METAGENOMIC AND METATRANSCRIPTOMIC INVESTIGATION OF MICROORGANISMS EXPOSED TO BENZALKONIUM CHLORIDE DISINFECTANTS

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METAGENOMIC AND METATRANSCRIPTOMIC INVESTIGATION OF MICROORGANISMS EXPOSED TO BENZALKONIUM CHLORIDE DISINFECTANTS

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To my father, Youngchul

whom I lost during this study

and I am forever indebted to

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SUMMARY

Benzalkonium chlorides (BACs) are widely used, broad-spectrum disinfectants and frequently detected in the environment, even at toxic levels for life. Since such disinfectants can induce broad resistance capabilities, BACs may fuel the emergence of antibiotic resistance in the environment. A substantial body of literature has reported that exposure to BACs causes antibiotic resistance; yet, other studies suggest that the resistance linkage is rare, unsystematic, and/or clinically insignificant. Accordingly, whether or not disinfectant exposure mediates antibiotic resistance and, if so, what molecular mechanisms underlie the resistance link remains to be clearly elucidated. Further, understanding how microbial communities degrade BACs is important not only for alleviating the possible occurrence of antibiotic resistance but also reducing the potential risks to environmental and public health.

An integrated strategy that combines metagenomics, metatranscriptomics, genetics, and traditional culture-dependent approaches was employed to provide novel insights into these issues. The integrative approach showed that a microbial community exposed to BACs can acquire antibiotic resistance through two mechanisms: i) horizontal transfer of previously uncharacterized efflux pump genes conferring resistance to BACs and antibiotics, which were encoded on a conjugative plasmid and co-selected together upon BACs and ii) selective enrichment of intrinsically multi-drug resistant organisms. Further, a microbial community adapts to BAC exposure via a variety of mechanisms, including selective enrichment of BAC-degrading species and amino acid substitutions and horizontal transfer of genes related to BAC resistance and degradation. The

metatranscriptomic data suggests that the BAC-adapted microbial community metabolized BACs by cooperative interactions among its members. More specifically, *Pseudomonas nitroreducens* cleaved (i.e., dealkylated) BACs, metabolized the alkyl chain (the dealkylated product of BACs), and released benzyldimethylamine (the other product of BACs), which was further metabolized by other community members (e.g., *Pseudomonas putida*).

Collectively, this study demonstrates the role of BACs in promoting antibiotic resistance and advances current understanding of a microbial community degrading BACs. The results of this work have important implications for (appropriate) usage of disinfectants and for assessing, predicting, and optimizing biological engineering processes treating BAC-bearing waste streams.

CHAPTER 1

INTRODUCTION

1.1. Summary

Benzalkonium chlorides (BACs) are popular "over-the-counter" surface disinfectants with a broad-spectrum of biocidal activity, which act primarily by disrupting the integrity of the cell membrane. Their extensive use and stability are responsible for BACs being readily detected in a variety of environmental settings where their concentrations are often toxic for life. Microorganisms can develop resistance to BAC toxicity by such mechanisms as efflux pumps, enzymatic degradation, and possibly cell envelope modification. Some of these mechanisms are also thought to confer resistance to clinically relevant antibiotics but the evidence in support of this hypothesis is anecdotal. Accordingly, if BACs indeed induce or co-select for antibiotic resistance, this would represent an important discovery for regulatory agencies due to the widespread use of BACs. In this chapter, current understanding and open questions concerning mode of action, bacterial sensitivity, resistance mechanism, and the possible link to antibiotic resistance are reviewed.

1.2. Physico-chemical properties and occurrence in the environment

BACs are the most commonly used members of quaternary ammonium compound (QAC) disinfectants. BACs contain a central, positively charged nitrogen atom with a benzyl group, two methyl groups (CH_3), and a long n-alkyl chain (C_8 to C_{18}) (Fig. 1.1.). Due to their amphiphilic property, BACs are also used as cationic surfactant agents. The

length of the alkyl chain affects not only physicochemical properties such as water solubility, the octanol/water partition coefficient, and adsorption/partition coefficient (41), but also an antimicrobial activity (43, 98). BACs have broad-spectrum biocidal activity (e.g., microbial, algal, fungal, and viral) and remain stable for both short and long-term usage (86). Accordingly, BACs are widely used as surface disinfecting agents in food processing lines (e.g., poultry facilities), dairy/agricultural settings, health care facilities, and domestic households and are also popular ingredients for over-the-counter products such as cosmetics, hand sanitizers, and pharmaceuticals (60).

Their extensive use and stability has caused QAC concentrations to often be higher than other conventional organic pollutants (e.g. polyaromatic hydrocarbons) in sediments (77) and, particularly, BAC concentrations were higher than other QAC representatives (87). BACs have been detected across diverse environments such as river sediment (5.8 μg/kg), estuarine sediment (1.5 μg/kg), surface water (1.9 μg/L), and wastewater influent (170 μg/L) (77, 87, 88). Notably, BAC concentrations were relatively high (up to 6.03 mg/L) in wastewater from clinical health care facilities (70). BACs may act as a causative agent to inhibit organisms that play key roles for ecologically important functions in natural environments and biological engineering systems (e.g., wastewater treatment plants). Given the high occurrence of BACs in the environment and the potential risks to public and environmental health, paying more attention to monitoring BACs persisting in the environment has been previously suggested (77).

1.3. Toxicity

QAC-family disinfectants are membrane active agents and their antimicrobial activities are mainly associated with the cationic surfactant property. QACs react

predominantly with the phospholipid components of the cytoplasmic (inner) membrane. The sequential mode of action is described as follows: i) attachment to cell membrane, ii) interaction of the hydrophobic nitrogen atom with acidic phospholipids, iii) integration of alkyl chains into lipid cores, iv) increase of osmotic pressure and decrease of membrane fluidity, and v) loss of structural integrity and physiological (e.g., osmoregulatory and respiratory) functionality (83, 92, 123). BACs also cause autolysis that contributes to cell lethality, in addition to the structural and functional disruption of the membrane (37, 57).

In addition to the interaction with the cell membrane, QACs were recently described to induce oxidative stress by generating reactive oxygen species (ROS, such as superoxide and hydrogen peroxide) (97), although the exact mechanism is not yet known. The ROS oxidize a wide range of cellular molecules such as DNA, RNA, protein, and lipid (17). Accordingly, bacteria subjected to BACs are known to induce cellular activities of ROS-scavenging antioxidants such as catalase, thioredoxin, and alkylhydroperoxide reductase (19, 84). Recently, Kohanski et al. proposed oxidative stress as a common mechanism that leads to bacterial cell death, which was observed with three major classes of antibiotics such as aminoglycosides, quinolones, and β-lactams (65). Accordingly, these findings imply that both BACs and antibiotics may have a common (secondary?) mode of action and thus, bacteria which have adapted and developed resistance to one antimicrobial agent may also exhibit resistance to the other.

Previously determined susceptibility data, based on minimum inhibitory concentration (MIC) values, for both Gram-positive and Gram-negative bacteria are listed in Table 1.1. Several studies hypothesized that Gram-negative bacteria are more resistant than Gram-positives (35, 83, 92) due to the role of the outer membrane, which

can prevent QACs from reaching the inner-membrane, the target of action. Although Gram-negative *Pseudomonas aeruginosa* showed extremely high-level resistance to BACs (12 to 1200 mg/L MIC), there is no systematic evidence supporting the higher resistance of Gram-negative as compared to Gram-positive bacteria (Table 1.1). In addition, the outer membrane reduced susceptibility to hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl), but not to the lower molecular weight BACs (C_{12}BDMA-Cl and C_{14}BDMA-Cl) in *P. aeruginosa*. Accordingly, it is thought that bacterial sensitivity to BACs involves many other factors in addition to the outer membrane.

Table 1.1. Minimum inhibitory concentrations of microorganisms to BACs

Organism	Gram-stain	MIC (mg/L)	References
Neisseria gonorrhoeae	Negative	1.75	(122)
Citrobacter freundii	Negative	10 - 20	(69)
Enterobacter cloacae	Negative	4	(47)
Escherichia coli	Negative	10 - 50	(35, 69)
Pseudomonas aeruginosa	Negative	12 - 1200	(35, 43, 69)
Pseudomonas fluorescens	Negative	20	(37)
Pseudomonas syringae	Negative	2.5	(134)
Sallmonella enterica	Negative	32	(13)
Sallmonella typhimurium	Negative	80	(35)
Serratia marcescens	Negative	0.5 – 2.5	(23, 127)
Stenotrophomonas maltophilia	Negative	10	(69)
Bacillus cereus	Positive	140	(35)
Leuconostoc sp.	Positive	4.3	(129)
Listeria monocytogenes	Positive	1 - 30	(1, 35)
Staphylococcus aureus	Positive	0.4 - 40	(35, 57)
Staphylococcus saprophyticus	Positive	2	(50)

1.4. Resistance mechanisms to BACs

Bacteria are previously known to resist BACs by exporting them out of cells using efflux pumps and transforming them to less toxic compounds. Cell envelope modification (i.e., presumably reducing BAC permeability) was frequently observed in bacteria adapted to BACs, with developed BAC resistance. Bacterial resistance to BACs has been a major challenge since BACs are commonly used disinfecting agents that are supposed to eradicate unwanted microorganisms (e.g., pathogens). Further, since some of the resistance mechanisms may also confer resistance to antibiotics, bacterial resistance to BACs has received increasing attention in terms of environmental and public health.

