease subtypes are indicated for simplicity, but many such subtypes may exist, and sets of overlapping risk loci may be associated with these multiple mechanistically distinct disease phenotypes.

the balance between pro- and anti-inflammatory T cells in CD and UC. The regulation of T cell differentiation is also a key target for host-gene-microbial interactions, as discussed below.

Mechanism-Based Disease Subtypes

GWAS have revealed a wealth of genes potentially involved in T1D, UC, and CD, but no single gene or set of genes is prognostic. How can we interpret this observation? Here, we argue for an important contributor to this observation—the concept that "diagnosis" does not equal "single phenotype." Without a distinct phenotype, genetic results are often difficult to interpret. This basic principle comes into sharp focus as one considers current genetic and pathogenesis studies of CD, UC, and T1D.

Why is a diagnosed "disease" an imprecise phenotype? It is not because patients have been misdiagnosed—the diagnoses of UC, CD, or T1D have stood the test of time to predict patient prognosis. However, we believe that there are many pathways to the same diagnosis. A diagnosis may be "clinically" precise but "mechanistically" imprecise. Thus, clinical diagnoses are poor phenotypes for genetic studies unless a single mechanism is responsible for the diagnosis, as in the case of a rare gene mutation in a monogenic disease. The complexity of GWAS results is consistent with the existence of multiple disease subtypes within T1D, UC, or CD, each based on a specific mechanism (Figure A19-2). Support for this idea comes from the observation that subsets of IBD patients respond differentially to mechanistically distinct interventions (Melmed and Targan, 2010).

Why do diagnostic categories group different mechanistic processes under the same moniker? Over many decades, pathologists have lumped patients with similar but nonidentical clinical and pathological signs and symptoms into diagnostic categories that predict outcome and complications. Indeed, this has enormous value clinically, but it emphasizes similarities between patients in outcome rather than differences in pathways that lead to a common endpoint. Complex diseases are diagnosed by summing up multiple factors that may be causes or mere consequences of the disease process. Disease "diagnosis" does not require the presence in the tissue of all of the abnormalities that may be "classically" seen in a given disease (Gianani et al., 2010; Odze, 2003). For example, at the polar extreme, CD is easily distinguished from UC by its classical ileal involvement (i.e., involvement of tissue at the end of the small intestine), fissures, granulomas, transmural inflammation (i.e., inflammation through the entire intestinal wall), fat wrapping of the intestine, patchy pathology, skip lesions, and patient presentation with bowel strictures or percutaneous fistulae. However, like UC, CD can be restricted to the colon, and the inflammatory infiltrates of CD and UC overlap. UC can be patchy, and the patient presentations of the two diseases can overlap extensively. Similarly, the genetics, pathology, and pathogenesis of IBD may differ between young and old patients with the same diagnosis (Imielinski et al.,

2009; Odze, 2003). Even when all classical aspects of a disease are present, the mechanism responsible for the pathology observed may differ from one person to another. Based on these considerations, it is no surprise that the genetics of T1D, CD, and UC are complex because different phenotypes may have been grouped into a single analysis.

This putative mechanistic heterogeneity is reflected in sometimes subtle, but quantifiable, characteristics of the disease process and pathology. Taking such differences into account can be used to identify disease subtypes that are more recognizable as molecularly defined pathological conditions and that more closely relate to specific pathogenetic mechanisms underpinned by distinct sets of genetic risk loci (Figure A19-3). For example, variations in the *ATG16L1* gene (i.e., hypomorphic expression in the mouse and homozygosity for the T300A variant in humans) result in abnormalities in Paneth cell granules and secretion (Cadwell et al., 2008, 2010). Paneth cells are innate immune epithelial cells positioned at the base of small intestinal crypts, where they secrete antimicrobial peptides and other factors that help shape the configuration of the intestine's bacterial community. Abnormalities in Paneth cells are observed in the subset of CD patients homozygous for the T300A allele, thus defining a pathologic subtype of CD (Figure A19-2B). If one used criteria including Paneth cell abnormalities in CD diagnosis, the frequency of the *ATG16L1 T300A* allele would be higher in patients with the "Paneth cell subtype" of CD than in the CD population as a whole (Figure A19-3). If multiple risk loci contribute to such Paneth cell changes, one might be able to detect gene-gene interactions in this subset of patients compared to other subsets.

A similar situation exists in T1D. Biopsy specimens of the pancreas are virtually impossible to obtain. Therefore, T1D is defined clinically by the downstream consequences of destroying the insulin-secreting b cells of the pancreatic islets, namely, high blood glucose and absolute insulin dependence, rather than by the mechanisms for their destruction. It is, therefore, possible that several different pathologic processes result in this disease. T1D patients diagnosed under age 10 years frequently exhibit islet inflammation or insulitis, whereas patients diagnosed over age 10 years exhibit insulitis less frequently. More recently, this histopathological heterogeneity has become even more evident (Gianani et al., 2010), thanks to the Juvenile Diabetes Research Foundation nPOD project (http://www.jdrfnpod.org). As for CD, the diagnosis of T1D may reflect the presence of more than one pathogenetic mechanism and, thus, represent more than one disease subtype, although in T1D the HLA effects are an essential common pathway.

The concept that disease diagnoses include mechanism-based disease subtypes has many implications for interpreting human genetic studies and for understanding the relationship between the microbiome and genetic susceptibility, as discussed below. Including disease subtypes within a single diagnosis would decrease the power to define causal alleles and to detect gene-gene interactions that contribute to a single disease subtype. In this view, the difficulty of interpreting

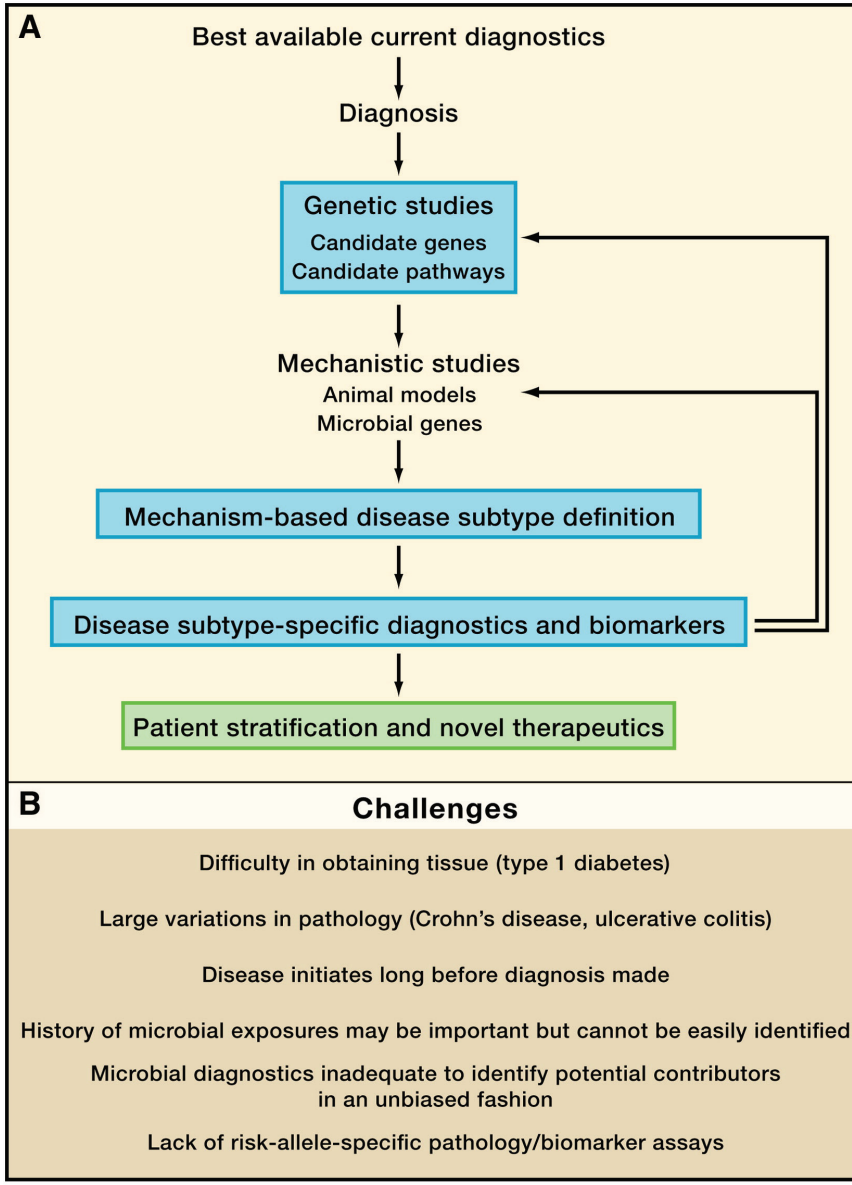


FIGURE A19-3 The iterative redefinition of mechanism-based disease subtypes. Here we present a conceptual workflow for breaking a broad disease diagnosis into its component subtypes by the iterative application of genetics and mechanistic studies. One output would be therapeutics based on disease subtype and patient stratification into groups more likely to respond to a given therapy or preventive strategy (A). (B) shows specific challenges for this process for type 1 diabetes and Crohn's disease.

how multiple small genetic effects sum to predispose an individual to a clinical diagnosis may partly reflect insufficient precision in selection of specific phenotypes to study.

It is important to recognize that it is the power and informativeness of GWAS themselves that drive the concept of mechanism-based disease subtypes (Figure A19-2). In the absence of candidate genetic mechanisms for defining disease subtypes, there is limited clinical utility in focusing on low-frequency characteristics or subtypes within a larger diagnostic category that predicts patient outcome. We, therefore, argue for iterative high-precision phenotyping of patients into mechanism-based subtypes in future studies; this will allow more accurate interpretation of genetic, pathogenesis, outcome, and therapeutic studies (Figure A19-3). Such definitions must be iteratively reassessed as risk alleles are defined and disease mechanisms are delineated so that the field is not limited by inflexible definitions of disease that may obscure mechanistic heterogeneity. This type of approach is a necessary presage to so-called stratified or personalized medicine. The genetic and pathological complexity of T1D, CD, and UC is particularly well suited for testing whether iteratively redefining disease diagnoses can enhance the value of genetic and pathogenesis studies. Importantly, precision in disease categorization would make defining the impact of host-gene-microbial interactions on disease processes more robust.

Host-Gene-Microbial Interactions in Metagenetics

Metazoan organisms are complex communities that include a core organism in combination with a veritable zoo of other organisms that live on or in the body—our microbiome. The microbiome includes eukaryotic viruses, Eukarya, bacteria viruses, Bacteria, Archaea, and, for many, helminths (Virgin et al., 2009; Kau et al., 2011; Garrett et al., 2010b; Spor et al., 2011). The importance of understanding the microbiome has been repeatedly emphasized, giving rise to a large number of international human microbiome projects (e.g., https://commonfund.nih.gov/hmp/, http://www.metahit.eu/) that have focused initially on the bacterial component of the microbiome. The host plus non-host genes of this polyglot and interactive community constitute our metagenome (Box A19-1). A critical emerging concept is that bacterial and viral interactions in the pathogenesis of inflammatory disease occur in a host gene-specific fashion (see below; Virgin et al., 2009; Cadwell et al., 2010; Bloom et al., 2011; Elinav et al., 2011). Understanding the metagenome is, therefore, highly relevant to understanding T1D, UC, CD, and other common multifactorial diseases.

Intestinal bacteria play a role in driving IBD, and emerging data support a similar view for T1D (Wen et al., 2008; Giongo et al., 2011; Roesch et al., 2009). The evidence that bacteria play a role in IBD includes two major observations: that surgical diversion of the fecal stream ameliorates inflammation (Sartor, 2008), and that antibiotics help some patients. In mouse models of colitis, viruses,

bacteria, or both acting together can contribute to the pathology via signaling through innate immune sensors and regulation of pro- and anti-inflammatory cytokines (Levine et al., 2011; Maloy and Powrie, 2011; Khor et al., 2011). For many years, enterovirus infection has been associated with T1D (e.g., Yeung et al., 2011; Oikarinen et al., 2011; Stene et al., 2010), and the major sensor for enterovirus RNA is the T1D susceptibility gene IFIH1, encoding MDA5 (Nejentsev et al., 2009; McCartney et al., 2011). The mechanisms for these associations between components of the microbiome and T1D, CD, or UC have proven elusive. The lack of integration among scientific disciplines and among training programs, together with limitations in technology, has substantially limited the understanding of metagenomic contributions to disease.

Adult mammals are permanently infected by many viruses, and they are populated by large site-specific bacterial and phage communities without overt negative effects (Virgin et al., 2009; Foxman and Iwasaki, 2011; Spor et al., 2011). Thus, the bacterial microbiota (and their phages) and the eukaryotic virome are two major (but not the only) contributors to the metagenome. The intestinal microbiome plays a critical role in mammalian physiology by synthesizing vitamins and harvesting energy from food (Spor et al., 2011; Kau et al., 2011). Further, the normal function of the innate immune system, which is critically involved in the pathogenesis of T1D, UC, and CD, is regulated by both chronic viral infections and resident bacterial communities (Barton et al., 2007; White et al., 2010; Virgin et al., 2009; Spor et al., 2011; Kau et al., 2011). The microbiome and metagenome vary from person to person based on host genetics, diet, exposure, geography (including westernization as approximated by the gross national product of a country), socioeconomic status, mode of delivery, gestational age at birth, breast feeding, antibiotic use, and additional factors (Virgin et al., 2009; Benson et al., 2010; Spor et al., 2011; Kau et al., 2011; Penders et al., 2006). Such variations could certainly provide environmental inputs that contribute to the incidence of T1D, UC, and CD, within the genetic foundation revealed by GWAS (Bach, 2002; Vehik and Dabelea, 2011; Ehlers and Kaufmann, 2010).

There are extensive interactions between host and non-host genes within the metagenome, and bacteria and eukaryotic viruses alter our physiology and fitness (Spor et al., 2011; Virgin et al., 2009; Hansen et al., 2010). These genetic interactions within the metagenome create a complex and poorly understood host-gene-microbial interaction matrix that can define phenotype. Host genes, such as those involved in innate and adaptive immunity (e.g., *NOD2*, *NLRP6*, *HLA*, *TLR2*, and *MYD88*), shape the bacterial microbiota (Spor et al., 2011; Elinav et al., 2011; Wen et al., 2008). Forward genetic screens in mice suggest that resistance to individual viruses involves hundreds of genes (Virgin et al., 2009), making it likely that many host genes regulate the microbiome (and thus the metagenome). Importantly, key interactions between members of the microbiome have been and are increasingly being reported. For example, murine norovirus can trigger an intestinal inflammatory process in mice with a mutation in *Atg16L1*. This process

can be treated with antibiotics and is thus presumed to be bacteria dependent (Cadwell et al., 2010) (Figure A19-4). The existence of such interactions indicates that it will be important to consider that the disease contributions of microbiome members (e.g., helminths and bacteria) are potentially dependent on each other.

Metagenetic influences on disease could occur in various ways. Familial disease clustering may reflect intrafamilial behavioral and dietary factors that define the metagenome. A major influence on the human metagenome may be vertical

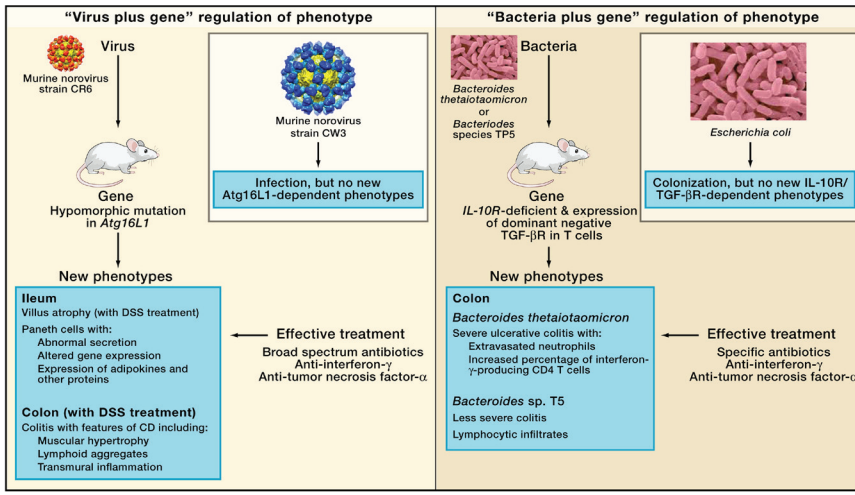


FIGURE A19-4 Microbe plus gene interactions determine inflammatory bowel disease phenotypes. (A) Two recent studies analyzed the capacity of two different strains of murine norovirus, MNV strain CR6 versus MNV strain CW3, to trigger phenotypes when orally inoculated into mice with a mutation in the Crohn's disease risk gene *Atg16L1* (Cadwell et al., 2008, 2010). This mutation results in decreased expression of Atg16L1 protein (hypomorphic, Atg16L1^{HM}). Even though MNV CW3 and MNV CR6 are closely related, they have different effects on intestinal pathology in Atg16L1^{HM} mice. Some of these interactions are observed only when mice are fed the chemical dextran sodium sulfate (DSS). (B) Two other studies analyzed the capacity of two different species of *Bacteroides* to trigger phenotypes in combination with mutations in the IL-10 receptor and T cell expression of a dominant-negative form of the TGF-β receptor (dnKO mice) (Bloom et al., 2011; Kang et al., 2008). dnKO mice are cured of their spontaneous colitis by treatment with antibiotics, but oral feeding of "cured" mice with fecal contents or specific bacteria reinduces disease. Even though *Bacteroides thetaiotaomicron* and *Bacteroides* sp. TP5 are closely related, they induce different forms of inflammation when fed to antibiotic-cured dnKO mice. In the same studies, dysbiosis (Box A19-1) with increases in the numbers of *Enterobacteriaceae* was noted in dnKO mice prior to curing the mice with antibiotics. However, *E. coli* inoculation did not trigger the pathologies seen with either *Bacteroides* species.

transmission of the maternal microbiome. In the controlled environment of mouse colonies, the bacterial microbiota is clearly maternally inherited. Furthermore, this microbiome can have profound pathological effects in mice carrying specific mutations, such that studies of host-gene functions must now consider contributions of the metagenome (see below).

The situation in humans is more complex (Hansen et al., 2011; Benson et al., 2010), although the importance of early environmental exposures has been well documented, including studies with mono- and dizygotic twins (Turnbaugh et al., 2009). Children delivered vaginally initially acquire a distinctly different intestinal bacterial microbiota than those born by caesarian section (Penders et al., 2006; Dominguez-Bello et al., 2010). In a meta-analysis, delivery by caesarean section increases the risk of T1D by 20% (Cardwell et al., 2008). An association with increased risk for T1D has also been reported for higher birth weight (Cardwell et al., 2010) and early infant diet (Pflüger et al., 2010) (Figure A19-5). Furthermore, changing microbial exposures and infections likely has a major

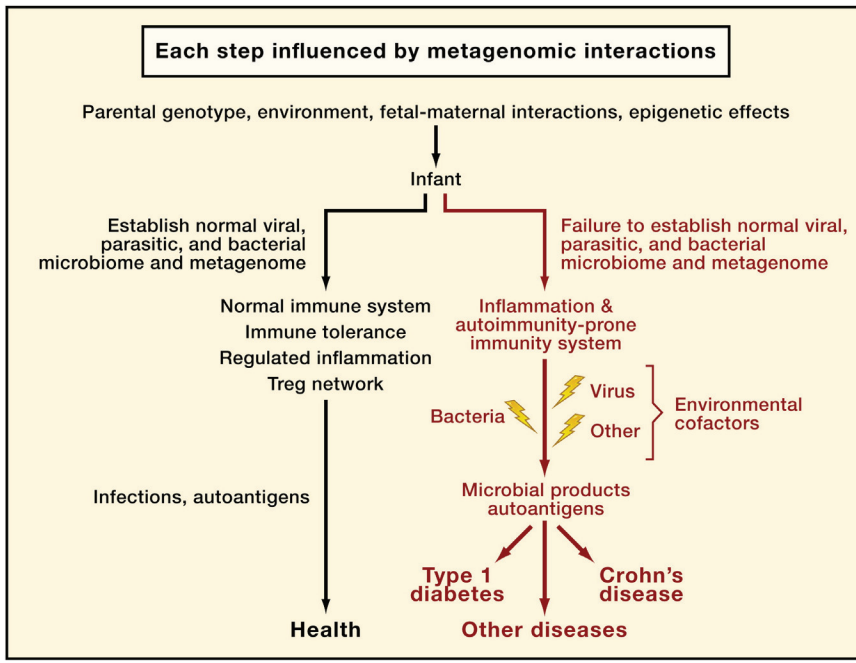


FIGURE A19-5 A metagenetic view of developing normal and pathological immune responses. This flowchart depicts stages in the development of normal immune responses or autoimmune and inflammatory diseases at which metagenetic interactions (i.e., gene-gene and gene-microbe interactions) might play a determining role. "Microbial products" refers to molecules that interact with host innate immune sensors and initiate inflammation.

influence on the incidence of other diseases. The dramatic rise in the incidence of allergy, asthma, and T1D in the last 60 years correlates with vast improvements in health care and sanitation (Bach, 2002; Vehik and Dabelea, 2011; Ehlers and Kaufmann, 2010). For example, severe rhinovirus infection before the age of 3 years coupled to an asthma-predisposing inherited host genome has been associated with increased risk of asthma (Foxman and Iwasaki, 2011). Thus, the metagenome could contribute to disease susceptibility and potentially explain a proportion of the familial clustering, the so-called "missing heritability" of multifactorial diseases.