1.4.1. Efflux pump

A relatively well-known resistance mechanism to BACs is the efflux pump system. Well-characterized efflux pumps from four families in phylogenetically diverse microorganisms have all been implicated in BAC resistance (Table 1.2). Included among these families are the major facilitator superfamily (MFS), multidrug and toxic compound extrusion family (MATE), resistance nodulation cell division family (RND), and small multidrug resistance family (SMR). The efflux pumps export a wide range of non-specific antimicrobials including BACs by mostly exchanging protons. Notably, while the characterized MFS, MATE, and RND-family efflux pumps related to BAC resistance are mostly chromosomally encoded, some SMR-family efflux pumps (*qacC*, *qacE*, *qacG*, *qacJ*, and *qacH*) are plasmid-borne (11, 50, 113). These genes also have been frequently found in other mobile elements such as integrons, which can be horizontally transferred to phylogenetically diverse organisms. These findings imply that bacteria can potentially

acquire BAC resistance by horizontal gene transfer in the presence of a selective BAC agent.

Table 1.2. Efflux pumps associated with resistance to both BACs and antibiotics. [a]AM, ampicillin; CA, carbenicillin; CE, cefoperazone; CF, cefotaxime; CU, cefuroxime; CH, chloramphenicol; CI, ciprofloxacin; CL, clindamycin; DA, daunomycin; DO, doxorubicin; EN, enoxacin; ER, erythromycin; FU, Flumequine; FO, fosfomycin; KA, kanamycin; LI, lincomycin; MI, minocycline; MT, Mitomycin C; MU, Mupirocin; NE, neomycin; NI, nitrofurantoin; NO, norfloxacin; NV, novobiocin; OF, ofloxacin; PI, piperacillin; PR, pristinamycin; PU, puromycin; RI, rifampicin; TE, tetracycline; TR, trimethoprim; VI, virginiamycin; ND, not determined.

Efflux pump	Family	Organism	Location	Antibiotic substrates[a]	Reference
YdhE	MATE	*Escherichia coli*	Chromosome	CH, NO, EN, FO, DO, TR	(102)
PmpM	MATE	*Pseudomonas aeruginosa*	Chromosome	NO, CI, OF	(47)
MepA	MATE	*Staphylococcus aureus*	Chromosome	NO, CI	(61)
EmeA	MFS	*Enterococcus faecalis*	Chromosome	NO, CI	(72)
EmrD	MFS	*Escherichia coli*	Chromosome	ND	(102)
MdfA	MFS	*Escherichia coli*	Chromosome	PU, TE, DA, RI, CH, NE, KA, CI, NO	(34)
SmfY	MFS	*Serratia marcescens*	Chromosome	NO, OF	(127)
MdeA	MFS	*Staphylococcus aureus*	Chromosome	VI, NV, MU	(54)
NorA	MFS	*Staphylococcus aureus*	Chromosome	NV, MU, NO	(54)
QacA	MFS	*Staphylococcus aureus*	Chromosome	ND	(112)
QacB	MFS	*Staphylococcus aureus*	Chromosome	ND	(112)
AheABC	RND	*Aeromonas hydrophila*	Chromosome	MI, PR, LI, ER, CE, CU	(53)
AcrAB	RND	*Escherichia coli*	Chromosome	CH, TE, MI, ER, NO, EN, DO, NV, RI,	(102)
OqxAB	RND	*Escherichia coli*	Chromosome	CH, CI, FU, MT, NO, TE, TR	(46)
YegMNOB	RND	*Escherichia coli*	Chromosome	NO, FO, NV	(102)
YhiUV	RND	*Escherichia coli*	Chromosome	ER, DO	(102)
MexAB	RND	*Pseudomonas syringae*	Chromosome	AM, CA, CH, CI, CL, DA, ER, MT, NI, NO, NV, PI, PU, TE, TR	(134)
SdeXY	RND	*Serratia marcescens*	Chromosome	TE, NO, AM	(23)
SugE	SMR	*Enterobacter cloacae*	Chromosome	ND	(48)
EmrE	SMR	*Escherichia coli*	Chromosome	ND	(102)
SepA	SMR	*Staphylococcus aureus*	Chromosome	ND	(99)
QacC	SMR	*Staphylococcus aureus*	Plasmid	ND	(113)
QacE	SMR	*Staphylococcus aureus*	Plasmid	ND	(113)
QacG	SMR	*Staphylococcus aureus*	Plasmid	ND	(49)
QacJ	SMR	*Staphylococcus aureus*	Plasmid	ND	(10)
QacH	SMR	*Staphylococcus saprophyticus*	Plasmid	ND	(50)

1.4.2. Enzymatic degradation

A few bacteria such as *Aeromonas hydrophila* K., *Bacillus niabensis* and *Thalassospira* sp. can metabolize BACs as a sole carbon and energy source (6, 111). These bacteria exhibited enzymatic degradation of 2 to 4 mg/L (*Bacillus niabensis* and *Thalassospira* sp.) and up to 100 mg/L (*Aeromonas hydrophila* K.) of BACs, which are toxic to many bacteria (Table 1.1). Metabolite analysis suggested that these bacteria transform BACs to benzyldimethylamine (BDMA) and a long chain alkyl group by dealkylation (Fig. 1.1). Since BDMA is about 500 times less toxic than BACs (145), enzymatic transformation of BACs to BDMA by dealkylation represents an important detoxification process. Although several enzymatic assays identified amine dehydrogenase and monooxygenase as being involved in cleaving C-N bonds of other QAC-family biocides (25, 30, 68, 78, 150), whether or not the same enzyme(s) catalyzes the cleavage of the C_{alkyl}-N bond of BACs and what gene encodes the enzyme remain currently unknown.

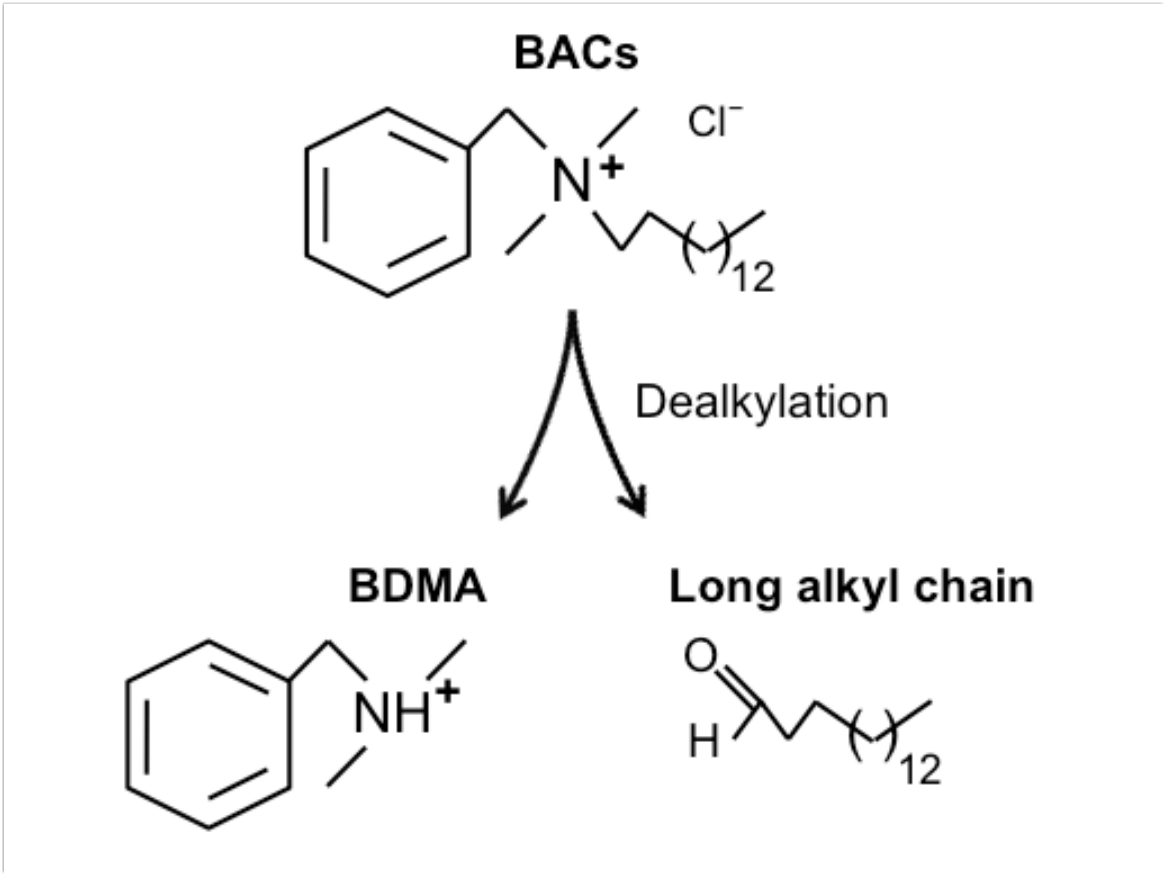


Figure 1.1. Enzymatic transformation of BACs (C_{14}BDMA-Cl)

1.4.3. Cell envelope modification

Previous studies frequently reported cell envelope modification in bacteria that were adapted and had developed resistance to BACs. The cell wall and outer membrane (Gram-negative bacteria) act as a barrier to prevent the entrance of BACs and access to the primary target site, the cytoplasmic (inner) membrane. The adaptive cell envelope modification involved physiological alterations in surface charge, hydrophobicity, roughness, and antigen types in the cell surface, and in fatty acid composition of the outer membrane. Highly BAC-resistant *Pseudomonas fluorescens* (4,000 mg/L BAC MIC) isolated from contaminated BAC stock solution showed reduced negative charge in cell surface (96). *Salmonella enterica* adapted to BACs showed increased surface hydrophobicity (13). Cell surface roughness in the *Salmonella enterica* was gradually reduced with longer BAC exposure and also showed an increase in saturated fatty acids, which may decrease cell membrane permeability and fluidity (85). Induction of type 1 and type 4 antigens was also described for the Gram-positive *Listeria monocytogenes* adapted to BACs (146). Although these studies have made an attempt to link specific phenotypic properties to the reduction of uptake, adsorption, and/or permeability of the cationic BAC agents, the evidence provided in support of these findings was not always conclusive. For instance, it remains speculative whether these phenotypes were directly selected by BAC exposure, occurred by chance (e.g., spontaneous responses), or selected by the culturing conditions used (but not BACs per se). Accordingly, the molecular mechanisms (e.g., changes in the underlying DNA sequences and/or gene expression levels) that facilitate the cell envelope modification and thus, mediate BAC resistance

remain to be clearly elucidated; for instance, by gene sequencing and genetic manipulations (e.g., mutants).

1.4. Link to antibiotic resistance

Antibiotics have a specific mode of action, typically on a single target, whereas disinfectants such as QACs act on non-specific, multiple sites. For this reason, QACs are thought to not share the mode of action with antibiotics and thus, not affect antibiotic resistance. Numerous studies have suggested that QACs rarely induce or co-select for antibiotic resistance (3, 26, 28, 69, 71, 131). However, other literature has indicated instead that bacteria subjected to QACs exhibit reduced antibiotic susceptibility (62, 79, 90, 118, 121, 141). In the latter case, the acquired antibiotic resistance induced by QAC exposure is thought to be mediated by advantageous mutations and horizontal transfer of genes involved in efflux pumps and cell envelope modification.

Numerous examples of organisms adapted to BACs that showed increased resistance capabilities to clinically relevant antibiotics are shown in Table 1.3. *Listeria monocytogenes* was adapted to BACs and showed gradually increased BAC resistance (2 to 8 fold increase in MIC) (118). These organisms also showed reduced susceptibility to ciprofloxacin and gentamycin with increased expression of efflux pump systems compared to the parent strain. The authors hypothesized that advantageous mutations accounting for the increased efflux activities were responsible for the increased antibiotic resistance. Decreased cell membrane permeability was also implicated as a mechanism of reduced sensitivity to chloramphenicol upon BAC exposure in *Pseudomonas aeruginosa* (79). Collectively, although adaptive mutations have been implicated for the increased efflux activity and impermeability of the cell envelope in the previous studies, further

investigation is necessary to determine what specific genetic modifications (e.g., changes in DNA sequence of the corresponding gene) underlie the BAC-induced antibiotic resistance. In addition, BAC-adapted *Pseudomonas aeruginosa* acquired 12-fold increase of BAC MIC and this was accompanied with a significant increase in ciprofloxacin resistance (256 fold) (90). This study provides genetic evidence supporting that the acquired resistance was due to a mutation in *gyrA* gene, a target site of ciprofloxacin; however, it remains to be clarified whether the point mutation was induced by BAC exposure or occurred spontaneously, given the different mode of action and target sites between BACs and ciprofloxacin.

Acquisition of resistance to both BACs and antibiotics can be mediated by horizontal transfer of multi-drug efflux pumps encoded in mobile genetic elements. Mobile genetic elements such as plasmids conferring resistance to BACs and antibiotics have been previously reported (154). The plasmid pSAJ1 carried by methicillin-resistant *Staphylococcus aureus* (MRSA) exhibited resistance to many antibiotics such as kanamycin, genamycin, tobaramycin, and amikacin as well as BACs. Further, some SMR-family efflux pump genes (e.g., *qacC*, *qacD*, *qacE*, *qacG*, *qacH*, and *qacJ*; see also Table 1.2) are also found in integrons, including class 1 integrons, which can also harbor a versatile repertoire of antibiotic resistance genes. Since the class 1 integrons can also be embedded in various mobile genetic elements (e.g., plasmids), they can be potentially transferred across diverse environmental habitats and phylogenetically divergent organisms. Although the previous findings collectively imply that BAC exposure can potentially propagate antibiotic resistance genes among broad hosts, this hypothesis remains to be rigorously tested.

Table 1.3. Reduced susceptibility to antibiotics in bacteria selected by BACs. [a]AM, ampicillin; CF, cefotaxime; CP, cefpodoxime; CT, ceftazidime; CR, ceftriaxone; CL, cephalexin; CH, chloramphenicol; CI, ciprofloxacin; FL, florfenicol; GE, gentamicin; PI, piperacillin; SU, sulfamethoxazole; TE, tetracycline; TR, trimethoprim.

Organism	Fold increase	Reduced susceptibilities to antibiotics[a]	Reference
Escherichia coli	2.9	AM, CT, CP, CH, CI, FL, SU, TE,	(130)
Leuconostoc sp.	4.3 - 16	GE	(129)
Listeria monocytogenes	2 - 8	CI, GE	(118)
Pseudomonas aeruginosa	1.1 - 2.2	CI	(110)
Pseudomonas aeruginosa	12	CI	(90)
Pseudomonas aeruginosa	16.7	CH	(79)
Salmonella enterica	3.3	AM, PI, CL, CP, CR, TE, CI, CH	(28)

1.5. Metagenomics and metatranscriptomics

Microbial consortia can carry out more complex tasks and be more robust to environmental fluctuations than individual populations (14). Related to the main topic of this thesis, almost all natural and engineered environments are constantly loaded with diverse microorganisms, which often act in concert to degrade complex organic compounds. Although considerable progress has been made in elucidating microbial sensitivity, resistance, degradation, and adaptation to BACs based on pure cultures, it remains unclear to what extent the findings and lessons learned from isolate-based studies can be translated to complex communities within natural or engineered systems. Further, the great majority of microorganisms resist cultivation under laboratory conditions, a phenomenon known as "the uncultivated majority" (2). Accordingly, understanding how whole microbial communities respond to and degrade BACs can provide better insights into assessment, prediction, and optimization of BAC detoxification processes in engineered or natural systems.

Metagenomics is a culture-independent approach to investigating the collective genomes of the entire microbial community. Thus, metagenomics can provide windows into the metabolic potential and phylogenetic diversity of the key community members, bypassing the need for cultivation, and potentially lead to the identification of previously uncharacterized/novel genes (e.g., bacteriorhodopsin) and species (7, 8, 132). Numerous studies have successfully applied metagenomics to natural ecosystems and engineering systems to address the phylogenetic diversity, metabolic potential, evolution, and adaptation of the target communities.

While metagenomics can recover the genes and organisms potentially involved in important metabolic processes, the activities in situ and how they change in response to environmental fluctuations remain elusive. Metatranscriptomics can take a snapshot of the transcriptional profiles of species and genes by randomly sampling the collective ribosomal and messenger RNA of the community expressed. Accordingly, metatranscriptomics provides insights into activities of ecologically important organisms (e.g., ones involved in global nutrient cycling), some of which had not been characterized by the traditional culture-dependent approach (39, 74, 149). Thus, application of metatranscriptomics can help narrow down the key "hero" species and genes for the functions of interest carried out by the whole microbial community. Hence, metagenomics coupled with metatranscriptomics can be a powerful tool to identify the presence, relative abundance, and dynamic activities of specific organisms and genes, which can be linked to the community physiological responses to BACs, under a given experimental framework (Fig. 1.2). Such an integrated approach has not been applied yet to the study for microbial communities subjected to BACs and thus, holds great potential to provide new insights into the issues discussed above compared to what has been done by culture-based approaches.