Metagenetic Effects on Immunity and Autoimmunity

GWAS point to a fundamental role for the immune system in the pathogenesis of T1D, UC, and CD. An emerging concept is that bacterial and viral interactions contribute to both normal immune physiologies and abnormal pathologic responses that occur in a host gene-specific fashion (Virgin et al., 2009; Cadwell et al., 2010; Bloom et al., 2011; Garrett et al., 2007; Elinav et al., 2011). The microbiome has significant effects on the development of the immune system (Lee et al., 2011; Mazmanian et al., 2008; Sartor, 2008; Ivanov et al., 2009) and on physiology, including susceptibilities to obesity and the metabolic syndrome (Kau et al., 2011). Serum IgE responses to antigenic challenge are lower in mice colonized with *Clostridium*, confirming that bacteria can have profound effects on systemic immune responses involved in allergy (Atarashi et al., 2011). Central nervous system (CNS) inflammation induced by autoantigen is limited in germ-free mice, but it can be restored by colonization with specific bacteria (Lee et al., 2011). In the non-obese diabetic (NOD) mouse model mouse model, autoimmune diabetes is regulated by a MyD88-dependent interaction of intestinal microbes with the innate immune system (Wen et al., 2008). Germ-free K/BxN T cell receptor transgenic mice are resistant to arthritis caused by autoantibodies to the self-antigen glucose-6-phosphate isomerase. When these animals are colonized with segmented filamentous bacteria (SFB), they regenerate TH17 responses in the small intestine, autoantibody production, and arthritis (Wu et al., 2010). Furthermore, the normal intestinal microbiome is essential for effective resistance to oral inoculation with *Toxoplasma gondii* and for generating appropriate $CD8^+$ T cell responses to influenza (Ichinohe et al., 2011; Benson et al., 2009).

The mechanisms responsible for these observations are under intensive investigation. One recent study shows that intestinal bacteria induce Tregs in an antigen-specific and T cell receptor-dependent fashion (Lathrop et al., 2011). This is a key observation because it provides a mechanism, in addition to thymic exposure to self-antigens, for how regulatory responses can be generated to blunt inflammation. Given the continuous presence of the stimulating antigens for these Tregs in the normal intestinal microbiome, such cells could have profound effects on both intestinal and systemic immune responses, including responses

to self-antigens. This is particularly important because it has been reported that T lymphocytes migrate to the intestine to accept differentiation signals regulating autoimmune responses (Esplugues et al., 2011). It was also shown that injection of *Staphylococcus aureus* or its superantigen *S. aureus* enterotoxin B (SEB) was able to induce these intestinal regulatory TH17 cells, which is consistent with SEB injection being immune tolerogenic (Esplugues et al., 2011). These studies suggest that variation in the metagenome between individual humans, between mice in different research facilities, or even between animals from different cages within the same facility could have profound effects on many aspects of the immune response. This concept has key implications for the interpretation of mouse studies. The microbiome is maternally inherited in mice, but it can differ among research facilities; there may even be significant microenvironmental variation between cages of mice or between mice born of different dams. Given that the microbiome influences immunity so extensively, experiments must control for these factors. Currently, this is neither consistently performed nor required by peer reviewers.

Host-Gene-Metagenome Interactions in UC and CD

Correlations between communities of intestinal bacteria and CD or UC have led to the concept of dysbiosis (Box A19-1) as a contributor to these diseases (e.g., Sartor, 2008). This important hypothesis emphasizes the potential role that changes in the bacterial microbiota have on disease. However, now this hypothesis needs to expand and include both nonbacterial components of the metagenome and highly specific interactions between individual bacteria or viruses and host genes, which have recently been identified as contributors to disease pathogenesis. The relative contribution of dysbiosis versus the contribution of single organisms within the microbiome to the etiology of complex inflammatory diseases is unresolved. A confounding element has been the reliance on antibiotic treatment to assess bacteria as causes for intestinal disease. Because antibiotics can treat enteric inflammatory disease triggered by viruses (Figure A19-4) (Cadwell et al., 2010), a broader approach—including proof that specific bacteria or viruses are both necessary and sufficient for a phenotype—will be required to understand metagenetics of disease. Specific risk alleles for CD or UC could affect IBD by altering bacterial populations or individual bacterial types (Maloy and Powrie, 2011; Garrett et al., 2010b; Spor et al., 2011). Data from numerous mouse models of transmissible colitis confirm this point and are discussed below. The complexity of these reciprocal interactions between host and non-host genes within the metagenome underlines the critical need for new concepts and methodologies in computational and systems biology that can deal with individual host-gene microbial interactions in the broader context of the metagenome.

IBD in humans and mice is associated with alterations in the balance between TH1, TH17, and Treg cells, and this balance is dependent on the metagenome

(Garrett et al., 2010b; Maloy and Powrie, 2011). The relevance of these studies to human CD and UC is strongly supported by the identification of genes regulating these pathways in GWAS on IBD (see above). The role of specific bacteria and helminths in regulating these T cell responses in both the small and large intestine is highly relevant to understanding the genetics and pathogenesis of IBD. Polysaccharide A synthesized by the common colonic commensal *Bacteroides fragilis* induces Tregs that secrete IL-10 and inhibit intestinal inflammation (Round and Mazmanian, 2010; Mazmanian et al., 2008). Similarly, a protein antigen secreted by the intestinal helminth *Heligmosomoides polygyrus* induces Foxp3$^+$ Treg cells in vitro and in vivo in mice (Grainger et al., 2010). Furthermore, enteric carriage of a community of *Clostridium* species induces IL-10-secreting Foxp3$^+$ Tregs in the colon, likely via induction of TGF-β (Atarashi et al., 2011). These findings are interesting in light of the ubiquity of *Bacteroides* and *Clostridia* as commensal organisms in human and mouse, and differences in human carriage of helminths across the world.

In mice, the presence of distant relatives of *Clostridia*, called SFB, drives resistance to the enteric pathogen *Citrobacter rodentium* and the induction of CD4$^+$ TH17 cells in the lamina propria of the small intestine (Ivanov et al., 2009; Gaboriau-Routhiau et al., 2009). The discovery that SFB influence CD4$^+$ T cell differentiation was made when investigators noticed differences in intestinal TH17 cell numbers between mice of the same strain purchased from different vendors, followed by the demonstration that co-housing of these mice resulted in induction of TH17 cells (Ivanov et al., 2009). SFB are highly evolved for their commensal relationship with the mouse intestine (Sczesnak et al., 2011). Similar organisms have not yet been reported in humans, but it seems likely that similarly coevolved organisms will play a role in human intestinal biology and immunoregulation. The discovery of the role for SFB in CD4$^+$ T cell responses is similar to the discovery of a virus-plus-gene trigger for an intestinal disease in mice with symptoms similar to those in CD (Cadwell et al., 2010). This finding occurred by comparing intestinal phenotypes in one strain of mice bred in two different facilities. Both of these findings underline the critical importance of directly analyzing the contributions of the entire microbiome, rather than individual components, in animal models of diseases.

Transmissible Colitis and Host-Gene-Metagenome Interactions

Recent studies have made the striking observation that genetically determined colitis is transmissible, revealing a key role for host genes in defining the microbiome and for metagenomic contributions to enteric disease. Mice lacking both Rag2 and the transcription factor T-bet develop colitis that can be transmitted from a mutant mother to wild-type fosterling mice (Garrett et al., 2007, 2010a). Although there are expansions of specific bacterial types in these mice, including *Klebsiella pneumoniae* and *Proteus mirabilis*, another cofactor, in addition to

these bacteria, is required to generate the colitis phenotype. This cofactor is not yet identified. Similarly, mice deficient in NLRP6, caspase- 1, IL-18, or ASC (all proteins that regulate the expression of proinflammatory cytokines such as IL-18) develop colitis that is transmissible to co-housed wild-type mice (Elinav et al., 2011). Recent studies in another mouse model of transmissible colitis, which has similarities to UC, provide an example of the specificity of host-gene-bacterial relationships and IBD (Figure A19-4) (Bloom et al., 2011; Kang et al., 2008). Mice lacking the IL-10 receptor and expressing a dominant-negative form of the TGF-β receptor in T lymphocytes develop IFN-γ- and TNF-α-dependent colitis (Kang et al., 2008). The disease is cured by antibiotic treatment and reinduced by co-housing diseased and cured animals or by simply feeding cured mice the common commensal bacteria *Bacteroides thetaiotaomicron* (*B. theta*) (Bloom et al., 2011). In the same mice, the related *Bacteroides* sp. TP5 induced a lymphocytic inflammatory infiltrate different from that induced by *B. theta*, indicating the remarkable specificity of host-gene-bacterial interactions (Figure A19-4). The authors noted dysbiosis in diseased animals with increased numbers of *Enterobacteriaceae*, but these bacteria did not induce disease despite being present in higher numbers in sick mice. This study shows that a single bacterial type can cause IBD-like pathology in the proper genetic setting, a bacteria-plus-gene interaction that triggers intestinal inflammation. Importantly, the observation that two closely related bacteria induce different pathologies in the same genetically susceptible host provides support for the concept that genes present in the non-host metagenome can determine a host phenotype.

A similar observation, in this case of a virus-plus-gene interaction that triggers IBD-like pathology, has been described in mice mutant for the CD risk gene *Atg16L1* (Figure A19-4) (Cadwell et al., 2008, 2010). Abnormal Paneth cells were observed in humans carrying the *ATG16L1 T300A* allele and mice hypomorphic for expression of Atg16L1 raised in a conventional clean barrier (Cadwell et al., 2008). Importantly, the phenotype of the mice varied between different facilities and could be induced in mutant mice, but not wild-type mice, by inoculation with a specific strain of murine norovirus (Karst et al., 2003; Thackray et al., 2007). When these mice were challenged with dextran sodium sulfate (DSS), they developed inflammatory phenotypes specific to the combination of Atg16L1 mutation and an individual norovirus strain (Figure A19-4). Virus-triggered pathologies could be treated by blocking TNF-α or IFN-γ or by treatment with antibiotics. Interestingly, infection with murine norovirus enhances signaling through Nod1 and Nod2 via the induction of type 1 IFN, potentially providing a direct link between enteric viral infection and NOD signaling pathways implicated in IBD risk (Kim et al., 2011). These data raise the possibility that patterns of viral infection and specific components of the bacterial metagenome act together to influence the penetrance of UC and CD susceptibility risk alleles in humans. Furthermore, these data show that closely related viruses can have quite different effects on the phenotype of a host genetically prone to a disease process. This finding further

supports the concept that genes in the non-host metagenome can determine host phenotypes.

Host-Gene-Metagenome Interactions in T1D

For T1D, recent observations fit with a "perfect storm" scenario in which numerous events combine to increase susceptibility to disease development in early childhood (Figure A19-1). These events include susceptibility alleles in HLA class II genes and INS that cause increased autoreactivity against insulin, its precursors, and other islet antigens; lowered IL-2, IL-10, and IL-27 production and signaling; altered T cell receptor signaling and regulation (via, for example, susceptibility alleles in *PTPN2*, *PTPN22*, *CTLA4*, and *IL2RA*); and increased type 1 IFN production and responsiveness (Todd, 2010; Robinson et al., 2011; Bluestone et al., 2010). The "perfect T1D storm" is generated when these factors combine with a permissive, modern environment of widespread vitamin D deficiency (Cooper et al., 2011) and other still unidentified environmental factors (Figure A19-5). In particular, the T1D susceptibility genes and candidates *IFIH1* (Nejentsev et al., 2009), *GPR183* (*EBI2*) (Heinig et al., 2010), *TLR7*, *TLR8* (Barrett et al., 2009), and *FUT2* (Smyth et al., 2011) strongly suggest an etiological role for virus-induced, type 1 interferon production. A common knockout mutation of *FUT2* in several populations causes the nonsecretor status (i.e., a lack of shedding of the A and B blood group antigens into saliva and intestinal secretions). This T1D-predisposing FUT2 genotype is also associated with increased risk of CD (McGovern et al., 2010; Franke et al., 2010), providing another direct mechanistic link between these two diseases and microbial infections. The *FUT2* nonsecretor genotype is associated with resistance to certain strains of norovirus and *Helicobacter pylori* (Smyth et al., 2011). Investigations of the mechanisms involved in the *FUT2* associations with chronic and infectious disease are urgently required, as is the case for many of the newly identified GWAS candidate genes.

Defining the Metagenome Now and in the Future

Technologies for analyzing human loci involved in complex diseases have, until recently, outstripped technologies for analyzing the metagenome. For example, single-nucleotide polymorphism (SNP)-based GWAS cover the entire human genome, although at low resolution, whereas most common tools and methods applied to the non-host metagenome focus on only one component, such as a particular bacteria, viruses, or phage. The non-host metagenome is so complex that researchers have focused on DNA sequencing, even though many organisms relevant to disease—including enteroviruses that have been linked to T1D and viruses that cause intestinal disease—have RNA genomes. Although our knowledge of the human gut metagenome is in its infancy, this metagenome can

now be explored in detail by deep, next-generation sequencing of both RNA and DNA, then stratified by host genotype, disease risk, or disease status. Investigators are increasingly using shotgun sequencing of RNA + DNA, which theoretically can detect any organism (e.g., Finkbeiner et al., 2008). However, studies to date have often relied on the DNA sequencing of 16S rRNA genes of bacteria. This standard and reliable method has identified dysbiosis in IBD and T1D (Wen et al., 2008; Roesch et al., 2009; Giongo et al., 2011; Sartor, 2008; Garrett et al., 2010b). Whether these changes are causal or secondary to disease is unclear.

An outstanding example of consequences of relying on the analysis of only a subset of the metagenome is the recent appreciation that bacterial phage viruses are a major and dynamic part of the intestinal microbiome (Reyes et al., 2010). This adds an enteric bacterial "virome" to the eukaryotic virome that lives in our tissues (Virgin et al., 2009). Bacteria are not the only cells, in addition to host cells, that can be infected by viruses with consequent changes in biology. For example, an RNA virus infects the eukaryotic pathogen *Leishmania* and regulates the host inflammatory responses during parasite infection (Ives et al., 2011). Thus, like bacteria and their phages, all Eukarya in the microbiome are candidates for viral infection that might alter biological processes.

The tools to detect and quantify the entire non-host metagenome at a reasonable cost will undoubtedly develop rapidly as metagenomic sequencing technologies and computational approaches to phylogeny and microbe detection are developed and applied. Similarly, sequencing the entire host genome is becoming more cost efficient and practical. This wealth of data will set the stage for metagenetics, but meaningful and robust analyses of the complex interactions within the metagenome will require new computational tools and new conceptualizations of gene-gene and gene-microbe interactions.

Conclusion: The Metagenetics of Mechanism-Based Disease Subtypes

Here we have argued that two factors need to be considered as key contributors to the genetics and pathogenesis of complex inflammatory diseases, such as T1D, CD, and UC: specific host-gene-microbial interactions and the mechanistic heterogeneity of phenotypes that constitute complex diseases. Although we have used the lens of T1D, CD, and UC research to support these concepts, it is clear that these ideas may apply to a broader array of diseases as well. The striking effects of the microbiome on systemic immunity and on diseases that affect both visceral and mucosal tissues suggest that any physiologic process may be altered by the microbiome and gene-specific interactions of the microbiome with the host. At a minimum, the diverse diseases that have been revealed by GWAS to share risk alleles are strong candidates for considering the metagenome, rather than only the host genome, as contributing to health or disease.

The concepts of mechanism-defined disease subtypes and host-gene-microbial interactions cooperate in important ways. For example, if the single

diagnosis of CD or T1D includes multiple mechanistic phenotypes (Figure A19-2 and Figure A19-3), a specific host-gene-microbial interaction (Figure A19-4) might contribute to only one of these phenotypes. In this setting, the impact of interactions between genes in the metagenome, of either microbial or host origin, would be obscured. This could, for example, obscure the role of a single microbe in causing one mechanism-based disease subtype rather than causing all cases of a disease. Failure to identify such an agent would prevent the use of approaches that treat or vaccinate against the agent (Figure A19-2 and Figure A19-5). It is logical and anticipated that stratifying patients for treatment with pathway-specific drugs will improve outcomes and success of phase II and III clinical trials (Figure A19-3). This paradigm is highly effective and increasingly used in the treatment of cancer, but it also seems likely to benefit those with germline-based predisposition to disease as well.

Deconvoluting the complex matrix of interactions within the metagenome that contribute to disease will require more complete analyses of the metagenome. It also requires an iterative redefinition of disease subtypes using markers that distinguish between patients based on the mechanism responsible for injury rather than the presence of tissue injury per se. This ambitious goal is daunting to consider, but data discussed herein from human studies, animal studies, and analyses of the microbiome lead us to the inescapable conclusion that complex interactions within the metagenome control phenotypes. We must face this complexity head-on to solve the puzzle of the etiology and pathogenesis of complex diseases.

We, therefore, argue for the inclusion of the metagenome in human genetic studies for these diseases. We view complex diseases as "metagenetic," reflecting the contributions of both host and non-host genes within the metagenome. The nonhost genes in the metagenome that are relevant to a disease might be viral, bacterial, or derived from additional members of the microbiome, which are still largely uncharacterized. Parasites likely play a critical role in some populations. These metagenetic interactions probably contribute to the development of disease at two levels (Figure A19-5). First, we envision the normal immune system developing via harmonious relationships within the metagenome. For example, the level of innate immunity in mice is regulated by chronic herpesvirus infection (Barton et al., 2007; White et al., 2010), and therefore acquisition of a specific chronic virus might predispose the host to either helpful or harmful responses to other components of the microbiome. It will be important to develop quantitative and robust ways to identify such a "normal" immune system. Second, once a poorly balanced immune system is generated, host-gene interactions, with either other host genes or the non-host metagenome, likely synergize to generate inappropriate levels of inflammation in response to microbial products (e.g., CD and UC) or to set the stage for development of HLA-dependent autoimmunity (T1D). Understanding this level of biological complexity will require the involvement of statisticians, computational biologists, geneticists, pathogenesis experts, virologists, bacteriologists, and parasitologists in an integrated fashion to identify

mechanistically important interactions. Such an integrated approach can then perhaps make sense of the metagenetics of complex diseases, to the advantage of us all.

Acknowledgments

The authors would like to acknowledge helpful conversations with Thad Stappenbeck, Ramnik Xavier, Emil Unanue, Jeff Gordon, Balfour Sartor, Adolfo Garcia-Sastre, and Dermot McGovern. We thank Tom Smith for providing the images of noroviruses used in Figure A19-4. H.W.V. is supported by the NCI, NIAID, NCRR, the Crohn's and Colitis Foundation of America, and the Broad Medical Foundation. J.A.T. is supported by the NIDDK, NIHR, the Wellcome Trust, the Juvenile Diabetes Research Foundation International, and the European Union.

References

Anderson, C.A., Boucher, G., Lees, C.W., Franke, A., D'Amato, M., Taylor,K.D., Lee, J.C., Goyette, P., Imielinski, M., Latiano, A., et al. (2011). Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat. Genet. 43, 246–252.

Arechiga, A.F., Habib, T., He, Y., Zhang, X., Zhang, Z.Y., Funk, A., and Buckner, J.H. (2009). Cutting edge: the PTPN22 allelic variant associated with autoimmunity impairs B cell signaling. J. Immunol. 182, 3343–3347.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331, 337–341.

Bach, J.F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. N. Engl. J. Med. 347, 911–920.

Barrett, J.C., Clayton, D.G., Concannon, P., Akolkar, B., Cooper, J.D., Erlich, H.A., Julier, C., Morahan, G., Nerup, J., Nierras, C., et al; Type 1 Diabetes Genetics Consortium. (2009). Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat. Genet. 41, 703–707.

Barton, E.S., White, D.W., Cathelyn, J.S., Brett-McClellan, K.A., Engle, M., Diamond, M.S., Miller, V.L., and Virgin, H.W., 4th. (2007). Herpesvirus latency confers symbiotic protection from bacterial infection. Nature 447, 326–329.

Benson, A., Pifer, R., Behrendt, C.L., Hooper, L.V., and Yarovinsky, F. (2009). Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. Cell Host Microbe 6, 187–196.

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M., Oh, P.L., Nehrenberg, D., Hua, K., et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. USA 107, 18933–18938.

Bloom, S.M., Bijanki, V.N., Nava, G.M., Sun, L., Malvin, N.P., Donermeyer, D.L., Dunne, W.M., Jr., Allen, P.M., and Stappenbeck, T.S. (2011). Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe 9, 390–403.

Bluestone, J.A., Herold, K., and Eisenbarth, G. (2010). Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 464, 1293–1300.