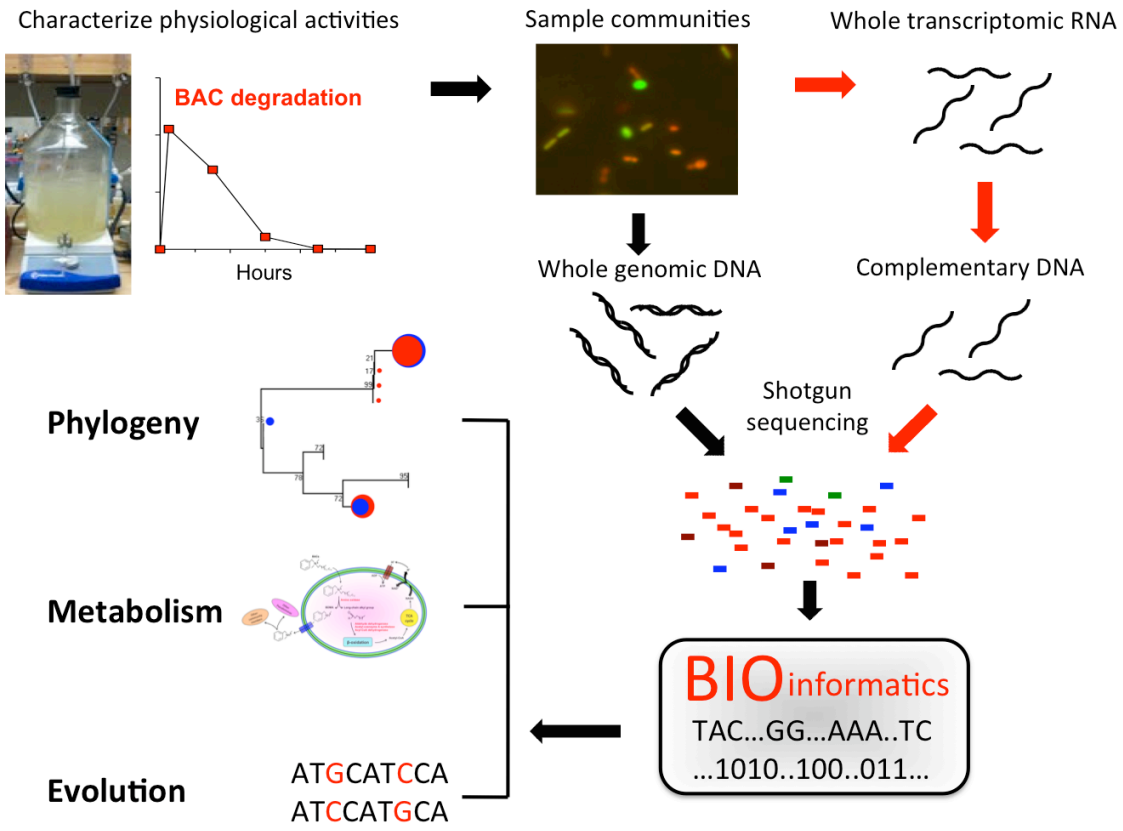


Figure 1.2. Graphical representation of metagenomic and metatranscriptomic approach to study microbial consortia degrading BACs

1.6. Thesis organization

This study was carried out under a collaborative, NSF-funded project entitled "Disinfectant-Induced Antibiotic Resistance: Relevance, Mechanisms and Practical Considerations", (NSF-CBET, Award #0967130, PI: S. G. Pavlostathis, Co-PI: K. T. Konstantinidis). The microbial communities analyzed in this study were originally established in (145) and maintained by the Pavlostathis laboratory. To characterize the phylogenetic diversity, function, evolution, and adaptation of these communities and their key isolates, comparative (meta-) genomic techniques were employed, as described previously (105, 106, 108). These techniques were also coupled with metatranscriptomics, genetics, and other traditional culture-dependent approaches to accomplish the following specific objectives based on the microbial communities maintained in the Pavlostathis laboratory.

Chapter 2 describes microbial community adaptation to BACs, including both resistance to and degradation of BACs. To this end, microbial communities exposed to varied concentrations of BACs for three years were sampled. Metagenomics quantitatively assessed the effect of BACs on the phylogenetic diversity and metabolic potential of the target communities and revealed the molecular mechanisms that facilitate microbial community adaptation to BACs. The latter findings were also confirmed based on studies of representative isolates from the target communities.

Chapter 3 examines time-course transcriptome responses of a microbial community degrading a high concentration of BACs (50 mg/L) as a sole source for carbon and energy. Metatranscriptomics coupled with genome sequencing of key isolates suggested specific species, their syntrophic interactions, and enzyme genes involved in

community BAC degradation. The results of this chapter complement the DNA-based findings from Chapter 2.

Chapter 4 addresses the issue of whether or not widely used BAC disinfectants induce antibiotic resistance, which has remained highly debatable for several decades based on previous literature. Using a combination of antimicrobial susceptibility testing, (meta-) genome analysis, and molecular genetic characterization of microbial communities and their isolates (i.e., BAC-exposed vs. unexposed or control), the underlying molecular mechanisms promoting antibiotic resistance in the presence of BACs were elucidated. This study provides convincing molecular evidence for the role of BAC disinfectants in propagating antibiotic resistance.

Chapter 5 summarizes the key findings of this research (chapters 2 through 4) on the effects and fate of BACs on microbial communities and discusses the implications of the conducted work for regulatory strategies and treatment of BAC-bearing waste stream and future works.

CHAPTER 2

MICROBIAL COMMUNITY ADAPTATION TO QUATERNARY AMMONIUM BIOCIDES AS REVEALED BY METAGENOMICS

This chapter was published in:

Seungdae Oh, Madan Tandukar, Spyros G. Pavlostathis, Patrick S. G. Chain, and Konstantinos T. Konstantinidis. Microbial community adaptation to quaternary ammonium biocides as revealed by metagenomics. *Environmental Microbiology.* DOI:10.1111/1462-2920.12154, 2013.

2.1. Summary

Quaternary ammonium compounds (QACs) represent widely used cationic biocides that persist in natural environments. Although microbial degradation, sensitivity, and resistance to QACs have been extensively documented, a quantitative understanding of how whole communities adapt to QAC exposure remains elusive. To gain insights into these issues, we exposed a microbial community from a contaminated river sediment to varied levels of benzalkonium chlorides (BACs, a family of QACs) for three years. Comparative metagenomic analysis showed that the BAC-fed communities were dramatically decreased in phylogenetic diversity compared to the control (no BAC exposure), resulting presumably from BAC toxicity, and dominated by *Pseudomonas* species (>50% of the total). Time-course metagenomics revealed that community adaptation occurred primarily via selective enrichment of BAC-degrading *Pseudomonas* populations, particularly *P. nitroreducens*, and secondarily via amino acid substitutions and horizontal transfer of a few selected genes in the *Pseudomonas* populations,

including a gene encoding a PAS/PAC sensor protein and ring-hydroxylating dioxygenase genes. *P. nitroreducens* isolates were reproducibly recovered from communities after prolonged periods of no-BAC exposure, suggesting that they are robust BAC-degraders. Our study provides new insights into the mechanisms and tempo of microbial community adaptation to QAC exposure and has implications for treating QACs in biological engineered systems.

2.2 Introduction

Quaternary ammonium compounds (QACs) are cationic surfactants commonly used as disinfectants, dispersants, and surface treatment agents, in a variety of environmental and clinical settings. QACs persist in natural and engineered biological systems, where they can be toxic for life. Concentrations of representative QACs such as dialkyldimethyalammonium compounds and benzalkonium chlorides (26 and 1.5 mg/kg, respectively) were found to be higher than other conventional organic pollutants (e.g., polycyclic aromatic hydrocarbons) in river sediment (77) and combined concentrations of dialkyldimethyalammonium compounds and benzalkonium chlorides were also significant (1.5 to 4 mg/L) in wastewater from hospitals (88). QACs are cell-membrane active agents and cause toxicity to organisms by primarily inhibiting cell membrane functionality. QACs significantly decreased the growth and heterotrophic activity (i.e., substrate utilization) of lake microbial communities (0.03 to 0.1 mg/L ditallowdimethylammonium chloride) (147, 151), activated sludge (28 mg/L trimethylammonium chloride causing 50% inhibition) and methanogenic and nitrate-reducing communities (50 mg/L didecyldimethylammonium chloride) (45, 144). Since the majority of QACs are released into wastewater streams through sewage disposal and

QACs can potentially be detoxified by microbial consortia (e.g., activated sludge) (103, 120, 135), microbial degradation of QACs is a desired process to reduce QAC toxicity in biological engineered systems and the environment.

A number of previous studies have characterized QAC degradation by pure or mixed cultures. Bacterium 5H2 and obligate/facultative methylotrophs can degrade tetramethyl ammonium chloride using an assortment of monooxygenase, demethylase, and amine dehydrogenase enzymes (25, 30). *Aeromonas hydrophila* species can transform benzalkonium chlorides into benzaldehyde through N-dealkylation (dehydrogenase) and N-demethylation (amine oxidase) (111). Alkyldimethylammonium salts are biologically converted to trimethylamine by N-dealkylation and further to dimethylamine and methylamine by N-demethylation in activated sludge systems (103). Despite several studies on the organisms, pathways, and enzymes involved in the QAC degradation, our understanding of whole community adaptation to QAC exposure is limited. Little is known about the relative importance of the molecular mechanisms responsible for adaptation, e.g., horizontal gene transfer vs. point mutations and clonal amplification, while most of the previous studies have been performed with pure cultures. Further, although the enzymes responsible for QAC degradation have been determined biochemically and/or enzymatically, the corresponding gene sequences encoding such enzymes remain unknown. In this study, we employed comparative metagenomics to provide new insights into the adaptation, diversity, and metabolic potential of microbial communities exposed to QACs.

Three microbial communities were developed in laboratory bioreactors under aerobic conditions, inoculated with a contaminated river sediment: one with

benzalkonium chlorides (BACs, a family of QACs) as a sole carbon and energy source (B community), one with BACs plus dextrin/peptone (DPB community), and a dextrin/peptone-fed one (DP community, control). Our previous study of the B community provided biochemical and kinetic evidence that BACs are transformed to benzyldimethylamine (BDMA), which is about 500 times less toxic than BACs and further to dimethylamine and benzoic acid by debenzylation (145). In the present study, isolates and community DNA samples were characterized from each of the three bioreactors following three years of exposure to BACs. Comparative analysis of the B metagenome against those originating from DPB and DP communities uncovered shifts in community phylogenetic structure and functional profile upon BAC exposure and key BAC-degrading populations. In addition, a new BAC-fed community was established with inoculum from the control DP community (i.e., the one that had been developed on DP for three years) and sampled over time to assess how fast and reproducibly community adaptation to BACs took place. Taken together, our results provide new insights into community dynamics, resilience, and molecular mechanisms of adaptation that occur upon BAC exposure.

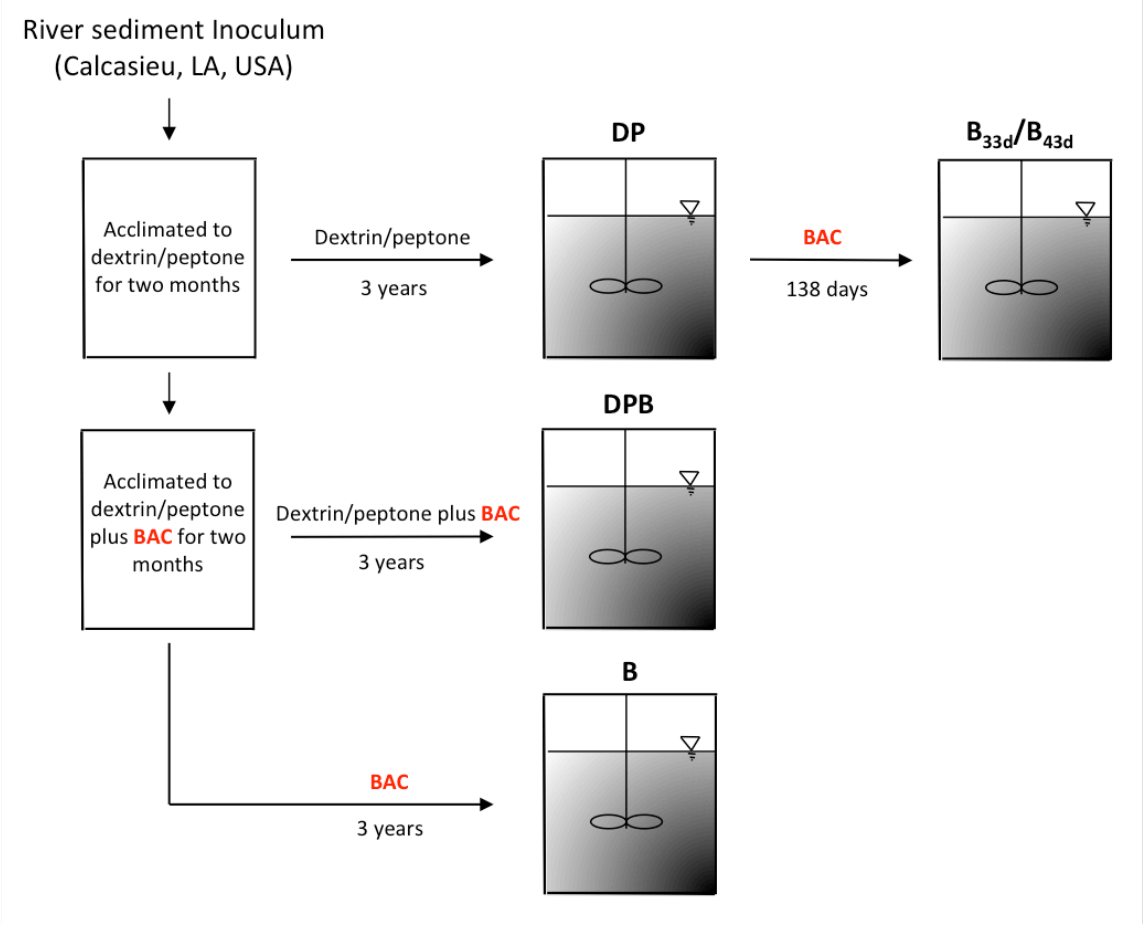


Figure 2.1. A schematic representation of the developmental history of each community. The substrates fed to and the age of each community are shown above and below the arrows, respectively.

2.3. Results

2.3.1. Long-term community adaptation to BACs

The communities analyzed in this study originated from a river sediment inoculum (Calcasieu River, LA), as described previously (145) and shown in Fig. 2.1. The control community (DP) was established with dextrin/peptone under aerobic conditions (fed-batch bioreactor). The DBP community was established as a subculture of the DP community by feeding dextrin/peptone plus BACs after two months of the establishment of the DP community. The majority of the carbon source in the DPB bioreactor was dextrin/peptone (2,200 mg COD/L - Chemical Oxygen Demand) rather than the BACs (150 mg COD/L). After two months of DPB community establishment, the B community was subsequently established using an inoculum from the DPB community by feeding BACs only as a sole carbon and energy source. The three microbial communities were maintained at room temperature in parallel for three years after their establishment, in aerobic fed-batch bioreactors with a 14 days residence time. BAC susceptibility of each evolved community was measured by minimum inhibitory concentrations (MICs) at the end of the 3rd year. The BAC MIC values were 100, 250, and 460 mg/L for the DP, DPB and B communities, respectively, revealing reduced BAC susceptibility for the BAC-fed DPB and B communities. The phenotypic differences observed called for further (metagenomic) investigations of the phylogenetic and functional differences among these communities that essentially originated from the same inoculum.

Table 2.1. Relative abundances of OTUs in the three microbial communities. The 911, 920, and 864 16S rRNA gene pyrosequences obtained from the DP, DPB, and B communities, respectively, were pooled together and clustered in a total of 168 OTUs. OTUs are listed by their average abundance in the three communities (high to low). Relative abundance (%) was defined as the number of pyrosequences from a community showing at least 97% nucleotide sequence identity to an OTU (only best matches were considered) divided by the total number of pyrosequences from the community. *NA-Not Available (i.e., not assignable to a taxon).

OTU	OTU lower taxonomic	Phylum	DP	DPB	B
1	Pseudomonas	Proteobacteria	0	1.4	58.6
2	Flavobacteriaceae	Bacteroidetes	0.5	43.3	0
3	Pseudochrobactrum	Proteobacteria	5.7	20	0
4	Enterobacteriaceae	Proteobacteria	0.1	23.2	0
5	Phycisphaerales	Planctomycetes	17	0	0
6	Sphingobacteriales	Bacteroidetes	12.2	0	0
7	Sphingobacterium	Bacteroidetes	7.1	3.5	0
8	Propionicimonas	Actinobacteria	10.3	0	0
9	Sphingobacteriales	Bacteroidetes	0	0	9.7
10	Sphingobacteriales	Bacteroidetes	7.1	0	0
11	Paucibacter	Proteobacteria	0	0	6.8
12	Caulobacteraceae	Proteobacteria	6	0	0
13	Flavobacterium	Bacteroidetes	0	0.3	5.6
14	Bacteroidetes	Bacteroidetes	0	0	5.8
15	Microbacterium	Actinobacteria	3	0	0
16	Enterobacteriaceae	Proteobacteria	0.5	1.7	0
17	Mitsuaria	Proteobacteria	0	0	2
18	Mycoplana	Proteobacteria	1.8	0	0
19	Pseudoxanthomonas	Proteobacteria	1.7	0	0
20	Rhodocyclus	Proteobacteria	1.7	0	0
21	Bosea	Proteobacteria	1.6	0	0
22	Mycobacterium	Actinobacteria	0	0	1.6
23	Propionicimonas	Actinobacteria	1.4	0	0
24	Flavobacteriaceae	Bacteroidetes	0	1.3	0
25	Mesorhizobium	Proteobacteria	1.3	0	0
26	Hylemonella	Proteobacteria	0.2	1	0
27	Elizabethkingia	Bacteroidetes	0	0	1.2
28	Cytophaga	Bacteroidetes	1.1	0	0
29	HN1	Chloroflexi	1.1	0	0
30	Sphingobacterium	Bacteroidetes	1	0	0
31	Alphaproteobacteria	Proteobacteria	1	0	0
32	Shinella	Proteobacteria	1	0	0
33	Acetobacteraceae	Proteobacteria	1	0	0