Bottini, N., Vang, T., Cucca, F., and Mustelin, T. (2006). Role of PTPN22 in type 1 diabetes and other autoimmune diseases. Semin. Immunol. 18, 207–213.

Brehm, M.A., Bortell, R., Diiorio, P., Leif, J., Laning, J., Cuthbert, A., Yang, C., Herlihy, M., Burzenski, L., Gott, B., et al. (2010). Human immune system development and rejection of human islet allografts in spontaneously diabetic NODRag1null IL2rgammanull Ins2Akita mice. Diabetes 59, 2265–2270.

Cadwell, K., Liu, J.Y., Brown, S.L., Miyoshi, H., Loh, J., Lennerz, J.K., Kishi, C., Kc, W., Carrero, J.A., Hunt, S., et al. (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 456, 259–263.

Cadwell, K., Patel, K.K., Maloney, N.S., Liu, T.C., Ng, A.C., Storer, C.E., Head, R.D., Xavier, R., Stappenbeck, T.S., and Virgin, H.W. (2010). Virus-plussusceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 141, 1135–1145.

Calderon, B., Carrero, J.A., Miller, M.J., and Unanue, E.R. (2011a). Cellular and molecular events in the localization of diabetogenic T cells to islets of Langerhans. Proc. Natl. Acad. Sci. USA 108, 1561–1566.

Calderon, B., Carrero, J.A., Miller, M.J., and Unanue, E.R. (2011b). Entry of diabetogenic T cells into islets induces changes that lead to amplification of the cellular response. Proc. Natl. Acad. Sci. USA 108, 1567–1572.

Cardwell, C.R., Stene, L.C., Joner, G., Cinek, O., Svensson, J., Goldacre, M.J., Parslow, R.C., Pozzilli, P., Brigis, G., Stoyanov, D., et al. (2008). Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: a meta-analysis of observational studies. Diabetologia 51, 726–735.

Cardwell, C.R., Stene, L.C., Joner, G., Davis, E.A., Cinek, O., Rosenbauer, J., Ludvigsson, J., Castell, C., Svensson, J., Goldacre, M.J., et al. (2010). Birthweight and the risk of childhood-onset type 1 diabetes: a meta-analysis of observational studies using individual patient data. Diabetologia 53, 641–651.

Clayton, D.G. (2009). Prediction and interaction in complex disease genetics: experience in type 1 diabetes. PLoS Genet. 5, e1000540.

Cooper, J.D., Smyth, D.J., Walker, N.M., Stevens, H., Burren, O.S., Wallace, C., Greissl, C., Ramos-Lopez, E., Hyppönen, E., Dunger, D.B., et al. (2011). Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. Diabetes 60, 1624–1631.

Cotsapas, C., Voight, B.F., Rossin, E., Lage, K., Neale, B.M., Wallace, C., Abecasis, G.R., Barrett, J.C., Behrens, T., Cho, J., et al; on behalf of the FOCiS Network of Consortia. (2011). Pervasive sharing of genetic effects in autoimmune disease. PLoS Genet. 7, e1002254.

Dendrou, C.A., Plagnol, V., Fung, E., Yang, J.H., Downes, K., Cooper, J.D., Nutland, S., Coleman, G., Himsworth, M., Hardy, M., et al. (2009). Cell-specific protein phenotypes for the autoimmune locus IL2RA using a genotype-selectable human bioresource. Nat. Genet. 41, 1011–1015.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. USA 107, 11971–11975.

Ehlers, S., and Kaufmann, S.H.; Participants of the 99(th) Dahlem Conference. (2010). Infection, inflammation, and chronic diseases: consequences of a modern lifestyle. Trends Immunol. 31, 184–190.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., and Flavell, R.A. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 145, 745–757.

Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T.,Wan, Y.Y., O'Connor, W., Jr., Rongvaux, A., Van Rooijen, N., Haberman, A.M., et al. (2011). Control of TH17 cells occurs in the small intestine. Nature 475, 514–518.

Fehrmann, R.S.N., Jansen, R.C., Veldink, J.H., Westra, H.J., Arends, D., Bonder, M.J., Fu, J., Deelen, P., Groen, H.J.M., Smolonska, A., et al. (2011). Trans-eQTLs reveal that independent genetic variants associated with a complex phenotype converge on intermediate genes, with a major role for the HLA. PLoS Genet. 7, e1002197.

Finkbeiner, S.R., Allred, A.F., Tarr, P.I., Klein, E.J., Kirkwood, C.D., and Wang, D. (2008). Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog. 4, e1000011.

Foxman, E.F., and Iwasaki, A. (2011). Genome-virome interactions: examining the role of common viral infections in complex disease. Nat. Rev. Microbiol. 9, 254–264.

Franke, A., McGovern, D.P., Barrett, J.C., Wang, K., Radford-Smith, G.L., Ahmad, T., Lees, C.W., Balschun, T., Lee, J., Roberts, R., et al. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat. Genet. 42, 1118–1125.

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G., et al. (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 31, 677–689.

Garrett, W.S., Lord, G.M., Punit, S., Lugo-Villarino, G., Mazmanian, S.K., Ito, S., Glickman, J.N., and Glimcher, L.H. (2007). Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 131, 33–45.

Garrett, W.S., Gallini, C.A., Yatsunenko, T., Michaud, M., DuBois, A., Delaney, M.L., Punit, S., Karlsson, M., Bry, L., Glickman, J.N., et al. (2010a). Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. Cell Host Microbe 8, 292–300.

Garrett, W.S., Gordon, J.I., and Glimcher, L.H. (2010b). Homeostasis and inflammation in the intestine. Cell 140, 859–870.

Gianani, R., Campbell-Thompson, M., Sarkar, S.A., Wasserfall, C., Pugliese, A., Solis, J.M., Kent, S.C., Hering, B.J., West, E., Steck, A., et al. (2010). Dimorphic histopathology of long-standing childhood-onset diabetes. Diabetologia 53, 690–698.

Giongo, A., Gano, K.A., Crabb, D.B., Mukherjee, N., Novelo, L.L., Casella, G., Drew, J.C., Ilonen, J., Knip, M., Hyöty, H., et al. (2011). Toward defining the autoimmune microbiome for type 1 diabetes. ISME J. 5, 82–91.

Glocker, E.O., Kotlarz, D., Boztug, K., Gertz, E.M., Schäffer, A.A., Noyan, F., Perro, M., Diestelhorst, J., Allroth, A., Murugan, D., et al. (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N. Engl. J. Med. 361, 2033–2045.

Goodman, A.L., Kallstrom, G., Faith, J.J., Reyes, A., Moore, A., Dantas, G., and Gordon, J.I. (2011). Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc. Natl. Acad. Sci. USA 108, 6252–6257.

Grainger, J.R., Smith, K.A., Hewitson, J.P., McSorley, H.J., Harcus, Y., Filbey, K.J., Finney, C.A., Greenwood, E.J., Knox, D.P., Wilson, M.S., et al. (2010). Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway. J. Exp. Med. 207, 2331–2341.

Hansen, E.E., Lozupone, C.A., Rey, F.E., Wu, M., Guruge, J.L., Narra, A., Goodfellow, J., Zaneveld, J.R., McDonald, D.T., Goodrich, J.A., et al. (2011). Pan-genome of the dominant human gut-associated archaeon Methanobrevibacter smithii, studied in twins. Proc. Natl. Acad. Sci. USA 108 (Suppl 1), 4599–4606.

Hansen, J., Gulati, A., and Sartor, R.B. (2010). The role of mucosal immunity and host genetics in defining intestinal commensal bacteria. Curr. Opin. Gastroenterol. 26, 564–571.

Heinig, M., Petretto, E., Wallace, C., Bottolo, L., Rotival, M., Lu, H., Li, Y., Sarwar, R., Langley, S.R., Bauerfeind, A., et al; Cardiogenics Consortium. (2010). A trans-acting locus regulates an anti-viral expression network and type 1 diabetes risk. Nature 467, 460–464.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. USA 108, 5354–5359.

Imielinski, M., Baldassano, R.N., Griffiths, A., Russell, R.K., Annese, V., Dubinsky, M., Kugathasan, S., Bradfield, J.P., Walters, T.D., Sleiman, P., et al; Western Regional Alliance for Pediatric IBD; International IBD Genetics Consortium; NIDDK IBD Genetics Consortium; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium. (2009). Common variants at five new loci associated with early-onset inflammatory bowel disease. Nat. Genet. 41, 1335–1340.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139, 485–498.

Ives, A., Ronet, C., Prevel, F., Ruzzante, G., Fuertes-Marraco, S., Schutz, F., Zangger, H., Revaz-Breton, M., Lye, L.F., Hickerson, S.M., et al. (2011). Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. Science 331, 775–778.

Kang, S.S., Bloom, S.M., Norian, L.A., Geske, M.J., Flavell, R.A., Stappenbeck, T.S., and Allen, P.M. (2008). An antibiotic-responsive mouse model of fulminant ulcerative colitis. PLoS Med. 5, e41.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J., and Virgin, H.W., 4th. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. Science 299, 1575–1578.

Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L., and Gordon, J.I. (2011). Human nutrition, the gut microbiome and the immune system. Nature 474, 327–336.

Khor, B., Gardet, A., and Xavier, R.J. (2011). Genetics and pathogenesis of inflammatory bowel disease. Nature 474, 307–317.

Kim, Y.G., Park, J.H., Reimer, T., Baker, D.P., Kawai, T., Kumar, H., Akira, S., Wobus, C., and Núñez, G. (2011). Viral infection augments Nod1/2 signaling to potentiate lethality associated with secondary bacterial infections. Cell Host Microbe 9, 496–507.

Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., Peterson, D.A., Stappenbeck, T., and Hsieh, C.S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature 10.1038/ nature10434.

Lee, Y.K., Menezes, J.S., Umesaki, Y., and Mazmanian, S.K. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. USA 108 (Suppl 1), 4615–4622.

Levine, B., Mizushima, N., and Virgin, H.W. (2011). Autophagy in immunity and inflammation. Nature 469, 323–335.

Long, S.A., Cerosaletti, K., Wan, J.Y., Ho, J.C., Tatum, M., Wei, S., Shilling, H.G., and Buckner, J.H. (2011). An autoimmune-associated variant in PTPN2 reveals an impairment of IL-2R signaling in CD4(+) T cells. Genes Immun. 12, 116–125.

Maloy, K.J., and Powrie, F. (2011). Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474, 298–306.

Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453, 620–625.

McCartney, S.A., Vermi, W., Lonardi, S., Rossini, C., Otero, K., Calderon, B., Gilfillan, S., Diamond, M.S., Unanue, E.R., and Colonna, M. (2011). RNA sensor-induced type I IFN prevents diabetes caused by a b cell-tropic virus in mice. J. Clin. Invest. 121, 1497–1507.

McGovern, D.P., Jones, M.R., Taylor, K.D., Marciante, K., Yan, X., Dubinsky, M., Ippoliti, A., Vasiliauskas, E., Berel, D., Derkowski, C., et al; International IBD Genetics Consortium. (2010). Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. Hum. Mol. Genet. 19, 3468–3476.

Melmed, G.Y., and Targan, S.R. (2010). Future biologic targets for IBD: potentials and pitfalls. Nat. Rev. Gastroenterol. Hepatol. 7, 110–117.

Nejentsev, S., Walker, N., Riches, D., Egholm, M., and Todd, J.A. (2009). Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 324, 387–389.

Odze, R. (2003). Diagnostic problems and advances in inflammatory bowel disease. Mod. Pathol. 16, 347–358.

Oikarinen, S., Martiskainen, M., Tauriainen, S., Huhtala, H., Ilonen, J., Veijola, R., Simell, O., Knip, M., and Hyo¨ ty, H. (2011). Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 60, 276–279.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van den Brandt, P.A., and Stobberingh, E.E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511–521.

Pflüger, M., Winkler, C., Hummel, S., and Ziegler, A.G. (2010). Early infant diet in children at high risk for type 1 diabetes. Horm. Metab. Res. 42, 143–148.

Rashid, S.T., Corbineau, S., Hannan, N., Marciniak, S.J., Miranda, E., Alexander, G., Huang-Doran, I., Griffin, J., Ahrlund-Richter, L., Skepper, J., et al. (2010). Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. J. Clin. Invest. 120, 3127–3136.

Reyes, A., Haynes, M., Hanson, N., Angly, F.E., Heath, A.C., Rohwer, F., and Gordon, J.I. (2010). Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466, 334–338.

Rivas, M.A., Beaudoin, M., Gardet, A., Stevens, C., Sharma, Y., Zhang, C.K., Boucher, G., Ripke, S., Ellinghaus, D., Burtt, N., et al. (2011). Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat. Genet. 10.1038/ng.952.

Robinson, T., Kariuki, S.N., Franek, B.S., Kumabe, M., Kumar, A.A., Badaracco, M., Mikolaitis, R.A., Guerrero, G., Utset, T.O., Drevlow, B.E., et al. (2011). Autoimmune disease risk variant of IFIH1 is associated with increased sensitivity to IFN-α and serologic autoimmunity in lupus patients. J. Immunol. 187, 1298–1303.

Roesch, L.F., Lorca, G.L., Casella, G., Giongo, A., Naranjo, A., Pionzio, A.M., Li, N., Mai, V., Wasserfall, C.H., Schatz, D., et al. (2009). Culture-independent identification of gut bacteria correlated with the onset of diabetes in a rat model. ISME J. 3, 536–548.

Rossin, E.J., Lage, K., Raychaudhuri, S., Xavier, R.J., Tatar, D., Benita, Y., Cotsapas, C., and Daly, M.J.; International Inflammatory Bowel Disease Genetics Constortium. (2011). Proteins encoded in genomic regions associated with immune-mediated disease physically interact and suggest underlying biology. PLoS Genet. 7, e1001273.

Round, J.L., and Mazmanian, S.K. (2010). Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc. Natl. Acad. Sci. USA 107, 12204–12209.

Sartor, R.B. (2008). Microbial influences in inflammatory bowel diseases. Gastroenterology 134, 577–594.

Sczesnak, A., Segata, N., Qin, X., Gevers, D., Petrosion, J.F., Huttenhower, C., Littman, D.R., and Ivanov, I.I. (2011). The genome of Th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. Cell Host Microbe 10, 260–272.

Smyth, D.J., Cooper, J.D., Howson, J.M.M., Clarke, P., Downes, K., Mistry, T., Stevens, H., Walker, N.M., and Todd, J.A. (2011). FUT2 non-secretor status links type 1 diabetes susceptibility and resistance to infection. Diabetes. Published online October 24, 2011. 10.2337/db11-0638.

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. Nat. Rev. Microbiol. 9, 279–290.

Stappenbeck, T.S., Rioux, J.D., Mizoguchi, A., Saitoh, T., Huett, A., Darfeuille-Michaud, A., Wileman, T., Mizushima, N., Carding, S., Akira, S., et al. (2011). Crohn disease: a current perspective on genetics, autophagy and immunity. Autophagy 7, 355–374.

Stene, L.C., Oikarinen, S., Hyöty, H., Barriga, K.J., Norris, J.M., Klingensmith, G., Hutton, J.C., Erlich, H.A., Eisenbarth, G.S., and Rewers, M. (2010). Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes 59, 3174–3180.

Thackray, L.B., Wobus, C.E., Chachu, K.A., Liu, B., Alegre, E.R., Henderson, K.S., Kelley, S.T., and Virgin, H.W., 4th. (2007). Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. J. Virol. 81, 10460–10473.

Todd, J.A. (2010). Etiology of type 1 diabetes. Immunity 32, 457–467. Turley, S.J., Lee, J.W., Dutton-Swain, N., Mathis, D., and Benoist, C. (2005). Endocrine self and gut non-self intersect in the pancreatic lymph nodes. Proc. Natl. Acad. Sci. USA 102, 17729–17733.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., et al. (2009). A core gut microbiome in obese and lean twins. Nature 457, 480–484.

Vehik, K., and Dabelea, D. (2011). The changing epidemiology of type 1 diabetes: why is it going through the roof? Diabetes Metab. Res. Rev. 27, 3–13.

Virgin, H.W., Wherry, E.J., and Ahmed, R. (2009). Redefining chronic viral infection. Cell 138, 30–50.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A., et al. (2008). Innate immunity and intestinal microbiota in the development of type 1 diabetes. Nature 455, 1109–1113.

White, D.W., Keppel, C.R., Schneider, S.E., Reese, T.A., Coder, J., Payton, J.E., Ley, T.J., Virgin, H.W., and Fehniger, T.A. (2010). Latent herpesvirus infection arms NK cells. Blood 115, 4377–4383.

Wu, H.J., Ivanov, I.I., Darce, J., Hattori, K., Shima, T., Umesaki, Y., Littman, D.R., Benoist, C., and Mathis, D. (2010). Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 32, 815–827.

Yeung, W.C., Rawlinson, W.D., and Craig, M.E. (2011). Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ 342, d35.

A20

FROM GENETICS OF INFLAMMATORY BOWEL DISEASE TOWARDS MECHANISTIC INSIGHTS[90]

Daniel B. Graham[91,92] *and Ramnik J.* ***Xavier***[91,93]

Advancements in human genetics now poise the field to illuminate the pathophysiology of complex genetic disease. In particular, genome-wide association studies (GWAS) have generated insights into the mechanisms driving inflammatory bowel disease (IBD) and implicated genes shared by multiple autoimmune and autoinflammatory diseases. Thus, emerging evidence suggests a central role for the mucosal immune system in mediating immune homeostasis and highlights the complexity of genetic and environmental interactions that collectively modulate the risk of disease. Nevertheless, the challenge remains to determine how genetic variation can precipitate and sustain the inappropriate inflammatory response to commensals that is observed in IBD. Here, we highlight recent advancements in immunogenetics

[90] Reprinted from *Trends in Immunology* 34(8), Graham, D. B. and R. J. Xavier. 2013. From genetics of inflammatory bowel disease toward mechanistic insights, pages 371-378, Copyright 2013, with permission from Elsevier.

[91] Broad Institute of MIT and Harvard, Cambridge, MA, USA.

[92] Department of Medicine, Massachusetts General Hospital, Boston, MA, USA.

[93] Gastrointestinal Unit, Center for the Study of Inflammatory Bowel Diseases, and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, MA, USA.

and provide a forward-looking view of the innovations that will deliver mechanistic insights from human genetics.

Genetics of IBD

Genetic predisposition to autoimmune and autoinflammatory diseases is well established, yet the interrelationship between multiple genetic components and environmental triggers remains to be elucidated. The observation that some individuals develop IBD at childhood and others during adulthood, suggests distinct environmental contributions to disease initiation versus disease progression. Although the genetic elements predisposing to IBD are present from birth, disease onset occurs later in life, and there is further need to understand how host genetic and epigenetic factors interact with environmental triggers at disease onset and how genetics influence immune regulatory networks that sustain disease. The most recent advancement in the field came from a meta-analysis of 15 prior GWASs to aid in the design of the Immunochip (Jostens et al., 2012). This custom-designed genotyping array contains high-density genomic coverage of single nucleotide polymorphisms (SNPs) implicated by GWASs to fine map disease loci and implicate new genes. In combination with imputed GWAS data, Immunochip validation studies on over 25 000 IBD cases identified 71 new loci for a total of 163 loci associated with IBD (Jostens et al., 2012). As a result, these studies substantially increase the estimated disease heritability for both Crohn's disease (CD) and ulcerative colitis (UC). Furthermore, they highlight 110 shared loci for both disease subtypes, while 30 are classified as specific for CD and 23 for UC (Jostens et al., 2012). IBD shares the largest number of loci with type 1 diabetes and shows substantial enrichment of overlap with ankylosing spondylitis, psoriasis, and susceptibility to mycobacterial infection (Jostens et al., 2012). Such overlap in the genetics of autoimmune/autoinflammatory diseases points to several common immune processes, such as regulation of mucosal immunity, in mediating inflammatory pathology. Together, the immediate impact of implicating 163 loci in IBD resulted in the identification of new candidate genes and pathways that may drive disease. However, taking human genetic studies a step further requires building new experimental systems to define the biological functions of these genes. For example, genetic studies in psoriasis patients implicated Act1 (*Traf3ip2*), an adaptor functioning downstream of the interleukin-17 receptor (IL-17R) (Ellinghaus et al., 2010; Huffmeier et al., 2010; Strange et al., 2010). The generation of a knockout mouse that modeled *TRAF3IP2* loss of function variants in humans revealed hyperactive TH17 responses driving IL-22-dependent inflammation (Wang et al., 2013). Thus, GWASs led to the development of a novel mouse model that provided key insight into disease mechanisms. Here, we discuss how advancements in genomics and functional genetics have contributed to our current understanding of IBD, and how these approaches can be applied to provide new mechanistic insights and therapeutic opportunities (Figure A20-1).