Table 2.1. (Continued)

34	Bradyrhizobiaceae	Proteobacteria	0	0	0.9
35	Bdellovibrio	Proteobacteria	0	0	0.9
36	Trichococcus	Firmicutes	0.8	0	0
37	Bacteroidetes	Bacteroidetes	0	0	0.8
38	Gemmatimonas	Gemmatimonadetes	0.8	0	0
39	Labrys	Proteobacteria	0	0	0.8
40	Sphingobacteriales	Bacteroidetes	0.8	0	0
41	Sphingobacterium	Bacteroidetes	0	0.7	0
42	Agrobacterium	Proteobacteria	0	0.7	0
43	Methylobacterium	Proteobacteria	0	0	0.6
44	Bacteria	NA	0	0	0.6
45	Prosthecobacter	Verrucomicrobia	0.6	0	0
46	Sphingobacteriales	Bacteroidetes	0.5	0	0
47	Actinomycetales	Actinobacteria	0.5	0	0
48	Sphingobacteriales	Bacteroidetes	0.5	0	0
49	Sphingobacteriales	Bacteroidetes	0.5	0	0
50	Sphingobacterium	Bacteroidetes	0.2	0.2	0
51	Sphingobacterium	Bacteroidetes	0.4	0	0
52	Bacteria	NA	0.4	0	0
53	Sphingobacteriales	Bacteroidetes	0.4	0	0
54	Flavobacteriaceae	Bacteroidetes	0	0	0.4
55	Bacteroidetes	Bacteroidetes	0.4	0	0
56	Bacteria	NA	0	0	0.4
57	Haloanella	Bacteroidetes	0	0.3	0
58	Mesorhizobium	Proteobacteria	0.3	0	0
59	Sphingobacteriales	Bacteroidetes	0.3	0	0
60	Alcaligenaceae	Proteobacteria	0	0.2	0
61	Paenibacillus	Firmicutes	0	0.2	0
62	Bacteria	NA	0.2	0	0
63	Chloracidobacteria	Acidobacteria	0.2	0	0
64	Thiobacillus	Proteobacteria	0.2	0	0
65	Bacteria	NA	0.2	0	0
66	Sphingobacteriaceae	Bacteroidetes	0.2	0	0
67	Propionicimonas	Actinobacteria	0.2	0	0
68	Bacteria	NA	0.2	0	0
69	OPB56	Chlorobi	0.2	0	0
70	CandidatusSolibacter	Acidobacteria	0.2	0	0
71	Pseudomonadaceae	Proteobacteria	0	0	0.2
72	Sphingomonadaceae	Proteobacteria	0.2	0	0
73	Enterobacteriaceae	Proteobacteria	0	0.1	0
74	Pseudochrobactrum	Proteobacteria	0	0.1	0
75	Gammaproteobacteria	Proteobacteria	0	0.1	0
76	Bacteria	NA	0	0.1	0
77	Proteobacteria	Proteobacteria	0	0.1	0
78	Defluvibacter	Proteobacteria	0	0.1	0

Table 2.1. (Continued)

79	Isosphaeraceae	Planctomycetes	0	0.1	0
80	Rhizobiales	Proteobacteria	0	0.1	0
81	Cohnella	Firmicutes	0	0.1	0
82	Pseudochrobactrum	Proteobacteria	0	0.1	0
83	Flavobacterium	Bacteroidetes	0	0.1	0
84	Cohnella	Firmicutes	0	0.1	0
85	Bacillales	Firmicutes	0	0.1	0
86	Stenotrophomonas	Proteobacteria	0	0.1	0
87	Paenibacillus	Firmicutes	0	0.1	0
88	Paenibacillus	Firmicutes	0	0.1	0
89	Rhizobiales	Proteobacteria	0	0.1	0
90	Clostridiales	Firmicutes	0	0.1	0
91	Streptococcus	Firmicutes	0	0.1	0
92	Enterobacteriaceae	Proteobacteria	0.1	0	0
93	Bacteroidetes	Bacteroidetes	0	0	0.1
94	Bacteria	NA	0.1	0	0
95	Alcaligenaceae	Proteobacteria	0.1	0	0
96	Sphingomonadaceae	Proteobacteria	0.1	0	0
97	Pigmentiphaga	Proteobacteria	0.1	0	0
98	Comamonadaceae	Proteobacteria	0.1	0	0
99	Bacteria	NA	0.1	0	0
100	Sphingobacterium	Bacteroidetes	0.1	0	0
101	Rhizobiales	Proteobacteria	0	0	0.1
102	Stenotrophomonas	Proteobacteria	0	0	0.1
103	Pseudomonadaceae	Proteobacteria	0	0	0.1
104	Bacteria	NA	0	0	0.1
105	Azotobacter	Proteobacteria	0	0	0.1
106	Bacteria	NA	0	0	0.1
107	Bacteria	NA	0	0	0.1
108	Roseomonas	Proteobacteria	0.1	0	0
109	Bacteria	NA	0.1	0	0
110	Bacteria	NA	0	0	0.1
111	Sphingobacteriales	Bacteroidetes	0.1	0	0
112	Bacteria	NA	0.1	0	0
113	Sphingobacteriales	Bacteroidetes	0	0	0.1
114	OPB56	Chlorobi	0.1	0	0
115	OPB56	Chlorobi	0.1	0	0
116	Bacillales	Firmicutes	0.1	0	0
117	Rhizobiales	Proteobacteria	0	0	0.1
118	Trichococcus	Firmicutes	0.1	0	0
119	Actinomyces	Actinobacteria	0.1	0	0
120	Xanthomonadaceae	Proteobacteria	0	0	0.1
121	Bacteria	NA	0	0	0.1
122	Azotobacter	Proteobacteria	0	0	0.1
123	Sphingobacteriales	Bacteroidetes	0.1	0	0

Table 2.1. (Continued)

124	Actinomycetales	Actinobacteria	0.1	0	0
125	Flavobacteriaceae	Bacteroidetes	0	0	0.1
126	Rhizobiales	Proteobacteria	0.1	0	0
127	OPB56	Chlorobi	0.1	0	0
128	Bacteria	NA	0.1	0	0
129	Devosia	Proteobacteria	0	0	0.1
130	Bacteria	NA	0.1	0	0
131	Alphaproteobacteria	Proteobacteria	0.1	0	0
132	Pseudomonadaceae	Proteobacteria	0	0	0.1
133	Pseudochrobactrum	Proteobacteria	0.1	0	0
134	Proteobacteria	Proteobacteria	0	0	0.1
135	Bacteria	NA	0	0	0.1
136	Sphingobacteriales	Bacteroidetes	0.1	0	0
137	Flavobacteriaceae	Bacteroidetes	0	0	0.1
138	Rhodocyclus	Proteobacteria	0.1	0	0
139	Bacteria	NA	0.1	0	0
140	CH21	Armatimonadetes	0.1	0	0
141	Pseudoxanthomonas	Proteobacteria	0.1	0	0
142	Bacteria	NA	0.1	0	0
143	Proteobacteria	Proteobacteria	0	0	0.1
144	Gemmata	Planctomycetes	0	0	0.1
145	Sphingomonadaceae	Proteobacteria	0.1	0	0
146	Proteobacteria	Proteobacteria	0.1	0	0
147	Granulicatella	Firmicutes	0.1	0	0
148	Sphingobacteriales	Bacteroidetes	0.1	0	0
149	Bacteria	NA	0.1	0	0
150	Proteobacteria	Proteobacteria	0.1	0	0
151	Actinomycetales	Actinobacteria	0	0	0.1
152	Bacteria	NA	0	0	0.1
153	Bacteria	NA	0.1	0	0
154	Alphaproteobacteria	Proteobacteria	0.1	0	0
155	Phyllobacteriaceae	Proteobacteria	0	0	0.1
156	Sphingobacteriales	Bacteroidetes	0.1	0	0
157	Tessaracoccus	Actinobacteria	0.1	0	0
158	Sphingobacteriales	Bacteroidetes	0.1	0	0
159	Trichococcus	Firmicutes	0.1	0	0
160	Bacteria	NA	0	0	0.1
161	Flavobacteriaceae	Bacteroidetes	0	0	0.1
162	Bacteria	NA	0	0	0.1
163	HN1	Chloroflexi	0.1	0	0
164	OPB56	Chlorobi	0.1	0	0
165	Alphaproteobacteria	Proteobacteria	0.1	0	0
166	Acinetobacter	Proteobacteria	0.1	0	0
167	Sphingobacteriales	Bacteroidetes	0.1	0	0
168	Veillonella	Firmicutes	0.1	0	0

2.3.2. Shifts in phylogenetic community structure upon BAC exposure

The phylogenetic community structure of the three communities was assessed using both metagenomic and 16S rRNA gene amplicon pyrosequencing approaches (Fig. 2.2A; all 16S rRNA gene-based OTU abundance data are available in Table 2.1). Best-match analysis of metagenomic reads against all available archaeal and bacterial genomes revealed a very small number of archaeal sequences (<0.1% of total) in the DP metagenome, indicating that the community comprised, almost entirely, bacteria. This analysis also revealed that *Proteobacteria* (71.8%) dominated the DP community, followed by *Actinobacteria* (18.1%), *Bacteroidetes* (2.9%), and *Firmicutes* (2.3%). The 16S rRNA gene pyrosequence data indicated that the dominant phyla were *Bacteroidetes* (34%), followed by *Proteobacteria* (26%), *Actinobacteria* (15.7%), and *Firmicutes* (1.3%). Despite the long-term maintenance under laboratory conditions, substantial diversity was revealed within the DP community (e.g., at least 90 distinct OTUs detected; Fig. 2.3) and relative abundance and phylogenetic diversity of dominant phyla were similar to those of the river sediment that served as inoculum (5, 12, 136).

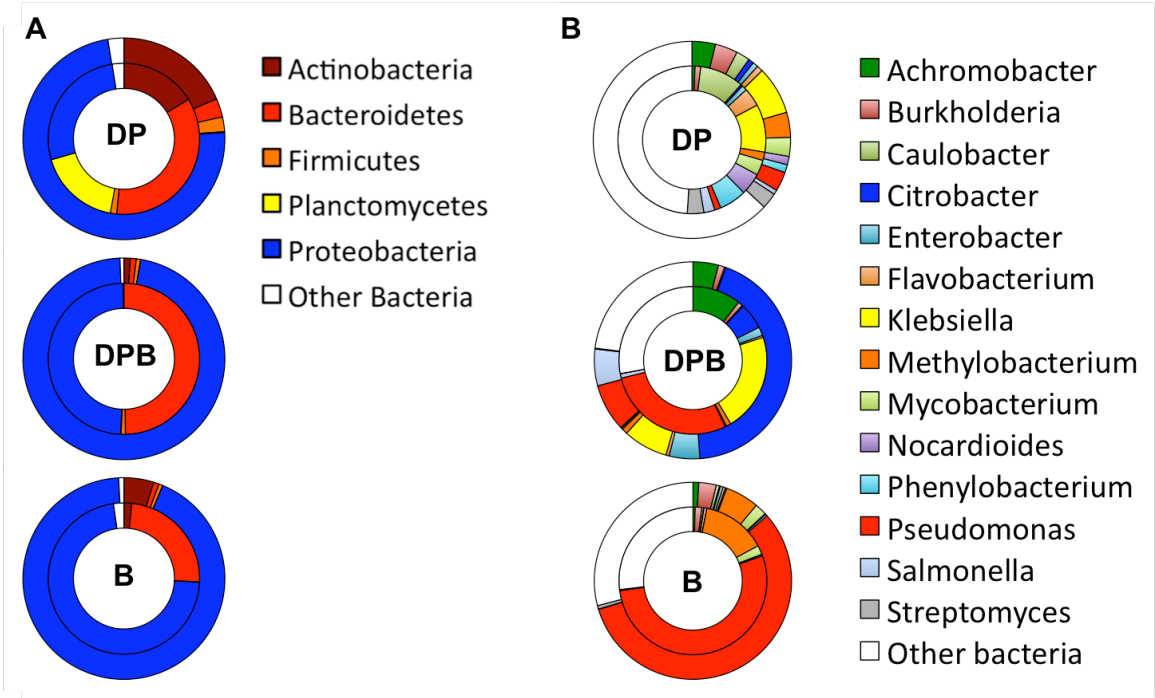


Figure 2.2. Phylogenetic composition of microbial communities. (**A**) Colors represent the major phyla (>2% of the total community; see key) based on best match Blastn analysis of metagenomic reads (outer circle) and 16S rRNA gene pyrosequences (inner circle). (**B**) Colors represent the major genera (>3% of the total) based on metagenomic reads (nucleotide level; outer circle) and amino acid sequences of annotated genes (amino acid level; inner circles). 'Other bacteria' represents the combined fraction of the remaining minor taxa.

Comparing the DP metagenome against the DPB and B ones revealed a dramatic decrease in community diversity in the BAC-fed communities. The DP metagenome contained many sequences assigned to minor genera (i.e., each <3% of the total; making up together 74.2% of the community) compared to those of the DPB (22.9%) and the B (33.7%) metagenome (Fig. 2.2B), revealing that BAC exposure led to the enrichment of a few dominant genera and/or inhibition of the other genera. Consistent with these interpretations, the Shannon diversity index and the number of OTUs (per 800 sequences; OTUs defined at the 97% sequence identity level) significantly decreased from the DP community (3.16±0.04 and 89.7±4.27, respectively) to the DPB (1.59±0.04 and 29.7±3.53, respectively) and the B (1.74±0.03 and 42.0±2.59, respectively) community (Fig. 2.3).

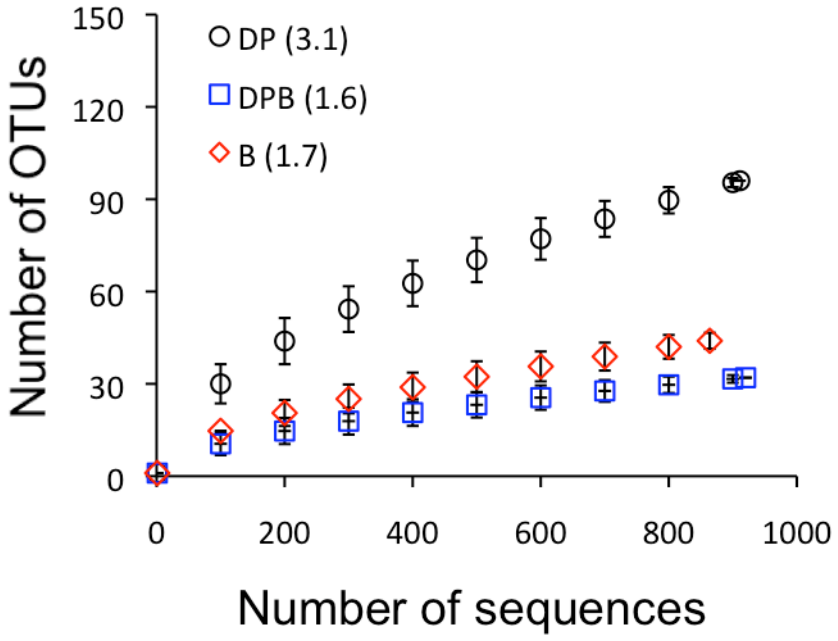


Figure 2.3. Rarefraction curves of 16S rRNA gene amplicon sequences recovered from each community. Curves represent the number of unique OTUs recovered (vertical axis), defined at the 97% nucleotide sequence identity level, for the number of sequences analyzed (horizontal axis) and reflect the extent of OTU diversity within each community (figure key) and what fraction of this diversity was sampled. Curves were produced in MOTHUR (125), using 1000 permutations. Error bars indicate one standard deviation from the mean. The Shannon diversity indices are shown in parentheses and were calculated using the following equation: $H=-\sum pi(ln(pi))$, where pi is the percentage of sequences assigned to an OTU divided by the total number of sequences. Note that the number of sequences obtained almost saturated the OTU diversity within the communities and that the DP community was more diverse than the BAC-fed ones.

In the DPB and the B metagenome, *Proteobacteria* were overrepresented (71.8% of total in DP vs. 96.5% and 92.3% in DPB and B, respectively), while *Actinobacteria* (18.1% of total in DP vs. 1.0% and 4.8% in DPB and B, respectively) and *Firmicutes* (2.3% of total in DP vs. 0.7% and 0.6% in DPB and B, respectively) were underrepresented (Fig. 2.2A). At the genus level (Fig. 2.2B), both BAC-fed communities were enriched in populations affiliated with the *Pseudomonas* genus (7.8% for DPB and 56.8% for B). The taxonomic affiliation of the longest 100 contigs assembled from the B metagenome revealed only five contigs that were not affiliated with *Pseudomonas* genera (Fig. 2.4). The DPB community was dominated by members of the *Enterobacteriaceae* family based on the metagenomic whole genome data, most notably, *Citrobacter* (43.5%), *Klebsiella* (7.3%), *Salmonella* (6.0%), and *Enterobacter* (5.0%), and *Achromobacter* (4.2%), in addition to *Pseudomonas* (7.8%).