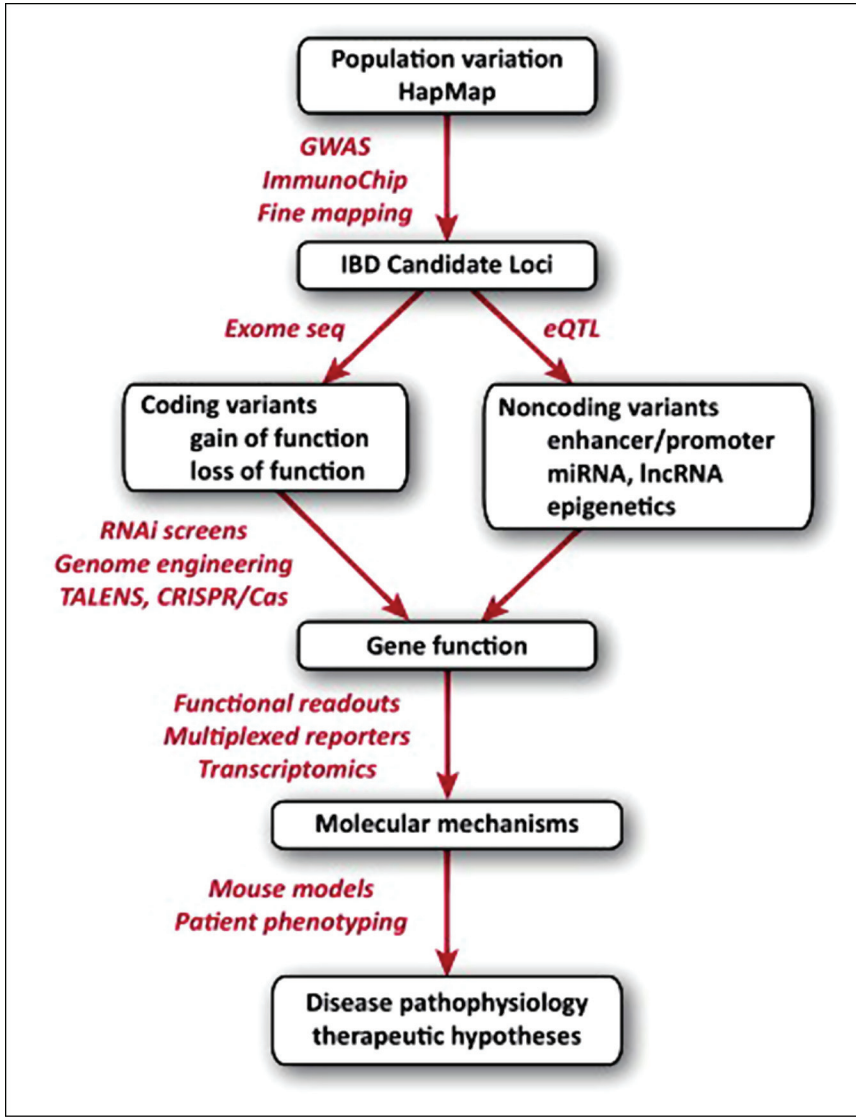


FIGURE A20-1 From genetics to disease mechanisms. Advancements in genomics technology have facilitated the discovery of 163 loci associated with inflammatory bowel disease (IBD). Identifying causal mutations within coding and noncoding regions of these loci has begun to reveal gene function and shed new light on disease mechanisms. Progressing from genetics to mechanistic insight is accelerating as a result of new technology in the field of functional genetics.

Genetic Variation and Functional Repercussions

Many SNPs implicated by GWASs are not directly causal with respect to phenotype, rather they exist in linkage disequilibrium with yet to be discovered variants that are functional. This point highlights the fact that GWASs cover relatively common genetic variants including SNPs with >1% frequency within the population and fail to capture rare or undiscovered variants. Such germline-encoded DNA variation can lead to nonsynonymous coding variants that change protein function and/or posttranslational regulation. However, the majority of the SNPs implicated by GWASs represent variation in noncoding regions of the genome. Thus, alterations in gene expression are likely to be important factors in immune dysregulation, and highlight the need to comprehensively characterize DNA and RNA regulatory elements.

Genetic variation impacts gene expression at the level of transcription, RNA stability, splicing, and epigenetic modification. Accordingly, new genomics tools have been developed to capture genetic regulation at multiple levels. Studies merging proteomics with genomics identified differential binding of transcription factors to SNPs in the *IL2RA* promoter that regulate gene expression (Butter et al., 2012). As an additional mechanism of transcriptional regulation, long noncoding (lnc) RNAs within the genome have been shown to play key roles in modulating gene expression (Derrien et al., 2012; Guttman et al., 2009). Recent studies have identified a critical requirement for lncRNA in maintaining pluripotency and enforcing lineage-specific gene expression profiles (Guttman et al., 2011). It is now clear that expression of lncRNAs is regulated in a cell type-specific manner. Thus, analysis of the human beta cell transcriptome identified tissue-specific lncRNAs that were transcriptionally dysregulated in type 2 diabetes (T2D) or that mapped to loci previously associated with T2D (Moran et al., 2012). In the context of host defense, the lncRNA NeST has been identified as an epigenetic regulator of interferon-gamma (IFN-γ) expression in CD8 T cells and determines susceptibility to viral and bacterial infection in mice (Gomez et al., 2013). However, the potential role for lncRNAs in regulating human inflammatory diseases remains largely unexplored.

Following transcription, mRNA splicing and stability are tightly regulated. In this context, inflammatory stimuli and microbial components induce a coordinated program of miRNA expression in monocytes that regulates inflammatory responses (Hasler et al., 2012). Consequently, variants in the gene encoding IL23R that associate with IBD are resistant to downmodulation by miRNA, which results in upregulation of IL23R expression (Zwiers et al., 2012). Similarly, variants in the 3′ UTR of genes encoding cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and IL-10 alter recognition by miRNAs, resulting in dysregulated expression (deJong et al., 2012). In addition to regulation at the level of mRNA stability, RNA splicing impacts gene function. SNPs in the 3′ untranslated region of *Ctla4* associate with type 1 diabetes (T1D) and correlate with reduced expression of a soluble CTLA-4 splice variant (Ueda et al., 2003). Accordingly,

transgenic mice with germline integration of a cassette encoding short hairpin RNA (shRNA) specifically targeting soluble CTLA-4 impaired T regulatory cell (Treg) function and resulted in accelerated onset of diabetes in the nonobese diabetic (NOD) system and severe colitis in the CD45RB^{hi} transfer model (Gerold et al., 2011).

Determining the impact of genetic variation on expression profiles has been a major focus in the field, as highlighted in studies of expression quantitative trait loci (eQTL) that aim to correlate SNPs with transcriptome data (Knight, 2012). Recent studies have begun to identify SNPs that alter transcription of neighboring genes (cis eQTLs) and distant genes (trans eQTLs). The analysis of 92 strains of inbred mice identified several thousand eQTLs associated with macrophage responses to lipopolysaccharide (LPS) (Orozco et al., 2012). Similarly, eQTL studies in human dendritic cells (DCs) identified 198 eQTLs associated with *Mycobacterium tuberculosis* infection (Barreiro et al., 2012). These findings are notable given the genetic overlap between susceptibility to *M. tuberculosis* infection and IBD and given the enrichment of IBD candidate genes in DCs (Jostens et al., 2012). In a direct example of eQTLs in IBD, SNPs associated with CD correlate with increased expression of caspase recruitment domain family member 9 (Card9), which has been suggested with potentiate inflammatory signaling cascades (Jostens et al., 2012). Similarly, SNPs associated with T1D have been shown to act in trans on an antiviral inflammatory network driven by interferon regulatory factor 7 (IRF7) in monocytes (Heinig et al., 2010). Although many of the eQTLs highlighted above are cell type-specific and the mechanistic basis of this specificity is not entirely clear, it is likely to be regulated by differential pathway activity and epigenetic effects. In this context, the Encyclopedia of DNA Elements (ENCODE) project continues to deposit rich datasets of genome-wide epigenetic modifications across multiple cell types. Recent chromatin mapping studies highlight SNPs associated with autoimmunity that are enriched within enhancer elements containing epigenetic modifications in specific cell types (Ernst et al., 2011; Maurano et al., 2012).

As the number of noncoding variants and SNPs associated with autoimmune disease has expanded, so has identification of coding variants. Exome sequencing at IBD loci has identified novel coding variants and helped pinpoint specific genes within these loci that are likely to impact disease (Rivas et al., 2011). For example, many IBD loci are gene-dense, and SNP signals are not able to precisely pinpoint the causative gene. A locus on chromosome 12 implicated by GWAS is comprised of the *LRRK2* gene and *MUC19*. Although a great deal of attention has focused on study of the kinase leucine-rich repeat kinase 2 (LRRK2), the discovery of new coding variants in *MUC19* indicates the need for more detailed analysis of this gene in IBD pathogenesis (Rivas et al., 2011). *MUC19* is a gel-forming apomucin, thus genetic variants of *MUC19* may contribute to mucosal barrier dysfunction by causing quantitative changes in its production or structural changes in the glycoprotein core. The barrier function of the mucous layer

acts in concert with its ability to retain antimicrobial effector molecules such as defensins and secreted IgA. Thus, B cell IgA production is a central pathway in mucosal immunity, which is consistent with a newly discovered role for the IBD candidate gene *BACH2* in class switch recombination (Muto et al., 2010). In fact, exome sequencing recently defined coding variants in *BACH2* and identified a distinct profile in severe UC characterized by mutations in both *BACH2* and IL10 (Christodoulou et al., 2012). Although B cell function was not directly tested in this study, *BACH2* and IL-10 can induce immunoglobulin class switching (Briere et al., 1994; Muto et al., 2010), thus highlighting the potential of gene–gene interactions to impact cell type-specific phenotypes. Collectively, these studies demonstrate the diverse mechanisms by which genetic variation impacts gene function and highlight the importance of genetic interactions.

Addressing Genetic Epistasis

The diversity of genetic backgrounds in human subjects complicates phenotyping endeavors, but highlights the notion that phenotypes derive from the aggregate effects of multiple genetic factors. Furthermore, IBD genes interact, as highlighted by the discovery of a microtubule-associated serine/threonine-protein kinase 3-regulated transcriptional program that broadly controls expression of inflammatory genes associated with nuclear factor-kappa-B (NFkB) activity, such as those induced by NOD2 and Toll-like receptors (TLRs) (Labbe et al., 2008, 2012). It is now possible to discern the impact of genetic perturbation on transcriptional programs by employing RNAi-mediated knockdown of candidate genes followed by RNAseq. Accordingly, this approach may provide a deeper understanding of how IBD genes interact with one another at the genetic level. Although genetic epistasis is thought to be an important component of disease, it is not captured well by GWASs (Zuk et al., 2012). Nevertheless, studies have shown that T1D risk alleles for human leukocyte antigen (HLA) class II and *Ctla4* statistically interact and support a role for gene–gene interactions (Howson et al., 2012). Epistasis is difficult to study systematically, because of the sheer number of possible genetic contributors. Furthermore, perturbing multiple genes simultaneously has practical limits, and addressing the issue of genetic epistasis in complex disease will ultimately require an unbiased discovery-based approach invoking GWASs on a much larger scale.

Exploring Gene-Environment Interactions

Following the logic that host genetic epistasis contributes to disease, emerging evidence indicates that host genetic interactions with microbes also shapes immunologic phenotypes. Twin studies suggest that infant and early childhood infections may be associated with IBD (Ng et al., 2012). In addition, stable interactions of commensal communities with the host shape immune development,

as demonstrated by the observation that segmented filamentous bacteria (SFB) promote development of Th17 cells in mice (Ivanov et al., 2009). Similarly, innate lymphoid cell (ILC) development is shaped by the microbiome (Sonnenberg and Artis, 2012), and ILCs accumulate in inflamed mucosal tissues (Bernink et al., 2013). Dietary factors also promote ILC development, as aryl hydrocarbon receptor (AHR) ligands induce lymphoid follicle development in the intestine by acting on ILCs (Kiss et al., 2011). Conversely, ILCs shape the composition of the microbiome by limiting dissemination of lymphoid resident commensals (Sonnenberg et al., 2012). These key observations begin to provide insight into the interactions between the host immune system, microbiome, and dietary metabolites (Fukuda et al., 2011).

In addition to stable host–commensal interactions, transient interactions between host and pathogens irrevocably change the immune system. Prior pathogen exposure elicits more robust responses to subsequent infection. While memory within the adaptive immune system provides antigen-specific protection upon rechallenge, the innate immune system can be "trained" by prior infection and poised to respond more robustly to a variety of pathogens (Cooper et al., 2009; O'Leary et al., 2006; Quintin et al., 2012; Sun et al., 2009). Thus, previous infection may condition the immune system to mount a pathologic response, which is not to suggest that IBD is caused by a particular pathogen. Rather, emerging evidence suggests a "multiple hit" model for conferring susceptibility to IBD (Cadwell et al., 2010). Similar to IBD patients bearing *ATG16L1* risk alleles, *Atg16l1* hypomorphic mice exhibit Paneth cell defects associated with abnormalities in granule packaging. Importantly, pathology associated with Paneth cell function depended on prior exposure to virus (Cadwell et al., 2010). Given the interconnection between host and microbes, the future challenge is to catalogue the metagenome and correlate host genotype to alterations in the microbiome. Still more challenging is the prospect of determining past history of pathogen infection in patients and identifying the immunologic consequences of prior infection. Notably, clues to prior pathogen exposure may be encoded in the specificity of gut IgA (Lindner et al., 2012).

Extrapolating Pathways from Human Genetic Data

To date, GWASs have identified 163 loci associated with IBD (Jostens et al., 2012). Using pathway analysis tools to examine genes within these loci immediately reveals patterns related to immunity. For example, cytokines and their respective receptors are abundantly represented. Upon closer examination, additional immunoregulatory pathways can be distilled and implicate genetic programs associated with Treg function, Th17 development, negative regulation of T cell receptor (TCR) signaling, innate pathogen sensing pathways, antigen presentation, and apoptosis (Figure A20-2). Although the function of caspases in eliciting apoptosis of T cells during activation-induced cell death is well

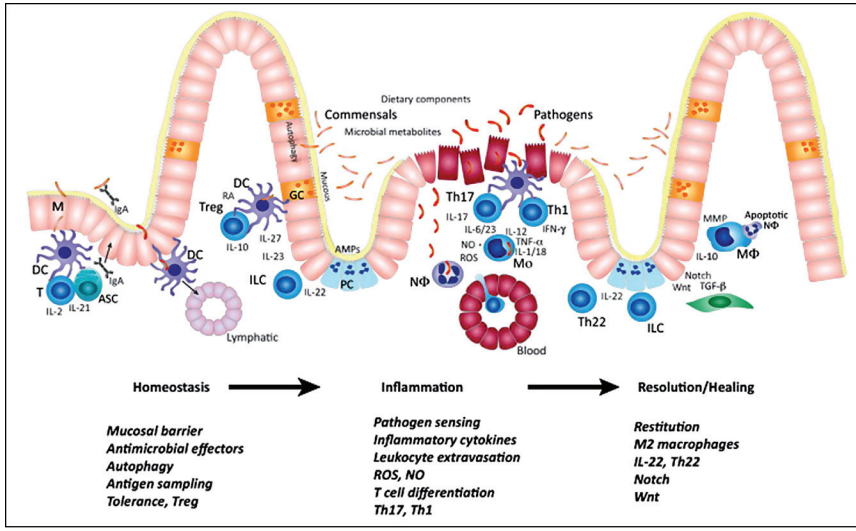


FIGURE A20-2 Inflammatory bowel disease (IBD) pathways and key cell types. Genetic studies have helped to identify critical biological processes and cell types that regulate intestinal inflammation. Genetic variation that perturbs any stage of immune homeostasis, inflammation, or resolution can result in the inappropriate inflammatory response to commensals that is observed in IBD. M cell (M), antibody secreting cells (ASC), dendritic cell (DC), retinoic acid (RA), T cell (T), T regulatory cell (Treg), innate lymphoid cell (ILC), goblet cell (GC), Paneth cell (PC), antimicrobial proteins (AMPs), neutrophil (Nϕ), inflammatory monocyte (Mo), macrophage (Mϕ), reactive oxygen species (ROS), nitric oxide (NO), and matrix metalloproteinase (MMP).

characterized, caspases have additional functions in inflammasome signaling and processing of IL-1 family cytokines. Given that dysregulated caspase activity and inflammasome function have been associated with IBD, study of additional caspase-dependent pathways and caspase substrates may reveal novel insights into IBD pathophysiology (Becker et al., 2013).

Gene annotation and functional associations have also highlighted key pathways that surpass the traditional boundaries of immunology and would not have been easily predicted by previous studies (Box A20-1). Paramount among these are cellular processes related to autophagy, redox signaling, and endoplasmic reticulum (ER) stress. Emerging evidence points to multiple pathway interactions; for example, ER stress and autophagy mutually regulate one another to maintain metabolic homeostasis during inflammation and to promote antimicrobial effector responses 45 and 46. In addition, macrophage exposure to bacterial lipopolysaccharide induces a metabolic switch to glycolysis resulting in accumulation of succinate that promotes IL-1β production by stabilizing hypoxia-inducible

BOX A20-1
Key Genes Implicated in IBD

Advancements in genomics have identified specific loci and rare coding variants associated with risk or protection from IBD. The genes identified by these studies highlight specific cellular pathways that may contribute to disease onset and/or progression (reviewed in Khor et al., 2011). Here, we highlight candidate IBD genes that implicate additional pathways that collectively suggest connections between cellular metabolism, inflammation, and mucosal microbial communities (Table A20-1).

TABLE A20-1 Candidate IBD Genes

Select IBD genes identified by ImmunoChip (Jostens et al., 2012)

Gene	Locus	Putative function
RNF186	1p36.13	Highly expressed in the intestine and contains a RING-type zinc finger that may function as a ubiquitin ligase. Association with IBD has been validated in several populations (Juyal et al., 2011; Yang et al., 2013). Evidence suggests genetic interaction with another IBD gene, HNF4A (Garrison et al., 2006).
SP110	2q37.1	Associated with primary immunodeficiency. Expressed in hematopoietic cells and contains a bromodomain with potential involvement in epigenetic regulation. Loss of function mutations can decrease IL-10 production by B cells (Bloch et al., 2012).
SP140	2q37.1	Expressed in hematopoietic cells and contains a bromodomain with potential involvement in epigenetic regulation.
MST1	3p21	Hepatocyte growth factor-like protein produced in the liver. Activates the receptor tyrosine kinase MST1R on epithelial cells (and some subsets of macrophages). Gain of function variants enhance macrophage motility (Hauser et al., 2012).
FUT2	19q13.3	Golgi protein expressed in gastrointestinal tract. Enzymatic activity generates a secreted oligosaccharide that functions as a substrate for synthesis of A and B blood group antigens. Loss of function mutations (nonsecretor phenotype) lack expression of blood group antigens in mucosal surfaces. Secretor status correlates with alterations in the microbiome (Rausch et al., 2011) and risk of IBD (Miyoshi et al., 2011) and T1D (Smyth et al., 2011).
SLC22A4	5q31.1	Ergothioneine transporter expressed in intestine and subsets of myeloid cells. May regulate cellular redox state, potentially linking metabolism with inflammatory responses (Kato et al., 2010).
GSDMB	17q12	May be involved in regulation of epithelial cell apoptosis (Saeki et al., 2009). It is also highly expressed in CD8 T cells.

continued

TABLE A20-1 Continued

Gene	Locus	Putative function
ORMDL3	17q12	Regulates the ER stress response associated with inflammation (McGovern et al., 2010).
TNFSF15	9q32	Expressed on endothelial cells and activated APCs. One of its receptors (TNFRSF25) promotes Treg expansion in a ligand-dependent manner (Khan et al., 2013).
TNFAIP3	6q23	Ubiquitin modifying enzyme expressed in myeloid cells. Negatively regulates NFkB signaling and inflammatory cytokines (Hammer et al., 2011).
SLC6A7	5q32	Proline transporter that may regulate cellular metabolic state and inflammation.
IL10RA	11q23	Receptor for IL-10 broadly expressed on hematopoietic cells. Transduces immunosuppressive signal through STAT3 and TYK2. Associated with early onset IBD (Moran et al., 2013).

Select IBD genes with coding variants identified by exome sequencing (Rivas et al., 2011)

Gene	Locus	Putative function
IL23R	1p31.3	Receptor for IL-23 expressed predominantly in T cells. Promotes differentiation of pathogenic Th17 cells (Ghoreschi et al., 2010).
CARD9	9q34.3	Expressed in myeloid cells where it promotes activation of NFkB and inflammatory cytokines downstream of pattern recognition receptors (PRRs) that are associated with immunoreceptor tyrosine-based activation motifs (ITAMs) or hemi-ITAMs (Hara et al., 2007). Promotes cytokine environment conducive to Th17 differentiation.
NOD2	16q21	Intracellular PRR specific for bacterial peptidoglycans and is expressed in myeloid cells. Activates NFkB and promotes inflammatory cytokines. Can induce bacterial killing in an autophagy-dependent manner (Homer et al., 2010).
IL18RAP	2q12	Accessory protein for IL-18 receptor expressed on NK and T cells. Promotes stimulatory effect of IL-18 on T cell IFN-γ^3 production (Cheung et al., 2005).
MUC19	12q12	Gel-forming mucin expressed in epithelial tissues. Potential role in barrier function and interaction with microbial communities.
CUL2	10p11.21	Component of E3 ubiquitin-protein ligase complex potentially linking proteosomal system with autophagy.
PTPN22	1p13.2	Protein tyrosine phosphatase that regulates T and B cell responses at the level of antigen receptor signaling (Rhee and Veillette, 2012).
C1orf106	1q32.1	Expressed in epithelial cells of the gastrointestinal tract. May promote epithelial integrity and barrier function.

factor 1 α subunit (HIF1-α) (Tannahill et al., 2013). Conversely, metabolism impinges upon the immune system by regulating tolerance. Tryptophan catabolism mediated by indoleamine 2,3-dioxygenase (IDO) generates kynurenine, which promotes IL-10 production by DCs and facilitates Treg differentiation, thus inhibiting Th17 responses (Nguyen et al., 2010). Th17 differentiation is also regulated by environmental factors such as NaCl derived from dietary sources. In this context, elevated levels of salt stimulate expression of serum/glucocorticoid regulated kinase (SGK1), which in turn induces expression of IL-23R to enhance Th17 differentiation (Wu et al., 2013). Further work elucidating Th17 cytokine networks has uncovered previously unrecognized connections between IL-17 and maintenance of epithelial barrier function (Reynolds et al., 2012), thus indicating an important role for the immune system in maintaining epithelial homeostasis and restitution. In particular, neutrophil influx into inflamed tissues initially amplifies inflammation, and later promotes an anti-inflammatory healing response through macrophage-dependent clearance of apoptotic neutrophils (Stark et al., 2005). In this context, mice deficient in the NADPH oxidase subunit P40phox exhibit an impairment in the healing response following acute colonic inflammation (Conway et al., 2012), thus revealing an additional role for reactive oxygen species (ROS) in epithelial restitution in addition to its known role as an antimicrobial agent. The importance of ROS in mucosal immunity is further supported by the discovery of rare variants of NCF4 and NCF2 that associate with IBD (Matute et al., 2009; Muise et al., 2012). These genetic studies implicating IBD genes in the ROS pathway suggest that neutrophils are key cell types in pathology, and further identify new functions for these cells that influence clinical manifestations of disease (Figure A20-3).

Pathway interactions identified by GWASs implicate several cell types in inflammatory pathology. By cross-referencing candidate disease genes and pathways with gene expression patterns in immune cell subsets, DCs and memory T cells feature prominently as central cell types contributing to IBD (Jostens et al., 2012). Consistent with this notion, Ly6C^{hi} monocytes in the inflamed colon generate inflammatory DCs and antigen presenting DCs that drive autoinflammatory T cell responses (Zigmond et al., 2012). Additional evidence implicates ILCs as key mediators of host defense and inflammatory pathology (Sonnenberg and Artis, 2012; Tait Wojno and Artis, 2012). Furthermore, expression of IBD genes identified by GWASs highlight the gut epithelium and innate immune mechanisms including barrier function, goblet cell secretion of mucins, and Paneth cell secretion of antimicrobial mediators (Bevins and Salzman, 2011). With several functional pathways implicated in IBD, the next challenge will be to place unannotated genes in pathways that drive disease and to elucidate regulatory networks.

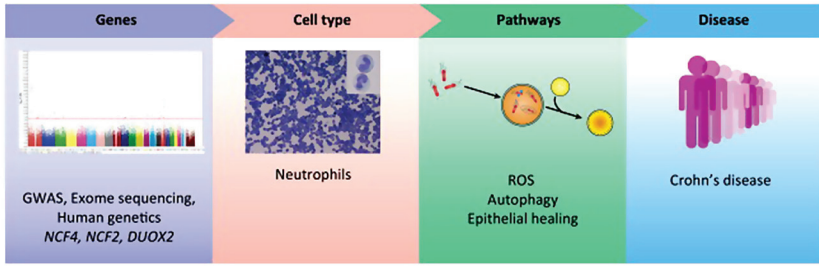


FIGURE A20-3 Molecular pathogenesis of inflammatory bowel disease (IBD): assembling the evidence. Identification of genes in the reactive oxygen species (ROS) pathway point to neutrophils as key cellular mediators of IBD, while functional studies implicated ROS in antibacterial defense, autophagy, and epithelial healing. Thus, genetics can be used to define a mechanism-based subset of IBD patients and guide treatment.

New Approaches in Assigning Function to Genes

Although many of the candidate IBD genes implicated by GWASs can be assembled into known pathways, a substantial fraction of these genes (>40%) are poorly characterized at the functional level. A significant challenge is to identify functions for candidate IBD genes and to determine how signaling pathways work together to regulate mucosal immune effector mechanisms. One can posit that candidate IBD genes with unknown function may control key immune functions that drive disease, and that assigning function to these genes will expand our understanding of immunoregulatory mechanisms. To meet the challenge of assigning functions to candidate IBD genes in relevant immune responses, high throughput RNAi screening approaches have been developed. Implementing RNAi screens has shown initial signs of success, but has yet to be systematically applied to assign IBD candidate genes with immunologic function. As a recent example, RNAi screening was used to identify mediators of innate pathogen recognition through the NOD2 complex. Here, screening approaches identified NOD2-dependent regulators of NFkB and further demonstrated a novel mechanism for the spatial assembly of the NOD2 signaling complex (Lipinski et al., 2012). In another recent study, genome-wide RNAi screening approaches identified 190 cofactors required for mediating endosomal pathogen sensing pathways mediated by TLR7 and TLR9 (Chiang et al., 2012).

Elucidating Mechanisms

Most functional genetic strategies aimed at assigning function to genes involve knockdown or overexpression of candidates. While this approach can establish the requirement, or define a regulatory role for a specific gene in a

defined biological process, it does not effectively capture how genetic variation in human populations impacts immune regulation. For example, coding variants may result in gain of function, loss of function, or exist on a functional continuum somewhere in between. These are important distinctions when attempting to infer disease mechanisms or design new therapeutics. By the same token, determining how genetic variation in risk alleles versus protective alleles impacts a cellular response requires mechanistic insight. The next challenge will be to develop reliable approaches to demonstrate the causal effect of a genetic variant on disease progression and to determine the underlying mechanism of action.

With accessibility of exome sequencing technology on the rise, genetic diversity can be quantified and increasing numbers of coding variants have been identified 60 and 61. It has been estimated that human genomes typically harbor up to 100 loss of function variants and approximately 20 genes that are nonfunctional (MacArthur et al., 2012). Due to limitations in the ability to predict which variants cause a given phenotype and which are benign, rigorous mechanistic studies must necessarily follow. IBD GWASs led to the identification of the autophagy gene *ATG16L1* and a putative loss of function coding variant thereof (T300A) (Hampe et al., 2007; Rioux et al., 2007). Knockdown of endogenous ATG16L1 in epithelial cells and overexpression of RNAi-resistant ATG16L1 T300A resulted in impaired antibacterial autophagy and formally demonstrated a role for this coding variant in a relevant biological readout (Kuballa et al., 2008). Although overexpression of coding variant cDNAs is ideally suited for high throughput analyses of many candidate mutations, overexpression can also mask subtle effects or exaggerate phenotypes. In gene replacement or exon replacement approaches, a coding variant is introduced into the endogenous locus so as to retain regulation at the level of chromatin remodeling, transcription, and splicing. Knockin mice have proven successful in this regard and can be cross-bred to introduce mutations at multiple loci. More recent innovations in genome engineering now enable efficient generation of isogenic human cells. Approaches that employ transcription activator like effectors (TALEs) allow for targeting endogenous genes to introduce coding variants (Bedell et al., 2012; Hockemeyer et al., 2011; Mouscou and Bogdanove, 2009; Reyon et al., 2012), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas) system has been recently adapted to target multiple genes simultaneously (Cong et al., 2013; Garneau et al., 2010; Mali et al., 2013).

Assessing Disease Relevance

In progressing from the identification of genetic loci by GWASs to defining functions for disease genes and variants, the next step towards determining the role for a candidate gene in disease pathology requires study in vivo. Here, multiple cell types and signaling pathways coordinately interact to mediate loss of immune tolerance and persistent inflammation. In this context, murine models have

earned a prominent position as a mainstay for determining if a candidate gene is necessary or sufficient for disease. Mouse models have been used to demonstrate that the ubiquitin modifying enzyme A20 limits inflammatory responses, because conditional deletion in DCs resulted in spontaneous colitis and spondyloarthritis (Hammer et al., 2011). Furthermore, the role of DCs in maintaining tolerance is highlighted by the observation that regulatory DCs pulsed with bacterial antigen can mitigate experimental colitis in mice (Yamanishi et al., 2012).

An emerging concept from mouse models and human genetic studies is that IBD cases are likely to be comprised of distinct subsets of mechanism-driven disorders (Virgin and Todd, 2011). In this context, systematic immunophenotyping of genotyped human cohorts will be required to define mechanism-based disease subsets. In disease-free individuals bearing a T1D risk allele at the *IL2R* locus, IL-2R expression was reduced and Treg function was impaired (Garg et al., 2012). Here, a rigorous approach with large sample sizes combined with rigorous quality control was implemented to address the effects of different genetic backgrounds. In addition, inclusion of healthy controls with disease risk genotypes can help to exclude complicating effects of chronic disease that may alter immune phenotypes.

Concluding Remarks

Human genetics has provided key insights into complex disease. Significant overlap in genes implicated across several autoimmune/autoinflammatory diseases indicates common immunologic mechanisms as well as unique disease-specific pathways that must be tightly regulated to balance host defense against the risk for pathological inflammation. Only with a deeper understanding of the mechanisms driving disease and their underlying genetic components will the goal of interpreting patient genotypes become feasible. Towards this end, the diagnostic power of genetics bears potential to stratify patient subsets based on disease mechanisms and treat them accordingly. Moreover, progress towards deciphering the genetic components of IBD pathophysiology will identify new points of entry for mechanism-based therapeutics. Although it remains a challenge to mitigate pathological inflammation without compromising host defense, advancements in genetics offer the opportunity to treat the underlying mechanisms that incite IBD rather than broadly suppressing immune function.

Acknowledgements

The authors are funded by the Center for the Study of Inflammatory Bowel Diseases (Massachusetts General Hospital), the Helmsley Trust, and NIH R01 DK092405.

References

Abecasis, G.R. et al. (2012) An integrated map of genetic variation from 1,092 human genomes. Nature 491, 56–65

Barreiro, L.B. et al. (2012) Deciphering the genetic architecture of variation in the immune response to Mycobacterium tuberculosis infection. Proc. Natl. Acad. Sci. U.S.A. 109, 1204–1209

Becker, C. et al. (2013) Complex roles of caspases in the pathogenesis of inflammatory bowel disease. Gastroenterology 144, 283–293

Bedell, V.M. et al. (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491, 114–118

Bernink, J.H. et al. (2013) Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat. Immunol. 14, 221–229

Bevins, C.L. and Salzman, N.H. (2011) Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat. Rev. Microbiol. 9, 356–368

Bloch, D.B. et al. (2012) Decreased IL-10 production by EBV-transformed B cells from patients with VODI: implications for the pathogenesis of Crohn disease. J. Allergy Clin. Immunol. 129, 1678– 1680

Briere, F. et al. (1994) Interleukin 10 induces B lymphocytes from IgA-deficient patients to secrete IgA. J. Clin. Invest. 94, 97–104

Butter, F. et al. (2012) Proteome-wide analysis of disease-associated SNPs that show allele-specific transcription factor binding. PLoS Genet. 8, e1002982

Cadwell, K. et al. (2010) Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 141, 1135–1145

Chang, J.S. et al. (2012) Endoplasmic reticulum stress response promotes cytotoxic phenotype of CD8alphabeta+ intraepithelial lymphocytes in a mouse model for Crohn's disease-like ileitis. J. Immunol. 189, 1510–1520

Cheung, H. et al. (2005) Accessory protein-like is essential for IL-18- mediated signaling. J. Immunol. 174, 5351–5357

Chiang, C.Y. et al. (2012) Cofactors required for TLR7- and TLR9- dependent innate immune responses. Cell Host Microbe 11, 306–318

Christodoulou, K. et al. (2012) Next generation exome sequencing of paediatric inflammatory bowel disease patients identifies rare and novel variants in candidate genes. Gut http://dx.doi.org/10.1136/ gutjnl-2011-301833

Cong, L. et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823

Conway, K.L. et al. (2012) p40phox expression regulates neutrophil recruitment and function during the resolution phase of intestinal inflammation. J. Immunol. 189, 3631–3640

Cooper, M.A. et al. (2009) Cytokine-induced memory-like natural killer cells. Proc. Natl. Acad. Sci. U.S.A. 106, 1915–1919

de Jong, V.M. et al. (2012) Post-transcriptional control of candidate risk genes for type 1 diabetes by rare genetic variants. Genes Immun. 14, 58–61

Derrien, T. et al. (2012) The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 22, 1775–1789

Ellinghaus, E. et al. (2010) Genome-wide association study identifies a psoriasis susceptibility locus at TRAF3IP2. Nat. Genet. 42, 991–995

Ernst, J. et al. (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473, 43–49

Fukuda, S. et al. (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547

Garg, G. et al. (2012) Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T cell function. J. Immunol. 188, 4644–4653

Garneau, J.E. et al. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468, 67–71

Garrison, W.D. et al. (2006) Hepatocyte nuclear factor 4alpha is essential for embryonic development of the mouse colon. Gastroenterology 130, 1207–1220

Gerold, K.D. et al. (2011) The soluble CTLA-4 splice variant protects from type 1 diabetes and potentiates regulatory T-cell function. Diabetes 60, 1955–1963

Ghoreschi, K. et al. (2010) Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. Nature 467, 967–971

Gomez, J.A. et al. (2013) The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-gamma locus. Cell 152, 743–754

Guttman, M. et al. (2009) Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458, 223–227

Guttman, M. et al. (2011) lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature 477, 295–300

Hammer, G.E. et al. (2011) Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. Nat. Immunol. 12, 1184–1193

Hampe, J. et al. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat. Genet. 39, 207–211

Hara, H. et al. (2007) The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. Nat. Immunol. 8, 619–629

Hasler, R. et al. (2012) Microbial pattern recognition causes distinct functional micro-RNA signatures in primary human monocytes. PLoS ONE 7, e31151

Hauser, F. et al. (2012) Macrophage-stimulating protein polymorphism rs3197999 is associated with a gain of function: implications for inflammatory bowel disease. Genes Immun. 13, 321–327

Heinig, M. et al. (2010) A trans-acting locus regulates an anti-viral expression network and type 1 diabetes risk. Nature 467, 460–464

Hockemeyer, D. et al. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat. Biotechnol. 29, 731–734

Homer, C.R. et al. (2010) ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. Gastroenterology 139, 1630–1641 1641.e1–2

Howson, J.M. et al. (2012) Evidence of gene-gene interaction and age-at-diagnosis effects in type 1 diabetes. Diabetes 61, 3012–3017

Huffmeier, U. et al. (2010) Common variants at TRAF3IP2 are associated with susceptibility to psoriatic arthritis and psoriasis. Nat. Genet. 42, 996–999

Ivanov, I.I. et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139, 485–498

Jostins, L. et al. (2012) Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 491, 119–124

Juyal, G. et al. (2011) An investigation of genome-wide studies reported susceptibility loci for ulcerative colitis shows limited replication in north Indians. PLoS ONE 6, e16565

Kato, Y. et al. (2010) Gene knockout and metabolome analysis of carnitine/organic cation transporter OCTN1. Pharm. Res. 27, 832–840

Khan, S.Q. et al. (2013) Cloning, expression, and functional characterization of TL1A-Ig. J. Immunol. 190, 1540–1550

Khor, B. et al. (2011) Genetics and pathogenesis of inflammatory bowel disease. Nature 474, 307–317

Kiss, E.A. et al. (2011) Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science 334, 1561–1565

Knight, J.C. (2012) Resolving the variable genome and epigenome in human disease. J. Intern. Med. 271, 379–391

Kuballa, P. et al. (2008) Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS ONE 3, e3391

Labbe, C. et al. (2008) MAST3: a novel IBD risk factor that modulates TLR4 signaling. Genes Immun. 9, 602–612

Labbe, C. et al. (2012) Genome-wide expression profiling implicates a MAST3-regulated gene set in colonic mucosal inflammation of ulcerative colitis patients. Inflamm. Bowel Dis. 18, 1072–1080

Lindner, C. et al. (2012) Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine. J. Exp. Med. 209, 365–377

Lipinski, S. et al. (2012) RNAi screening identifies mediators of NOD2 signaling: implications for spatial specificity of MDP recognition. Proc. Natl. Acad. Sci. U.S.A. 109, 21426–21431

MacArthur, D.G. et al. (2012) A systematic survey of loss-of-function variants in human protein-coding genes. Science 335, 823–828

Mali, P. et al. (2013) RNA-guided human genome engineering via Cas9. Science 339, 823–826

Matute, J.D. et al. (2009) A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. Blood 114, 3309–3315

Maurano, M.T. et al. (2012) Systematic localization of common disease-associated variation in regulatory DNA. Science 337, 1190–1195

McGovern, D.P. et al. (2010) Genome-wide association identifies multiple ulcerative colitis susceptibility loci. Nat. Genet. 42, 332–337

Miyoshi, J. et al. (2011) Ectopic expression of blood type antigens in inflamed mucosa with higher incidence of FUT2 secretor status in colonic Crohn's disease. J. Gastroenterol. 46, 1056–1063

Moran, C.J. et al. (2013) IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. Inflamm. Bowel Dis. 19, 115–123

Moran, I. et al. (2012) Human beta cell transcriptome analysis uncovers lncRNAs that are tissue-specific, dynamically regulated, and abnormally expressed in type 2 diabetes. Cell Metab. 16, 435–448

Moscou, M.J. and Bogdanove, A.J. (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326, 1501

Muise, A.M. et al. (2012) NADPH oxidase complex and IBD candidate gene studies: identification of a rare variant in NCF2 that results in reduced binding to RAC2. Gut 61, 1028–1035

Muto, A. et al. (2010) Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch. EMBO J. 29, 4048–4061

Ng, S.C. et al. (2012) Role of genetic and environmental factors in British twins with inflammatory bowel disease. Inflamm. Bowel Dis. 18, 725–736

Nguyen, N.T. et al. (2010) Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. Proc. Natl. Acad. Sci. U.S.A. 107, 19961–19966

O'Leary, J.G. et al. (2006) T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat. Immunol. 7, 507–516

Orozco, L.D. et al. (2012) Unraveling inflammatory responses using systems genetics and gene-environment interactions in macrophages. Cell 151, 658–670

Quintin, J. et al. (2012) Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe 12, 223–232

Rath, E. and Haller, D. (2012) Unfolded protein responses in the intestinal epithelium: sensors for the microbial and metabolic environment. J. Clin. Gastroenterol. 46 (Suppl.), S3–S5

Rausch, P. et al. (2011) Colonic mucosa-associated microbiota is influenced by an interaction of Crohn disease and FUT2 (Secretor) genotype. Proc. Natl. Acad. Sci. U.S.A. 108, 19030–19035

Reynolds, J.M. et al. (2012) Cutting edge: regulation of intestinal inflammation and barrier function by IL-17C. J. Immunol. 189, 4226–4230

Reyon, D. et al. (2012) FLASH assembly of TALENs for high-throughput genome editing. Nat. Biotechnol. 30, 460–465

Rhee, I. and Veillette, A. (2012) Protein tyrosine phosphatases in lymphocyte activation and autoimmunity. Nat. Immunol. 13, 439–447 Review

Rioux, J.D. et al. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat. Genet. 39, 596–604

Rivas, M.A. et al. (2011) Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat. Genet. 43, 1066–1073

Saeki, N. et al. (2009) Distinctive expression and function of four GSDM family genes (GSDMA-D) in normal and malignant upper gastrointestinal epithelium. Genes Chromosomes Cancer 48, 261–271

Sanjana, N.E. et al. (2012) A transcription activator-like effector toolbox for genome engineering. Nat. Protoc. 7, 171–192

Smyth, D.J. et al. (2011) FUT2 nonsecretor status links type 1 diabetes susceptibility and resistance to infection. Diabetes 60, 3081–3084

Sonnenberg, G.F. and Artis, D. (2012) Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. Immunity 37, 601–610

Sonnenberg, G.F. et al. (2012) Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science 336, 1321–1325

Stark, M.A. et al. (2005) Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity 22, 285–294

Strange, A. et al. (2010) A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-C and ERAP1. Nat. Genet. 42, 985–990

Sun, J.C. et al. (2009) Adaptive immune features of natural killer cells. Nature 457, 557–561

Tait Wojno, E.D. and Artis, D. (2012) Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine. Cell Host Microbe 12, 445–457

Tannahill, G.M. et al. (2013) Succinate is an inflammatory signal that induces IL-1b through HIF-1a. Nature 496, 238–242

Ueda, H. et al. (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 423, 506–511

Virgin, H.W. and Todd, J.A. (2011) Metagenomics and personalized medicine. Cell 147, 44–56

Wang, C. et al. (2013) The psoriasis-associated D10N variant of the adaptor Act1 with impaired regulation by the molecular chaperone hsp90. Nat. Immunol. 14, 72–81

Wu, C. et al. (2013) Induction of pathogenic T17 cells by inducible salt-sensing kinase SGK1. Nature http://dx.doi.org/10.1038/nature11984

Yamanishi, H. et al. (2012) Regulatory dendritic cells pulsed with carbonic anhydrase I protect mice from colitis induced by CD4+CD25- T cells. J. Immunol. 188, 2164–2172

Yang, S.K. et al. (2013) Genome-wide association study of ulcerative colitis in Koreans suggests extensive overlapping of genetic susceptibility with Caucasians. Inflamm. Bowel Dis. 19, 954–966

Zigmond, E. et al. (2012) Ly6C(hi) monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen- presenting cells. Immunity 37, 1076–1090

Zuk, O. et al. (2012) The mystery of missing heritability: genetic interactions create phantom heritability. Proc. Natl. Acad. Sci. U.S.A. 109, 1193–1198

Zwiers, A. et al. (2012) Cutting edge: a variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production. J. Immunol. 188, 1573–1577

A21

ANTIMICROBIAL PEPTIDES AND THE MICROBIOME

Michael ***Zasloff***[94]

Antimicrobial peptides are widely distributed throughout nature, present in all animals and plants. In general they are short (less than 50 amino acids), cationic, and amphipathic. Most can target a broad range of microbes, including bacteria, fungi, viruses, and protozoa. Because they generally act by disturbing membrane permeability, most are microbicidal and kill the target within seconds to minutes (Zasloff, 2002b).

Antimicrobial peptides have traditionally been considered components of the innate immune system that protect the "milieu interieur" from microbial invasion. Indeed, there is a large body of data that unequivocally supports this role for antimicrobial peptides. In humans, for example, areas of the body that we regard as normally "sterile," such as the urinary tract and the distal divisions of the airway, are kept free of microbes, in states of health, by the orchestrated elaboration of suites of antimicrobial peptides and proteins (Zasloff, 2002b, 2007). In the setting of wounds, we still believe that effective repair and healing requires the elimination of microbes within the wound environment, and antimicrobial peptides and proteins again play a role here (Lai and Gallo, 2009; Sorensen et al., 2003).

In the context of this meeting, the question arises as to whether antimicrobial peptides and proteins influence the commensal microbiome of humans, or function solely to prevent microbial invasion.

Lessons from a Frog

The African clawed frog, *Xenopus laevis*, is an aquatic creature. Its world as such is somewhat indeterminate with respect to the micro-organisms it can encounter. The skin of this amphibian, like that of other frogs, is invested with granular glands, neuroendocrine structures that synthesize at least a dozen antimicrobial peptides, along with proteins that create a hydrophobic gel on the skin surface when the gland discharges its contents. The wound is subsequently covered with a hydrophobic salve containing a cocktail of antimicrobial peptides at a concentration about 50–100 fold greater than required to kill all micro-organisms that might interfere with healing (Zasloff, 1987).

Yet, the skin of a healthy *Xenopus laevis*, like other amphibians, is populated by microbes, including organisms that cause lethal systemic infections in this animal (Culp, 2007). *Aeromonas hydrophila*, for instance, causes "red-leg," a devastating hemorrhagic septic infection. Surprisingly, *A. hydrophila* is relatively

[94] MedStar Georgetown Transplant Institute, Georgetown University, Washington, DC.

resistant to the action of the skin's antimicrobial peptides (Rollins-Smith et al., 2002), as are several of the other gram-negative bacteria present on the skin that are also associated with red leg, including Morganella and Serratia species (Zasloff, 1987). The population of its skin by organisms that are relatively resistant to the action of the skin peptides would seem to be a straightforward consequence of the selective pressure imposed by the antimicrobial arsenal. It is likely that continuous low level discharge of the widely distributed granular glands maintains a relatively restricted diversity of resistant bacteria on its skin. In addition, several of the amphibian skin commensals are known to themselves secrete antimicrobial agents that must act to further reduce species diversity (Harris, 2006).

Invasion by these organisms resulting in disease occurs with low probability, generally believed to occur when homeostasis is disturbed ("stress"), possibly resulting in a breakdown of the full defensive capacity of the skin. Thus a balance of sort has evolved in the frog between itself and its selected commensals: organisms that comprise the skin microbiome can survive low-level assault by the antimicrobial peptides released onto the surface, but which, should these defenses fail, can nonetheless cause disease.

That these granular glands are positioned to deal with commensal microbes can be inferred from a fascinating simple experiment. If the granular glands of a frog are fully discharged by an appropriate dose of noradrenaline (or an electrical discharge), and the animal is placed into a tank of sterile water containing broad-spectrum antibiotics, antimicrobial peptides will not reappear within the granular glands. In contrast, if the animals are returned to a tank containing its normal commensal microbes the glands fully regenerate within days (Mangoni et al., 2001).

Human Epidermis and Its Microbial Inhabitants

The micro-organisms that populate human skin, with whom we have co-evolved, also exhibit evidence of the pressure exerted on their survival by antimicrobial peptides. The best example is seen in the case *Staphylococcus aureus*. Human epidermis is invested with an array of cationic antimicrobial peptides and proteins, most of which are transcribed initially by the more basal keratinocyte layers (Gallo et al., 2011; Schroder and Harder, 2006). Some are secreted constitutively, like HBD1, while others are expressed after injury or infection, such as LL-37, human defensins 2 and 3. These peptides, like most cationic antimicrobial peptides, target the cytoplasmic membrane of microbes through electrostatic attraction, since the bacteria display negatively charged phospholipids on the outer leaflet of their cytoplasmic membrane (Zasloff, 2002b). Once bound to the membrane, antimicrobial peptides cause damage (by a variety of mechanism) and generally kill the microbe. In response, the modern strains *S. aureus* appear to have developed a means of enzymatically reducing the net negative surface charge of its cytoplasmic membrane by coupling phosphatidyl glycerol with lysine (Andra

et al., 2011). Presumably the existing strains of *S. aureus* have developed a degree of resistance to cationic antimicrobial peptides that permits them to survive on the skin, but not sufficient to normally resist the action of defensins that are present within the neutrophils and other phagocytic cells, or induced in the skin in high concentration on injury. Thus a détente of sorts exists between host and microbe. *Organisms within our microbiome that exist in proximity to sites of secretion of antimicrobial proteins and peptides have evolved mechanisms of relative resistance to their action.*

Human Diseases Where Failed Antimicrobial Defenses Lead to an Altered Microbiome

Atopic Dermatitis: A Failure to Contain the Growth of S. aureus

Several of these antimicrobial proteins keep certain organisms at a very low relative abundance on the skin surface. Psoriasin and RNAse 7 are two very abundant proteins that are constitutively secreted onto the skin (Glaser et al., 2011; Koten et al., 2009). Both of these antimicrobial agents were first discovered in a survey of antimicrobial substances present in the isolated skin scales from individuals suffering from psoriasis. Christophers, an astute clinician, had noted that although psoriatic lesions are inflamed and physically defective "barriers," they rarely suffer bacterial infection (Glaser et al., 2005). Schroder and colleagues, following up on this clinical clue, surmised that the skin of the psoriatic might compensate for the barrier defect by over expressing antimicrobial peptides and proteins (Glaser et al., 2005; Harder and Schroder, 2005). Indeed, this is the case, now realized to be a consequence of the intense expression of IL-17 by lymphocytes within the dermis (Martin et al., 2013). It was from psoriatic scales that several human antimicrobial peptides were first purified and identified (Harder and Schroder, 2005).

Psoriasin is active against *E. coli*, while RNase 7 is most active against *E. faecalis*. If either organism is applied to the surface of unwashed human adult skin, within several minutes these bacteria die. If an antibody that inactivates either of the antimicrobial proteins is applied to the skin prior to application of bacteria, the corresponding microbes remain viable on the skin (Glaser et al., 2005; Koten et al., 2009). These studies teach us that the microbiome on the skin surface is constrained on the undamaged skin of a "healthy" human by the presence of certain antimicrobial agents. In addition, we should appreciate that excessive washing of the skin with strong detergents will remove the antimicrobial shield and possibly permit the establishment of an alternate microbiome.

Following injury the orchestration of batteries of antimicrobial peptides is initiated, releasing molecules not normally seen on healthy skin. It is not surprising that the skin microbiome of the injury site changes in the setting of acute injury (Zeeuwen et al., 2012).

One of the common complications of atopic dermatitis is infection of the skin lesions by *S. aureus*. The damaged epidermis is unable to restrain the invasion of this microbe due to a failure of expression of antimicrobial peptides and proteins such as LL-37 (Ong et al., 2002; Zasloff, 2002a). The precise mechanism responsible for the depressed expression of epidermal antimicrobial defenses is not entirely understood, although several of the cytokines expressed by lymphocytes within the dermis appear to play a role (Ong et al., 2002).

Cystic Fibrosis: A Failure to Prevent a Microbiome from Establishing Itself in the Airway

There are areas of the body that are generally regarded as "sterile," such as the bronchi and more distal branches of the airway. Few, if any microorganisms can normally be seen microscopically in fluids sampled from a healthy human airway.

The epithelial lining of the normal human airway distal to the trachea is covered by a micron-thick fluid layer secreted by the underlying cells. The height of the fluid layer, its ionic composition, and pH are maintained by the action of epithelial ion and water channels. Into to this fluid layer the epithelial cells secrete antimicrobial proteins and peptides, such as lysozyme, lipocalin, lactoferrin, LL-37, and human beta defensins. The cocktail of antimicrobial peptides and proteins present within the airway surface fluid layer creates a protective barrier that has the capacity to rapidly kill most microbes that are inhaled. *In this anatomical compartment of humans, antimicrobial proteins and peptides actively suppress the establishment of a microbiome.*

In cystic fibrosis, however, the airway becomes populated by a dense microbiome that chronically colonizes the bronchial tree during the life of the affected individual. Organisms such as *Ps. aeruginosa*, *S. aureus*, and *Burkholderia cepacia* can together reach densities of 10^{10} cells/gram of sputum despite chronic intensive antibiotic therapy. In CF the antimicrobial activity of the surface fluid layer is depressed (Goldman et al., 1997; Smith et al., 1996) and rather than restrict the growth of inhaled bacteria provides a growth medium. The inflammation that occurs within the airway of the individual with CF can be explained as being a secondary response to the presence of bacteria in the airway. The influx of neutrophils that characterizes the inflammatory response represents an attempt by the immune system to defend the airway from the CF microbiome, a futile response that ultimately destroys the physical structure of the bronchi. *In CF, a pathological microbiome establishes itself in the airway.*

The etiology of the chronic infections in CF has been elucidated through the study of a genetically engineered pig in which the porcine CFTR has been replaced with a common human CFTR mutation (Ostedgaard et al., 2011). These animals will develop pulmonary inflammation and bronchial infection within several months of life (Ostedgaard et al., 2011). Longitudinal study of these animals from birth reveals that they have a defect in the capacity of their airway

surface fluid to kill bacteria. The cause of this defect appears to be a failure of the epithelium to maintain the normal pH of the airway fluid, permitting it to become excessively acidic (Pezzulo et al., 2012), an effect of a perturbation caused by the defective CFTR. By simply making a sample of airway fluid more alkaline, antimicrobial activity is restored. *Antimicrobial peptides and proteins normally prevent a microbiome from establishing itself in the airway.*

Crohn's Disease: Unable to Keep the Microbiome at a Distance

The human gastrointestinal tract is home to a complex microbiome, which differs in density and diversity throughout the various regions. An area of great interest and considerable investigation are the mechanisms that exist that permit us to contain great numbers of microbes within an organ, such as the ileum or colon that is lined by a single celled layer, and yet normally appears relatively free of inflammation.

Like the airway, the surface of the epithelium of the small and large intestine is covered by a thin layer of fluid, secreted from the underlying epithelial cells. This layer is itself covered by a layer of mucous, secreted by goblet cells. Microbes present in the lumen of the intestine, were they to attempt to invade the epithelial layer, would first come in contact with the mucous layer, and then as they penetrated deeper, would enter the fluid layer. As in the airway, antimicrobial agents are secreted into the fluid layer. Some of these, such as beta-defensins, are the products of the common enterocyte. In the small intestine, specialized Paneth cells that lie at the base of the crypts, secrete high concentrations of a cocktail of antimicrobial peptides and proteins that flood the overlying surface fluid layer. As a consequence of the mucous barrier, the bactericidal submucous fluid layer, and the rapid regeneration of the epithelial layer, bacteria generally cannot gain a foothold on the epithelial surface, nor invade the layer and enter the lamina propria. Although the GI tract harbors a complex microbiome, the organisms are normally kept at bay from the epithelium through the action of these defenses (Salzman et al., 2007). *Thus in the human intestine, antimicrobial peptides and proteins create an antimicrobial shield that permits containment of the intestinal microbiome.*

In Crohn's disease the normal barrier defenses of the small intestine fail. Commensal organisms are no longer spatially restricted, and can access the epithelium and the lamina propria of the intestine (Swidsinski et al., 2005). This occurs in part through a failure of the antimicrobial defenses of the Paneth cell. Normally abundant antimicrobial peptides, such as human defensin 5, are present in reduced amounts, resulting in the reduced antibacterial strength of the antimicrobial barrier (Wehkamp et al., 2005a). As in cystic fibrosis, the failure of antimicrobial defense of the barrier results in microbes gaining access to the epithelium, subsequent invasion, and secondary inflammation (Wehkamp et al., 2005b).

The secretion of high local concentrations of antimicrobial peptides by the Paneth cells likely influences the diversity of bacteria that find themselves in

contact with the mucous layer, rather than the planktonic microbes that live within the intestinal lumen, where the concentrations of antimicrobial peptides would be too low to exert antimicrobial activity. In mice engineered to express human defensin 5 in the small intestine, the bacteria that populate the mucous layer of the small intestine differ from those seen in the wild-type animals, reflecting the selective pressure imposed by the human antimicrobial peptide (Salzman et al., 2003). In individuals with Crohn's disease genetic polymorphisms that influence levels of Paneth cell antimicrobial peptide expression appear to be associated with differences in the commensal microbiome of the ileum (Zhang et al., 2012). *It is likely that as in the skin, the antimicrobial agents secreted from the intestinal wall influence the diversity of the organisms that populate the immediate luminal surface and ultimately the inflammatory state of the intestine.*

The ability of humans to coexist with environmental microbes and to support a diverse microbiome is in part a consequence of the existence of antimicrobial peptides and proteins. The antimicrobial barrier is generally clinically "silent" in states of health, creating a chemical barrier without the need for a degree of inflammation that we recognize clinically by the classic signs of "redness, heat, and swelling." At the same time, these substances exert selective pressure on the organisms that comprise our microbiome, influencing microbial ecology. Many questions regarding the antimicrobial barrier still remain poorly explored and likely would provide insights into a deeper understanding of our microbiome. For example: Does the antimicrobial barrier change with age, acquired illness, or nutritional status? Are there significant genetic differences in the strength of the barrier between individuals? What can we do to strengthen this barrier, and what practices should we avoid? Hopefully these and other questions will be answered in the future.

References

Andra, J., T. Goldmann, C. M. Ernst, A. Peschel, and T. Gutsmann. 2011. Multiple peptide resistance factor (MPRF)-mediated resistance of *Staphylococcus aureus* against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. *Journal of Biological Chemistry* 286(21):18692-18700.

Culp, C. E., Falkinham, J.O., Belden, L.K. 2007. Identification of thf natural bactfrial microflora on the skin of eastern newts, bullfrog tadpoles and redback salamanders. *Herpetologia* 63:66-71.

Gallo, R. L., M. Kulesz-Martin, and J. R. Bickenbach. 2011. Montagna symposium 2010: Small molecules: Skin as the first line of defense. *Journal of Investigative Dermatology* 131(11):2166-2168.

Glaser, R., J. Harder, H. Lange, J. Bartels, E. Christophers, and J. M. Schroder. 2005. Antimicrobial psoriasin (s100a7) protects human skin from *Escherichia coli* infection. *Nature Immunology* 6(1):57-64.

Glaser, R., B. Koten, M. Wittersheim, and J. Harder. 2011. Psoriasin: Key molecule of the cutaneous barrier? *Journal der Deutschen Dermatologischen Gesellschaft* 9(11):897-902.

Goldman, M. J., G. M. Anderson, E. D. Stolzenberg, U. P. Kari, M. Zasloff, and J. M. Wilson. 1997. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88(4):553-560.

Harder, J., and J. M. Schroder. 2005. Psoriatic scales: A promising source for the isolation of human skin-derived antimicrobial proteins. *Journal of Leukocyte Biology* 77(4):476-486.

Harris, R. N., James, T.Y., Lauer, A., Simon, M.A., and Patel, A. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3:53-56.

Koten, B., M. Simanski, R. Glaser, R. Podschun, J. M. Schroder, and J. Harder. 2009. Rnase 7 contributes to the cutaneous defense against *Enterococcus faecium*. *PloS One* 4(7):e6424.

Lai, Y., and R. L. Gallo. 2009. Amped up immunity: How antimicrobial peptides have multiple roles in immune defense. *Trends in Immunology* 30(3):131-141.

Mangoni, M. L., R. Miele, T. G. Renda, D. Barra, and M. Simmaco. 2001. The synthesis of antimicrobial peptides in the skin of rana esculenta is stimulated by microorganisms. *FASEB Journal* 15(8):1431-1432.

Martin, D. A., J. E. Towne, G. Kricorian, P. Klekotka, J. E. Gudjonsson, J. G. Krueger, and C. B. Russell. 2013. The emerging role of il-17 in the pathogenesis of psoriasis: Preclinical and clinical findings. *Journal of Investigative Dermatology* 133(1):17-26.

Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *New England Journal of Medicine* 347(15):1151-1160.

Ostedgaard, L. S., D. K. Meyerholz, J. H. Chen, A. A. Pezzulo, P. H. Karp, T. Rokhlina, S. E. Ernst, R. A. Hanfland, L. R. Reznikov, P. S. Ludwig, M. P. Rogan, G. J. Davis, C. L. Dohrn, C. Wohlford-Lenane, P. J. Taft, M. V. Rector, E. Hornick, B. S. Nassar, M. Samuel, Y. Zhang, S. S. Richter, A. Uc, J. Shilyansky, R. S. Prather, P. B. McCray, Jr., J. Zabner, M. J. Welsh, and D. A. Stoltz. 2011. The deltaf508 mutation causes CFTR misprocessing and cystic fibrosis-like disease in pigs. *Science Translational Medicine* 3(74):74ra24.

Pezzulo, A. A., X. X. Tang, M. J. Hoegger, M. H. Alaiwa, S. Ramachandran, T. O. Moninger, P. H. Karp, C. L. Wohlford-Lenane, H. P. Haagsman, M. van Eijk, B. Banfi, A. R. Horswill, D. A. Stoltz, P. B. McCray, Jr., M. J. Welsh, and J. Zabner. 2012. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 487(7405):109-113.

Rollins-Smith, L. A., J. K. Doersam, J. E. Longcore, S. K. Taylor, J. C. Shamblin, C. Carey, and M. A. Zasloff. 2002. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Developmental & Comparative Immunology* 26(1):63-72.

Salzman, N. H., D. Ghosh, K. M. Huttner, Y. Paterson, and C. L. Bevins. 2003. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 422(6931):522-526.

Salzman, N. H., M. A. Underwood, and C. L. Bevins. 2007. Paneth cells, defensins, and the commensal microbiota: A hypothesis on intimate interplay at the intestinal mucosa. *Seminars in Immunology* 19(2):70-83.

Schroder, J. M., and J. Harder. 2006. Antimicrobial skin peptides and proteins. *Cellular and Molecular Life Sciences* 63(4):469-486.

Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85(2):229-236.

Sorensen, O. E., J. B. Cowland, K. Theilgaard-Monch, L. Liu, T. Ganz, and N. Borregaard. 2003. Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *Journal of Immunology* 170(11):5583-5589.

Swidsinski, A., J. Weber, V. Loening-Baucke, L. P. Hale, and H. Lochs. 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *Journal of Clinical Microbiology* 43(7):3380-3389.

Wehkamp, J., N. H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R. E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R. W. Feathers, H. Chu, H. Lima, Jr., K. Fellermann, T. Ganz, E. F. Stange, and C. L. Bevins. 2005a. Reduced paneth cell alpha-defensins in ileal Crohn's disease. *Proceedings of the National Academy of Sciences of the United States of America* 102(50):18129-18134.

Wehkamp, J., M. Schmid, K. Fellermann, and E. F. Stange. 2005b. Defensin deficiency, intestinal microbes, and the clinical phenotypes of Crohn's disease. *Journal of Leukocyte Biology* 77(4):460-465.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from xenopus skin: Isolation, characterization of two active forms, and partial CDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America* 84(15):5449-5453.

Zasloff, M. 2002a. Antimicrobial peptides in health and disease. *New England Journal of Medicine* 347(15):1199-1200.

Zasloff, M. 2002b. Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389-395.

Zasloff, M. 2007. Antimicrobial peptides, innate immunity, and the normally sterile urinary tract. *Journal of the American Society of Nephrology* 18(11):2810-2816.

Zeeuwen, P. L. J. M., J. Boekhorst, E. H. van den Bogaard, H. D. de Koning, P. M. C. van de Kerkhof, D. M. Saulnier, I. I. van Swam, S. A. F. T. van Hijum, M. Kleerebezem, J. Schalkwijk, and H. M. Timmerman. 2012. Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biology* 13(11):R101.

Zhang, T., R. A. DeSimone, X. Jiao, F. J. Rohlf, W. Zhu, Q. Q. Gong, S. R. Hunt, T. Dassopoulos, R. D. Newberry, E. Sodergren, G. Weinstock, C. E. Robertson, D. N. Frank, and E. Li. 2012. Host genes related to paneth cells and xenobiotic metabolism are associated with shifts in human ileum-associated microbial composition. *PloS One* 7(6):e30044.

Appendix B

Agenda

Microbial Ecology in States of Health and Disease

March 18–19, 2013
500 Fifth Street, NW
Washington DC

DAY ONE: MONDAY, MARCH 18, 2013

7:30–8:00: Registration and Continental Breakfast

8:00–8:15: Welcoming Remarks: David Relman, James Hughes, and Lonnie King

8:15–9:00: **KEYNOTE:** Indigenous microbes and the ecology of chronic diseases
Martin Blaser, New York University

9:00–9:15: Discussion

9:15–9:30: BREAK

SESSION I: HOST–MICROBE INTERACTIONS IN HUMANS, ANIMALS, AND PLANTS

Moderator: Rima Khabbaz, Centers for Disease Control and Prevention

9:30–10:00: Plant–microbe interactions in root endophyte and rhizosphere communities of *Populus*
Gerald Tuskan, Oak Ridge National Laboratory

10:00–10:30: Origins and maintenance of host–microbe interactions
Angela Douglas, Cornell University

10:30–11:00: Annual cycles of extreme dietary change shape gut microbiota and their hibernator hosts
Hannah Carey, University of Wisconsin, Madison

11:00–11:30: Symbiont microbiota and the development and maturation of the mammalian immune system
Gérard Eberl, Pasteur Institute

11:30–12:00: Discussion

12:00–1:00: LUNCH

SESSION II: EMERGING INSIGHTS INTO HOST–MICROBE INTERACTIONS

Moderator: David Relman, Stanford University

1:00–1:30: Community ecology and the human vaginal microbiome
Larry Forney, University of Idaho

1:30–2:00: Host defense and immunomodulation of mucosal candidiasis
Paul Fidel, Jr., Louisiana State University

2:00–2:30: Interactions between the mammalian virome, disease susceptibility genes, and the phenome
Herbert "Skip" Virgin, Washington University

2:30–3:00: BREAK

3:00–3:30: Microbial community dynamics and disruption—Health and disease in coral reefs and the human lung
Forest Rohwer, San Diego State University

3:30–4:00: Interactions and functions of the gut microbiota in a model vertebrate host
Karen Guillemin, University of Oregon

4:00–4:30: The influence of the mucosal immune system and gut microbiota on inflammatory bowel diseases (IBDs)
Richard Blumberg, Harvard Medical School

4:30–5:30: Discussion

5:30–6:00: Concluding Remarks

6:00: ADJOURN DAY ONE

DAY TWO: TUESDAY, MARCH 19, 2013

8:00–8:30: Registration and Continental Breakfast

8:30–8:45: Welcoming Remarks and Summary of Day One: David Relman

SESSION III: STRUCTURE AND FUNCTION OF HOST-ASSOCIATED MICROBIAL COMMUNITIES

Moderator: Queta Bond, Burroughs Wellcome Fund

8:45–9:15: The application of ecological concepts to host-associated microbial communities
Brendan Bohannan, University of Oregon

9:15–9:45: Nutrition and the infant gut microbiota—Health and disease in the first 1,000 days
David Mills, University of California, Davis

9:45–10:15: Roles of the microbiota in the control and pathogenesis of infections
Yasmine Belkaid, National Institute of Allergy and Infectious Diseases

10:15–10:45: BREAK

10:45–11:15: Host–microbe interactions and the genetic architecture of IBD and other complex diseases
Ramnik Xavier, Massachusetts General Hospital

11:15–11:45: Host–mycobiome interactions in gut homeostasis and pathogenesis
David Underhill, Cedars-Sinai Medical Center

11:45–12:15: Discussion

12:15–1:15: LUNCH

SESSION IV: RESEARCH CHALLENGES AND OPPORTUNITIES

Moderator: Jesse Goodman, Food and Drug Administration

1:15–1:45: Metabolism, interspecies interactions, and novel approaches to disease diagnostics and treatment
Michael Fischbach, University of California, San Francisco

1:45–2:15: Fecal transplantation as a treatment option for recurrent *Clostridium difficile* infection
Josbert J. Keller, University of Amsterdam

2:15–2:45: BREAK

2:45–3:15: Innate antimicrobial mechanisms in disease prevention and treatment
Michael Zasloff, Georgetown University

3:15–3:45: The skin microbiome and chronic disease states
Julie Segre, National Human Genome Research Institute

3:45–4:30: Discussion

4:30–4:45: Concluding Remarks

4:45: ADJOURN

Appendix C

Acronyms

AIDS	acquired immune deficiency syndrome
AMD	acid mine drainage
AMP	antimicrobial peptide
ATP	adenosine triphosphate
BAM	bacteriophage adherence to mucus
CA-MRSA	community-acquired methicillin-resistant *S. aureus*
CBER	Center for Biologics Evaluation and Research
CD	Crohn's disease
CDC	U.S. Centers for Disease Control and Prevention
DOE	U.S. Department of Energy
DIO	diet-induced obesity
DNA	deoxyribonucleic acid
FDA	U.S. Food and Drug Administration
FMT	fecal microbiota transplantation
GE	gastroesophageal
GEJ	gastroesophageal junction
GI	gastrointestinal
GIO	gene-induced obesity
HIV	human immunodeficiency virus

HMO	human milk oligosaccharides
HMP	Human Microbiome Project
IBD	inflammatory bowel disease
iNKT	invariant natural killer T
IOM	Institute of Medicine
MAMP	microbe-associated molecular pattern
MIO	microbe-induced obesity
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *S. aureus*
NASH	nonalcoholic steatohepatitis
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NO	nitric oxide
NSF	National Science Foundation
OPC	oropharyngeal candidiasis
OTU	operational taxonomic unit
PAMP	pathogen-associated molecular pattern
PAT	pulsed antibiotic treatment
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
POP	pattern of pathogenesis
PRR	pattern-recognition receptor
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RYGB	Roux-en-Y gastric bypass
SCFA	short-chain fatty acid
SIV	simian immunodeficiency virus
STAT	subtherapeutic antibiotic treatment
TMAO	trimethylamine N-oxide
VVC	vulvovaginal candidiasis

Appendix D

Glossary

16S ribosomal RNA (rRNA): A component of the small subunit of prokaryotic ribosomes. Significant insights into species richness, structure, composition, and membership of microbial communities have been gained through analysis of small-subunit rRNA gene sequences; these sequences contain hypervariable regions that can provide species-specific signature sequences. PCR amplification with primers that hybridize to highly conserved regions in bacterial or archaeal 16S rRNA genes (or eukaryotic microbial 18S rRNA genes) followed by cloning and sequencing yields an initial description of species present in a microbial community.

Acquired immune deficiency syndrome: An infectious disease caused by the human immunodeficiency virus (HIV). There are two variants of the HIV virus, HIV-1 and HIV-2, both of which ultimately cause AIDS.

Antibiotic: Class of substances that can kill or inhibit the growth of some groups of microorganisms. Originally antibiotics were derived from natural sources (e.g., penicillin from molds), but many currently used antibiotics are semisynthetic and modified with additions of man-made chemical components. *See Antimicrobial.*

Antimicrobial: In this document, the term *antimicrobial* is used inclusively to refer to any agent (including an antibiotic) used to kill or inhibit the growth of microorganisms (bacteria, viruses, fungi, or parasites). This term applies whether the agent is intended for human, veterinary, or agricultural applications.

Antimicrobial resistance: Most commonly, this refers to infectious microbes that have acquired the ability to survive exposures to clinically relevant concentrations of antimicrobial drugs that would kill otherwise sensitive organisms of the same strain. The phrase is also used to describe any pathogen that is less susceptible than its counterparts to a specific antimicrobial compound (or combination thereof).

Archaea: Any of various single-celled prokaryotes genetically distinct from bacteria, often thriving in extreme environments.

ATP: Short for adenosine triphosphate, an organic compound that serves as a source of energy for many metabolic processes.

Bacteria: Microscopic, single-celled organisms that have some biochemical and structural features different from those of animal and plant cells.

Biogeography: The study of biodiversity in space and time.

***Clostridium difficile*:** A bacterium that can cause symptoms ranging from diarrhea to life-threatening inflammation of the colon.

Commensal relationship: An intimate, although generally benign, relationship between a resident microorganism and its host—probably the product of a long evolutionary interplay between the microorganism and the host. The relationship need not be symbiotic. *See Commensalism*.

Commensalism: Two (or more) species coexist, one deriving benefit from the relationship without harm or obvious benefit to the other.

Crohn's disease: A type of inflammatory bowel disease (IBD), resulting in swelling and dysfunction of the intestinal tract, especially the small intestine.

Disease: In medicine, disease is often viewed as an observable change of the normal network structure of a system resulting in damage to the system.

Diversity: A measure of how much variety is present in a community, irrespective of the identities of the organisms present; consists of richness and evenness.

DNA (deoxyribonucleic acid): Any of various nucleic acids that are usually the molecular basis of heredity are constructed of a double helix held together by hydrogen bonds between purine and pyrimidine bases that project inward from two chains containing alternate links of deoxyribose and phosphate and that in eukaryotes are localized chiefly in cell nuclei.

DNA sequencing: Determining the order of nucleotides in DNA.

Dysbiosis: Most commonly refers to a disruption in the normal homeostatic and beneficial relationship between microbes and their host, including disruptions in microbial community structure and function. Alterations in microbial community structure, involving Bacteria, Archaea, and/or Eukarya, can occur in any body habitat but have been best described in the gut where they have been associated with a number of disease states including, for example, inflammatory bowel disease.

Ecology: The scientific study of the relationship between living things and their environments.

Emerging infectious diseases: Infections that are rapidly increasing in incidence or geographic range.

***Escherichia coli*:** A straight rod-shaped gram-negative bacterium that is used in public health as an indicator of fecal pollution (as of water or food) and in medicine and genetics as a research organism and that occurs in various strains that may live as harmless inhabitants of the human lower intestine or may produce a toxin causing intestinal illness.

Eukaryotic: One of the three domains of life. The two other domains, Bacteria and Archaea, are prokaryotes and lack several features characteristic of eukaryotes (e.g., cells containing a nucleus surrounded by a membrane and whose DNA is bound together by proteins [histones] into chromosomes). Animals, plants, and fungi are all eukaryotic organisms.

Evenness: The distribution of individuals across types.

Fecal microbiota transplantation: An emerging therapy for the treatment of *Clostridium difficile* colitis. Also known as a stool transplant, it is the process of transplanting fecal bacteria from a healthy individual into a recipient patient suffering from a *Clostridium difficile* infection.

Function: An activity or "behavior" associated with a community, e.g., nitrogen fixation or resistance to invasion.

Gene transfer: The transfer of individual genes or their components, islands of genes, entire organisms, or communities of organisms from parent to offspring (vertical transfer) or between individuals not in a direct lineage (horizontal transfer).

Genome: The complete set of genetic information in an organism. In bacteria, this includes the chromosome(s) and plasmids (extra-chromosomal DNA molecules that can replicate autonomously within a bacterial cell).

Genomics: The study of genes and their associated functions.

Glycan: A polysaccharide or oligosaccharide, especially one that is attached to a glucoconjugate, as a glycoprotein, glycolipid, and proteoglycan.

***Helicobacter pylori*:** A species of spiral or straight gram-negative bacteria with multiple sheathed flagella found in the gastric mucosa of humans and other animals and associated with gastric and peptic ulcers as well as gastric cancers.

Host: Animal or plant that harbors or nourishes another organism.

Host defense: The protection an organism is afforded against infections. Types 1. Nonimmunologic—e.g., mucocutaneous or integumental barriers, cilia, microvilli; mechanical—e.g., urinary outflow, vascular perfusion of tissues; native flora, which outcompete pathogens; 2. Immunologic—e.g., chemotaxis, phagocytosis, immunoglobulins, complement, T cell defense.

Host–pathogen interactions: The interactions taking place between a pathogen and its host. By definition, all pathogens damage their host to some extent.

Human Genome Project: An international scientific research project with a primary goal of determining the sequence of chemical base pairs that make up DNA, and of identifying and mapping the approximately 20,000–25,000 genes of the human genome from both a physical and functional standpoint. A working draft of the genome was announced in 2000 and a complete one in 2003, with further, more detailed analysis still being published.

Human immunodeficiency virus: A retrovirus that causes AIDS by infecting helper T cells of the immune system. The most common serotype, HIV-1, is distributed worldwide, while HIV-2 is primarily confined to West Africa.

Human Microbiome Project: An international project aiming to characterize the microbial communities found at several different sites on the human body, including nasal passages, oral cavities, skin, gastrointestinal tract, and urogenital tract, and to analyze the role of these microbes in human health and disease. The Human Microbiome Project is one of several international efforts designed to take advantage of metagenomic analysis to study human health and expects to continue the practice established by the Human Genome Project of international collaboration to generate a rich, comprehensive, and publicly available data set.

Hyphae: The fine, branching tubes that make up the body (or mycelium) of a multicellular fungus.

Immune system: A complex network of interacting cells, cell products, and cell-forming tissues that protects the body from pathogens and other foreign substances, destroys infected and malignant cells, and removes cellular debris: the system includes the thymus, spleen, lymph nodes and lymph tissue, stem cells, white blood cells, antibodies, and lymphokines.

Infection: The invasion of the body or a part of the body by a pathogenic agent, such as a microorganism or virus. Under favorable conditions the agent develops or multiplies, the results of which may produce injurious effects. Infection should not be confused with disease.

Inflammatory bowel disease (IBD): A term covering a group of disorders in which the intestines become inflamed (red and swollen), probably as a result of an immune reaction of the body against its own intestinal tissue. IBD includes Crohn's disease and ulcerative colitis.

Invasion: An ecological event characterized by the establishment of a foreign organism in a new community and the persistence and spread of this organism.

Koch's postulates: Koch's postulates must be satisfied in order to state that a particular microbe causes a specific infectious disease. They include the following: (1) The parasite occurs in every case of the disease in question and under circumstances that can account for the pathological changes and clinical course of the disease; (2) The parasite occurs in no other disease as a fortuitous and non-pathogenic parasite; (3) After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

Latency: Of an infection, a period in which the infection is present in the host without producing overt symptoms. The time that elapses between a stimulus and a response to it.

Lysogeny: The biological process in which a bacterium is infected by a bacteriophage that integrates its DNA into that of the host such that the host is not destroyed.

Metabolic syndrome: A name for a group of risk factors related to obesity—such as extra weight around the middle and upper parts of the body and insulin resistance—that occur together and increase the risk for coronary artery disease, stroke, and type 2 diabetes.

Metagenome: The sum of all genes and genetic elements and their modifications in the somatic and germ cells of a host plus all genes and genetic elements in all microorganisms that live on or in that host at a given time. The metagenome has transient elements (e.g., during infection with a pathogen) and more persistent elements (e.g., infection with latent eukaryotic virus; presence of commensal bacteria).

Metagenomics: A culture-independent method used for functional and sequence-based analysis of total environmental (community) DNA (note that this is not the same as amplifying, cloning, and sequencing the 16S rRNA-encoding gene, although metagenomic sequences [e.g., generated via modern sequencing methods] can be probed for 16S rRNA-encoding genes or other phylogenetic markers).

Microbe: A microscopic living organism, such as a bacterium, fungus, protozoan, or virus.

Microbiome: The gene complement of a community; the collective genomes of the microorganisms that reside in an environmental niche.

Microbiota/community: A collection of microorganisms existing in the same place at the same time.

Mucosal immunity: Nonsusceptibility to the pathogenic effects of foreign microorganisms or antigenic substances as a result of antibody secretions of the mucous membranes. Mucosal epithelia in the gastrointestinal, respiratory, and reproductive tracts produce a form of IgA that serves to protect these ports of entry into the body.

Mutualism: An interspecies relationship in which both (or all) members benefit.

Nash equilibrium: In game theory, a solution concept in which players in a game are aware of the strategies of the other players but do not deviate from their own, because they do not have anything to gain; it will be disadvantageous to deviate (to cheat).

Operational taxonomic unit (OTU): Taxonomic level of sampling selected by the user to be used in a study, such as individuals, populations, species, genera, or bacterial strains.

Opportunistic pathogen: An infectious microorganism that is normally a commensal or does not harm its host but can cause disease when the host's resistance is low.

Pan-genome: The set of all of the genes that are found in members of a single species.

Parasite: An organism that diminishes the reproductive fitness of another organism or benefits from another organism without reciprocity.

Parasitism: A relationship between two organisms where one organism benefits at the expense of the other.

Pathogen: In medicine, any organism that causes disease. In biological terms, a pathogen is a microorganism that has the inherent capacity to cross anatomical barriers and resist host defenses that ordinarily restrict most other microorganisms.

Pathogenic: Capable of causing disease.

Phylogenomic: The use of evolutionary information in the prediction of gene function.

Phylogeny: The evolutionary development and history of a species or higher taxonomic grouping of organisms.

Polymerase chain reaction (PCR): A scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Pure culture: Technique in which single cells of a particular microbial type are grown in isolation from other organisms.

Pyrosequencing: A method of DNA sequencing based on the "sequencing by synthesis" principle. It differs from Sanger sequencing, in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. The sequence of solutions that produce chemiluminescent signals allows the determination of the sequence of the template.

Resilience: The rate at which a community recovers to its native structure following a perturbation.

Resistance: The ability of a community to resist change to its structure after an ecological challenge. See *Antimicrobial resistance*.

Rhizosphere: The region of the soil in contact with the roots of a plant. It is directly influenced by root secretions and associated soil microorganisms.

Richness: Number of types (e.g., species) in a community.

RNA (ribonucleic acid): Any of various nucleic acids that contain ribose and uracil as structural components and are associated with the control of cellular chemical activities.

rRNA (ribosomal RNA): A nucleic acid found in all living cells. Plays a role in transferring information from DNA to the protein-forming system of the cell. More specifically, rRNA sits in the ribosome, decoding the mRNA into various amino acids and assisting in translation.

Sanger sequencing: A method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

"Shotgun" sequencing: Sequencing of a genome that has been fragmented into small pieces.

Similarity: A measure that determines the similarity of two or more communities, typically based on shared members, total richness, and sometimes abundance of members.

***Staphylococcus aureus*:** A facultatively anaerobic, gram-positive coccus and is the most common cause of staph infections. It is frequently part of the skin flora found in the nose and on skin.

Structure: The composition of the community and the abundance of individual members.

Superorganism: A living system of a superior degree of complexity, consisting of many organisms; a collection of agents that can act in concert to produce phenomena governed by the collective.

Symbiont: An organism in a symbiotic relationship. In cases in which a distinction is made between the two interacting organisms, the symbiont is the smaller of the two and is always a beneficiary in the relationship, while the larger organisms is the host and may or may not derive a benefit.

Syntrophic interactions: Interactions in which organisms do more together than alone.

Temporal stability: The ability of a community to maintain its native structure.

Torpor: The dormant, inactive state of a hibernating animal.

Ulcerative colitis: A serious chronic inflammatory disease of the large intestine and rectum characterized by recurrent episodes of abdominal pain and fever and chills and profuse diarrhea.

***Vibrio fischeri*:** A gram-negative rod-shaped bacterium found globally in marine environments. *V. fischeri* has bioluminescent properties, and is found predominantly in symbiosis with various marine animals, such as the bobtail squid. It is heterotrophic and moves by means of flagella.

Virome: The sum of all viruses living in the tissues of the host or infecting organisms in the microbiome.

Virulence: A quantitative estimate of the ability of one organism to harm another.

Virulence factor: Intrinsic characteristic of an infectious bacterium that facilitates its ability to cause disease.

Virus: A small infectious agent that can only replicate inside the cells of another organism. Viruses are too small to be seen directly with a light microscope. Viruses infect all types of organisms, from animals and plants to bacteria and archaea.

***Yersinia pestis*:** A gram-negative rod-shaped bacterium belonging to the family Enterobacteriaceae. It is a facultative anaerobe that can infect humans and other animals. Human *Y. pestis* infection takes three main forms: pneumonic, septicemic, and the notorious bubonic plagues.

Appendix E

Speaker Biographies

Yasmine Belkaid, Ph.D., obtained her Ph.D. in 1996 from the Pasteur Institute in France on innate responses to Leishmania infection. Following a postdoctoral fellowship at the National Institute of Allergy and Infectious Diseases (NIAID) on immune regulation during Leishmania infection, she joined the Children's Hospital Research Foundation in Cincinnati as an assistant professor in 2002. In 2005, she joined the Laboratory of Parasitic Diseases as a tenure-track investigator and was appointed as a senior investigator in 2008. Since 2008, she has worked as an adjunct professor at the University of Pennsylvania. Her work focuses on immune regulation at sites colonized by commensals such as the gut and the skin. More particularly her work has demonstrated that (1) regulatory T cells play a major role during infections, (2) commensals control host defense in both the skin and the gastrointestinal (GI) tract, (3) dietary factors control the induction of effector and regulatory responses in the GI tract, and (4) in order to protect tissue integrity, the GI tract is a major site of induction of T cells and dendritic cells with regular functions.

Martin J. Blaser, M.D., has served as the Frederick King Professor of Medicine and Chair of the Department of Medicine and as Professor of Microbiology at the New York University (NYU) School of Medicine since April 2000. Dr. Blaser's research has focused on bacterial pathogenesis and ecology. He has studied the role of *Campylobacter* and *Helicobacter* species, among other organisms, in human disease. Much work since 1985 has involved the gastric bacterium *H. pylori*, linking colonization to inflammation and to gastric cancer. His studies identified the two (vacA and cagA) major host-interaction genes and showed differential disease risk associated with particular alleles. He developed a conceptual

framework involving unique dynamic equilibria between bacterial populations and colonized hosts, which has become a general model of persistence for coadapted microbes. His work identified protective roles of *H. pylori* against esophageal adenocarcinoma, and allergic disorders, including asthma. These led to studies of the composition and function of the human microbiome, with a major focus now on human microbiome changes due to social and medical progress and their downstream health consequences. He holds 24 U.S. patents and has authored more than 500 original articles. In 2011, he was elected to the Institute of Medicine and currently chairs the Advisory Board for Clinical Research at the National Institutes of Health (NIH).

Richard S. Blumberg, M.D., trained in internal medicine (The New York Hospital, 1982), infectious diseases (Massachusetts General Hospital, 1986), and gastroenterology and hepatology (Brigham and Women's Hospital, 1989). He is currently Senior Physician in Medicine and Gastroenterology at Brigham and Women's Hospital (BWH) where he leads the Division of Gastroenterology, Hepatology and Endoscopy, is Professor of Medicine at Harvard Medical School and co-Director of the Harvard Digestive Diseases Center. In addition, Dr. Blumberg serves on the Executive Advisory Committee of the Department of Medicine and is the incoming Chair of the Biomedical Research Institute at BWH. He has served as a member of the Immunology Sciences Study Section of NIAID, a member on the National Commission of Digestive Diseases of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), scientific consultant to the Human Microbiome Project (National Human Genome Research Institute), a member of the Vaccine Branch External Advisory Board (National Cancer Institute), and Chair of the External Scientific Consultants for the Intestinal Stem Cell Consortium Initiative (NIDDK) and is currently on the Board of Scientific Councilors (NIAID). He served as the Chair of the National Scientific Advisory Committee of the Crohn's & Colitis of America (2002–2005) and President of the Society for Mucosal Immunology (2007–2009). Dr. Blumberg is an elected member of the American Association of Clinical Investigation and the Association of American Physicians and the recipient of a MERIT Award from NIH (2005), the William Beaumont Prize from the American Gastroenterological Association (2012), and the Distinguished Scientific Achievement Award from the Crohn's and Colitis Foundation of America (2012). He is an NIH-funded investigator whose research program focuses on mucosal immunology and was Scientific Founder, Syntonix Pharmaceuticals (now Biogen-Hemophilia) that developed long-acting therapeutic agents successful in the treatment of chronic diseases such as hemophilia A and B.

Brendan Bohannan, Ph.D., is Professor of Environmental Studies and Biology, and Director of the Institute of Ecology and Evolution at the University of Oregon. His research is focused on understanding the causes and consequences

of microbial biodiversity. His recent work can be divided into three topical areas: the fundamental drivers of biodiversity and how these apply to microorganisms, the response of microbial biodiversity to environmental change (including global change), and microbial biodiversity in human-dominated environments. Professor Bohannan is a founding member of the Microbial Ecology and Theory of Animals Center for Systems Biology (an NIH Center of Excellence focused on the application of ecological theory to host–microbe systems) and the Biology and the Built Environment Center (a national center for the study of the microbial ecology of buildings). Professor Bohannan received a Ph.D. in microbiology from Michigan State University and postdoctoral training in Ecology at the University of Chicago, before joining the Stanford University faculty in 1999. He has been a member of the University of Oregon faculty since 2006.

Hannah V. Carey, Ph.D., is a Professor in the Department of Comparative Biosciences at the University of Wisconsin School of Veterinary Medicine. She received her Ph.D. in zoology from the University of California, Davis, and completed postdoctoral training at the University of Nevada and the Ohio State University. Her research interests are in gastrointestinal and liver physiology, hibernation biology, and the translation of hibernation to biomedicine, including organ preservation, ischemia-reperfusion injury, and severe blood loss. Current projects focus on the symbiosis between hibernating mammals and their gut microbes. Dr. Carey's research has been supported by the National Institutes of Health, the National Science Foundation, the U.S. Department of Agriculture, the U.S. Army Research Office, and the Defense Advanced Research Projects Agency. She is the North American editor of the *Journal of Comparative Physiology B*, is on the editorial board of the *American Journal of Physiology: Gastrointestinal and Liver Physiology*, and is a consulting editor for *Physiology* and the *Journal of Applied Physiology*. Dr. Carey is a past President of the American Physiological Society and past Chair of the Nutrition and Obesity Section of the American Gastroenterological Association, and he was a Program Director at the National Science Foundation from 2010 to 2011, working in the Division of Integrative Organismal Systems.

Angela Douglas, Ph.D., is the Daljit S. and Elaine Sarkaria Professor of Insect Physiology at Cornell University. Her research expertise is animal–microbial symbioses, especially the nutritional and immunological interactions between beneficial microorganisms and their animal hosts. Her current research focus is insect–bacterial symbioses: gut microbiota in *Drosophila* and obligate intracellular mutualists in plant-sap feeding insects. Dr. Douglas has a B.A. degree in zoology (Oxford, 1978) and a Ph.D. in microbiology (Aberdeen, 1981), and she was a postdoctoral researcher at Oxford and East Anglia (1981–1986) before her appointment to a Royal Society Research Fellowship (1996–2006), and subsequently as a faculty member at University of York, with promotion to Personal

Chair in 2003. She moved to Cornell University in 2008. In addition to research publications and reviews, she has written three books, including *The Symbiotic Habit* (2010), Princeton University Press.

Gérard Eberl, Ph.D., received his doctorate from the University of Lausanne in 1995 and completed postdoctoral training at the Ludwig Institute of Cancer Research in Lausanne and at the Skirball Institute at New York University. In 2005, he started the Lymphoid Tissue Development Unit at the Pasteur Institute in Paris and became Senior Investigator in 2010. In 2006, the Laboratory received a Marie Curie Excellence Grant to decipher the role of intestinal symbionts in the development of lymphoid tissues and cells. This work lead to the discovery of a new type of lymphoid cells, termed "innate lymphoid cells" or ILCs, which play a fundamental role in the intestinal homeostasis with microbes and the regulation of adaptive immunity. In 2010 and 2011, Dr. Eberl received awards from the Institut de France and the Fondation de France for his work on the constructive interaction between symbionts and the immune system.

Paul L. Fidel, Jr., Ph.D., received his doctorate from the University of Oklahoma in 1988 in the discipline of microbiology/immunology. He then did postdoctoral work at Wayne State University School of Medicine in Detroit, Michigan, in the Department of Immunology and Microbiology. He was appointed Assistant Professor in the Department of Medicine, Division of Infectious Disease, in 1990. In 1995, Dr. Fidel went to the Louisiana State University (LSU) Health Sciences Center in New Orleans at the rank of Associate Professor of Microbiology, Immunology, and Parasitology. He rose to the rank of Professor in 1999. In 2001 he was named recipient of the Carl Baldridge Endowed Chair in Oral and Craniofacial Biology and appointed the Director of the Center of Excellence in Oral and Craniofacial Biology and Associate Dean for Research at the LSU School of Dentistry. He has authored nearly 150 publications and has served as Principal Investigator for upward of 20 investigator-initiated or center research grants totaling more than $33 million (predominantly NIH). Dr. Fidel's research interests include immune defense mechanisms against oral and vaginal candidiasis. His scientific contributions have included a number of paradigm changes and new hypotheses to explain host resistance and susceptibility to mucosal *Candida* infections.

Michael Fischbach, Ph.D., is an Assistant Professor in the Department of Bioengineering and Therapeutic Sciences at University of California, San Francisco (UCSF), and a member of the California Institute for Quantitative Biosciences (QB3). Dr. Fischbach is a recipient of the NIH Director's New Innovator Award, a Fellowship for Science and Engineering from the David and Lucille Packard Foundation, a Medical Research Award from the W.M. Keck Foundation, and the Young Investigator Grant for Probiotics Research from the Global Probiotics Council. His laboratory uses a combination of genomics and chemistry to identify

and characterize small molecules from microbes, with an emphasis on the human microbiome. Dr. Fischbach received his Ph.D. in chemistry from Harvard in 2007, where he studied the role of iron acquisition in bacterial pathogenesis and the biosynthesis of antibiotics. Before going to UCSF, he spent 2 years as an independent Fellow at Massachusetts General Hospital, coordinating a collaborative effort based at the Broad Institute to develop genomics-based approaches to the discovery of small molecules from microbes. Dr. Fischbach is a member of the scientific advisory boards of Schiff Nutrition, Second Genome, and Warp Drive Bio and a consultant for Achaogen, Agraquest, and Genentech.

Larry Forney, Ph.D., has scientific expertise in the evolutionary ecology of prokaryotes, and his research program focuses on studies of the human microbiome, the adaptive evolution of prokaryotes, microbial community dynamics, and the biogeography of microorganisms. Although formally trained in microbial physiology, his interests have expanded over the years to encompass various other topics pertinent to understanding the development and distribution of microbial diversity on temporal and spatial scales. He has pioneered the development of molecular microbial ecology methods to understand the extent and distribution of microbial diversity and to characterize within species genetic diversity and the evolution of novel microbial traits. His accomplishments in microbial evolution and ecology and scientific leadership have been recognized through various awards and by appointment as a Fellow of the American Academy of Microbiology. Dr. Forney is currently director of the Institute for Bioinformatics and Evolutionary Studies and director of an NIH-funded Center of Biomedical Research Excellence. In these roles he leads a vibrant community of scientists, mathematicians, and statisticians at the University of Idaho who conduct interdisciplinary research on a range of topics related to evolutionary and computational biology with colleagues on campus and across the globe.

Karen Guillemin, Ph.D., received her bachelor's degree at Harvard and Radcliffe Colleges and her Ph.D. from the Department of Biochemistry at Stanford University Medical School, where she worked with Dr. Mark Krasnow studying *Drosophila* development. She stayed at Stanford University to pursue postdoctoral studies with Dr. Stanley Falkow in the Department of Microbiology and Immunology, where she worked on genomic approaches to characterizing bacterial–host interactions, focusing in the human pathogen, *Helicobacter pylori*. In 2001, she joined the faculty of the Institute of Molecular Biology at the University of Oregon, where she is currently a Professor. As an independent principal investigator, she has developed a research program that uses zebrafish to investigate the role of host-associated microbial communities in animal development. In 2012 she became the director of the Microbial Ecology and Theory of Animals Center for Systems Biology, a Center of Excellence for Systems Biology funded by NIH/National Institute of General Medical Sciences (NIGMS).

Josbert Keller, M.D., Ph.D., attended medical school at the University of Amsterdam/Academic Medical Center (AMC) and was a Ph.D. Fellow at the Department of Pathology at the AMC, performing research on gastrointestinal polyposis syndromes in collaboration with the Department of Gastroenterology at the Johns Hopkins University Hospital in Baltimore. During his GI fellowship at the AMC, he initiated the FECAL trial, comparing the efficacy of donor feces infusion with conventional antibiotic therapy for patients with longstanding recurrent *Clostridium difficile* infection. The study was funded by The Netherlands Organisation for Health Research and Development, and the results were recently published. Since 2009, he works at the Haga Teaching Hospital (Hagaziekenhuis) in The Hague, the Netherlands. Dr. Keller is the Secretary of the Netherlands Society of Gastroenterology (NVGE).

David Mills, Ph.D., is the Peter J. Shields Endowed Chair in the Departments of Viticulture & Enology and Food Science & Technology at the University of California, Davis. Dr. Mills studies lactic acid bacteria (LAB) and bifidobacteria used in food fermentations or active as probiotics. Dr. Mills is the founder of the LAB Genomics Consortium and is a founding member of the UC Davis Milk Bioactives and Functional Glycobiology Programs. Dr. Mills is a past Chair of the Food Microbiology Division for the American Society for Microbiology, where he has also served as a Waksman Foundation Lecturer. Dr. Mills currently serves as an associate editor for the journal *Microbiology*. In 2010 Dr. Mills was awarded the Cargill Flavor Systems Award from the American Dairy Science Association.

Forest Rohwer, Ph.D., is a Professor of Biology at San Diego State University (SDSU), California. He received his B.A. from the College of Idaho, with emphases in history, biology, and chemistry. His Ph.D. is from the SDSU/University of California, San Diego, Joint Doctoral program in molecular immunology, where he studied Interleukin-2 signal transduction. Dr. Rohwer then moved to Scripps Institution of Oceanography and developed metagenomic approaches to study marine viruses with Farooq Azam. In 2002, he started his lab at SDSU, where the main areas of study are viral metagenomics and coral reef microbiology. Dr. Rohwer is a Fellow of the American Academy for Advancement of Science and the Canadian Institute for Advanced Research. In 2008 he received the Young Investigators Award from the International Society of Microbial Ecology. Current major fields of research are the human virome and coral reef microbiology.

Julie Segre, Ph.D., received her Ph.D. in 1996 from the Massachusetts Institute of Technology (MIT) in the laboratory of Dr. Eric Lander and the newly formed genome center. Dr. Segre then performed postdoctoral training with Elaine Fuchs, an expert in skin biology, at the University of Chicago. Dr. Segre joined the National Human Genome Research Institute of NIH in 2000 and was promoted to a Senior Investigator with tenure in 2007. Dr. Segre's laboratory utilizes

high-throughput sequencing and develops algorithms to study the microbial diversity of human skin in both health and disease states, with a focus on eczema and primary immune deficiencies. Dr. Segre published the first topographical map of human skin bacterial diversity, followed recently by a study of fungal diversity. Dr. Segre's laboratory also maintains an active interest in developing genomic tools to track hospital-acquired infections of multidrug-resistant organisms, including the NIH's recent Klebsiella pneumoniae outbreak. Dr. Segre's research is based on active collaborations with the NIH Intramural Sequencing Center and the clinical departments of Infection Control, Microbiology, and Dermatology. Dr. Segre is a leader in the NIH Roadmap Human Microbiome Project, communicating with multiple media sources to promote the concept of humans as ecological landscapes. Dr. Segre is the 2013 recipient of the Service to America Medal.

Gerald Tuskan, Ph.D., is Group Leader of the Plant Systems Biology Group in the Biosciences Division at Oak Ridge National Laboratory. Dr. Tuskan received a Ph.D. in 1984 from Texas A&M University in genetics. Dr. Tuskan led the International Populus Genome Consortium, which sequenced, assembled, annotated, and published the *Populus* genome. This paper has been cited more than 1,600 times and has been one of the top cited papers in *Science* during the past 5 years. Subsequent to that, and as Co-Lead of the Plant Genome Program at the Joint Genome Institute, Dr. Tuskan provided leadership in the sequencing and assembly of other plant genomes including *Brachypodium*, *Eucalyptus*, *Physcomitrella*, *Salix*, and *Setaria*. Dr. Tuskan has more than 154 publications in the areas of genetics and genomics of perennial plants, including 45 publications, with nearly 800 citations that exclusively relate to U.S. Department of Energy (DOE) missions in the area of biomass production and bioenergy. In the past 10 years, Dr. Tuskan has led or co-led 14 proposals to DOE, the U.S. Department of Agriculture, and the National Science Foundation, which have resulted in more than $323 million in funding. During the past 5 years, Dr. Tuskan has had more than 25 invention disclosures, 6 of which have been moved forward to patent applications.

David M. Underhill, Ph.D., is a Professor in the F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute at Cedars-Sinai Medical Center in Los Angeles. He is also the Director of the medical center's Ph.D. Program in Biomedical Science and Translational Medicine and is on the faculty of the David Geffen School of Medicine at University of California, Los Angeles. He currently holds the Janis and William Wetsman Family Chair in Inflammatory Bowel Disease Research. Dr. Underhill's laboratory studies the cellular and molecular basis for microbial recognition by the innate immune system. He has made important contributions to defining Toll-like receptor ligands, characterizing Toll-like receptor signaling pathways, and in characterizing C-type lectin signaling pathways.

His laboratory has been particularly instrumental in developing an understanding of how the process of phagocytosis participates in directing inflammatory signaling in macrophages and dendritic cells. The laboratory has typically focused on understanding how bacterial and fungal pathogens are recognized, but it has more recently been exploring how commensal bacteria and fungi interact with the innate immune system and how this affects inflammatory diseases such as inflammatory bowel disease.

Herbert W. "Skip" Virgin, M.D., Ph.D., received his A.B., M.D., Ph.D., internal medicine residency, and postdoctoral training at Harvard and the Brigham and Women's Hospital and subsequently trained in infectious diseases. In 2006 he became Edward Mallinckrodt Professor and Head of the Department of Pathology and Immunology. He is the head of the Midwest Regional Center for Excellence in Biodefense and Emerging Infectious Diseases Research. Dr. Virgin is the author of the definitive chapter on viral pathogenesis in *Fields Virology* and of reviews on the virome, AIDs, autophagy, and personalized medicine/metagenomics in *Cell* and *Nature*. He is on the Board of Reviewing Editors for *Science* magazine, is a member of the American Society for Clinical Investigation and the American Association of Physicians, and is a Fellow of the American Academy of Arts and Sciences and the American Academy for Microbiology. His laboratory is known for taking genetic, genomic, and structural approaches to defining mechanisms of immunity, chronic viral infection, viral virulence, and the pathogenesis of cancer, infection, and inflammatory diseases. Recent work has focused on pathogen discovery, mucosal immunity, the role of interferons and autophagy in immunity, and the importance of the virome and virome–gene interactions in normal immunity and disease susceptibility.

Ramnik Xavier, Ph.D., is a clinical gastroenterologist and molecular biologist and is Chief of Gastroenterology at Massachusetts General Hospital. The overall goal of his laboratory is to understand the function of mediators and effectors involved in innate and adaptive immunity. A second major focus is to understand the function of genes associated with Crohn's disease/ulcerative colitis and risk of autoimmunity using genomics, computational tools, and model systems. Recent findings in the laboratory have contributed to elucidating the role of autophagy in Crohn's disease and the discovery of novel immune regulatory genes. In future studies, Dr. Xavier's team hopes to gain insights into the cell–cell interactions and regulatory networks that define functional modules at the host cell–microbe interface in the intestinal mucosa. Dr. Xavier completed his clinical training in internal medicine, followed by subspecialty training in gastroenterology and hepatology, at Massachusetts General Hospital. His laboratory is located at the Center for Computational and Integrative Biology, of which he is a founding member, at Massachusetts General Hospital. Dr. Xavier is the Kurt Isselbacher Associate Professor of Medicine at Harvard Medical School, is a Senior Associate Member

of the Broad Institute of Harvard and MIT, and sees patients at the Crohn's and Colitis Center at Massachusetts General Hospital.

Michael Zasloff, M.D., Ph.D., has centered his scientific interests on the innate immune systems of animals during the past 25 years. Dr. Zasloff received his M.D. and Ph.D. in the Medical Scientist training program at NYU School of Medicine. In the 1980s, Dr. Zasloff was Chief, Human Genetics Branch, National Institutes of Child Health and Human Development, at NIH. In 1988 Dr. Zasloff founded Magainin Pharmaceuticals, Inc., a publicly traded biotechnology company. In the same year he joined the faculty of the University of Pennsylvania School of Medicine as the Charles E.H. Upham Professor of Pediatrics and Genetics and assumed the position of Director of the Division of Human Genetics of the Children's Hospital of Philadelphia. In July 1992, Dr. Zasloff left Penn and joined Magainin on a full-time basis, and he served as Executive Vice President and President of the Magainin Research Institute, a basic research division of the company. From July 1996 through November 2000, Dr. Zasloff was Vice Chairman of the Board of Magainin Pharmaceuticals. In 2002, Dr. Zasloff was named Dean of Research and Translational Science at Georgetown University, tasked with the integration of the basic science conducted at Georgetown with the clinical environment of the Medical Center. Since 2004, Dr. Zasloff has been actively engaged in studies of innate immunity within the Transplant Institute of the Department of Surgery. His research interests remain focused on the role of antimicrobial peptides and aminosterols in health and disease, and application to the prevention and treatment of disease.