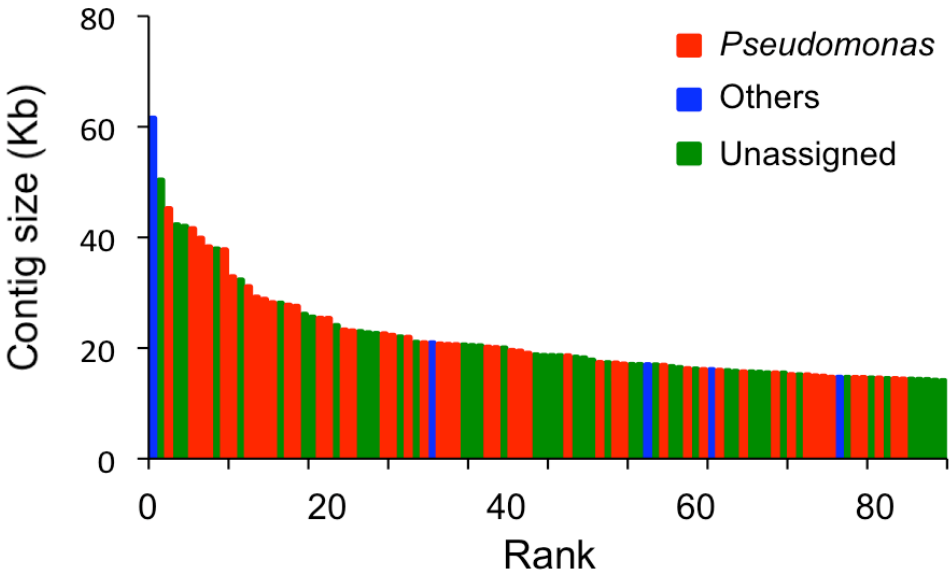


Figure 2.4. Phylogenetic affiliation of the longest contigs assembled from the B metagenome. The longest 100 contigs were ranked by their length. Note that 48 of the contigs were phylogenetically affiliated with *Pseudomonas* based on best match Blastp analysis of the encoded protein sequences against all available genome sequences in NCBI database; five with other genera and the remaining 47 remained unassigned at the genus level.

Consistent with the results based on the metagenomes, analysis of 16S rRNA gene pyrosequences also revealed the dominance of *Pseudomonas*-affiliated OTUs (OTU ID: 1 in Table 2.1) in the B (58.6%) community and *Enterobacteriaceae* (OTU ID: 4) in the DPB (23.2%) community. Further, the estimated average genome size (AGS) and G+C% content of the BAC-fed communities were consistent with those of the genera found to be abundant in the corresponding 16S rRNA gene and metagenomic datasets (Table 2.2). The DPB and the B metagenome showed larger AGS (5.0 Mbp and 4.8 Mbp, respectively) than the DP one (3.6 Mbp), reflecting the relatively larger genome size of *Citrobacter* (~4.5 Mbp; average of all available *Citrobacter* genomes in GenBank) and *Pseudomonas* (~6.5 Mbp), which dominated the DPB and B communities, respectively. The G+C% content of the DPB and the B metagenomes were 52.4% and 60.0%, which were comparable to those of the sequenced *Citrobacter* (~52%) and *Pseudomonas* (~64%), respectively.

Table 2.2. Statistics of the metagenomes used in this study. [a] Only contigs and genes longer than 300 bases were counted. [b]The average genome size (AGS) of a metagenome was estimated as described previously (117)

Metagenome characteristic	DP	DPB	B	B_{33d}	B_{43d}
Sequences (Mb)	357	1,145	717	1,068	1,471
G+C content (%)	56.6	52.4	60.0	59.6	62.4
No. of contigs[a]	15,557	44,037	31,336	31,226	45,424
N50 of contigs (b)[a]	772	969	1,791	1,737	2,165
No. of ORFs[a]	14,141	45,570	41,128	39,062	51,711
Avg genome size (Mb)[b]	3.6	5.0	4.8	-	-

2.3.3. Community metabolic adaptation to BACs

Functional annotation of genes using the Pfam database revealed that the BAC-fed communities were significantly enriched ($P < 10^{-4}$ by Kolmogorov-Smirnov test) in functions associated with i) transformation of BACs and their byproducts, and ii) antimicrobial resistance (Fig. 2.5). An array of genes encoding monooxygense, dehydrogenase (formaldehyde, aldehyde, formate, amine, and acyl-CoA), citrate lyase, amine oxidase, and aromatic-ring opening dioxygenase was consistently overrepresented, 1.3 to 12 fold, in BAC-fed communities compared to the control DP community. Several of these genes may encode the enzymes that have been biochemically associated with the proposed BAC degradation pathways (25, 78, 101, 111, 150). Genes associated with antimicrobial resistance were also overrepresented, at least 1.6 fold, in BAC-fed communities, including efflux pumps (e.g., HlyD secretion protein family and small multidrug resistance protein), cell envelope modifications, chaperones, and oxidative stress defense systems. Genes related to modification and biosynthesis of the cell envelope, the primary target of BAC toxicity, such as lipid A 3-O-deacylase (2.2 and 1.9 fold in the B and the DPB communities, respectively), SmpA/OmlA protein family (2.5 and 2.1 fold), lipocalin (4.3 fold in both communities) and carbohydrate-selective porin (12.3 fold in both communities) were also enriched. Finally, genes associated with oxidative stress defense systems, which are likely involved in mediating BAC-induced oxidative damage (97), such as glutathione S-transferase (3.1 and 3.4 fold for the N-terminal domain and 7.1 and 5.5 fold for the C-terminal domain, respectively), glutathione synthetase (4 and 5.5 fold), glutaredoxin (2.2 and 2.5 fold), and chaperone

(POTRA domain, ShlB-type, 2.0 fold in both communities) were significantly more abundant in BAC-fed vs. DP communities.

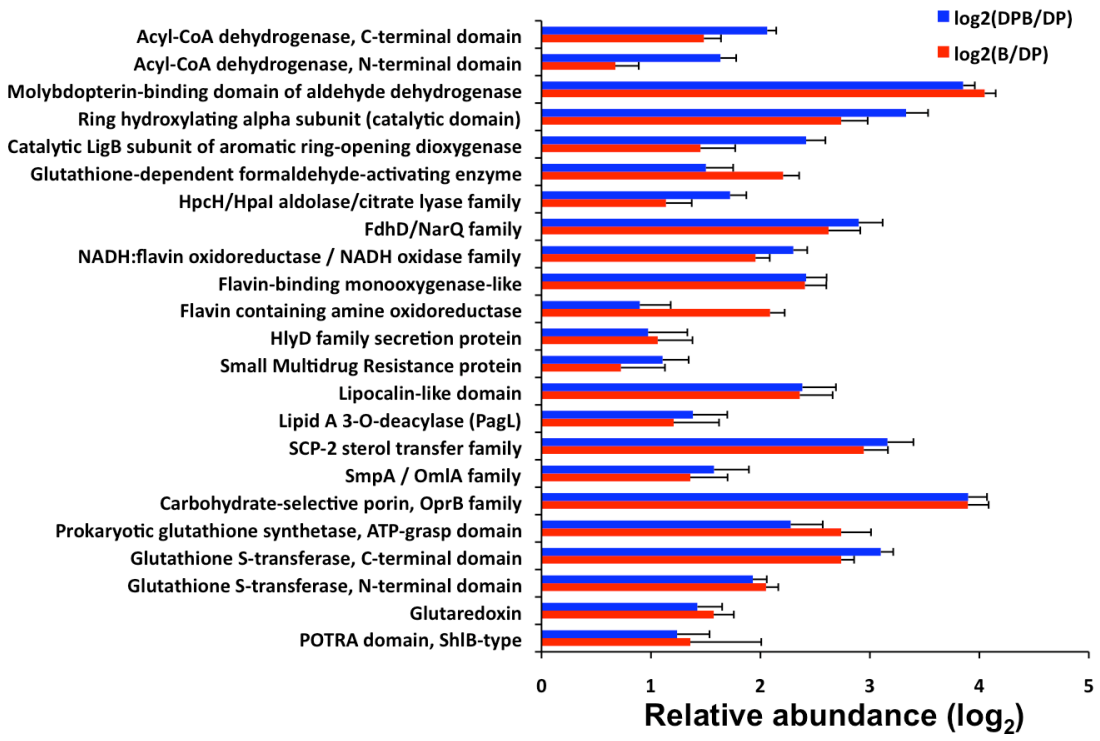


Figure 2.5. Functional annotation of genes enriched in the BAC-fed communities. The Pfam protein families significantly enriched ($P < 10^{-4}$, Kolmogorov-Smirnov test) in the BAC-fed (i.e., DPB or B) relative to the control (i.e., DP) communities are shown. The bar represents the mean fold change (logarithmic scale) and the error bar represents one standard deviation from the mean based on 100 replicate subsamples (See details in Materials and Methods).

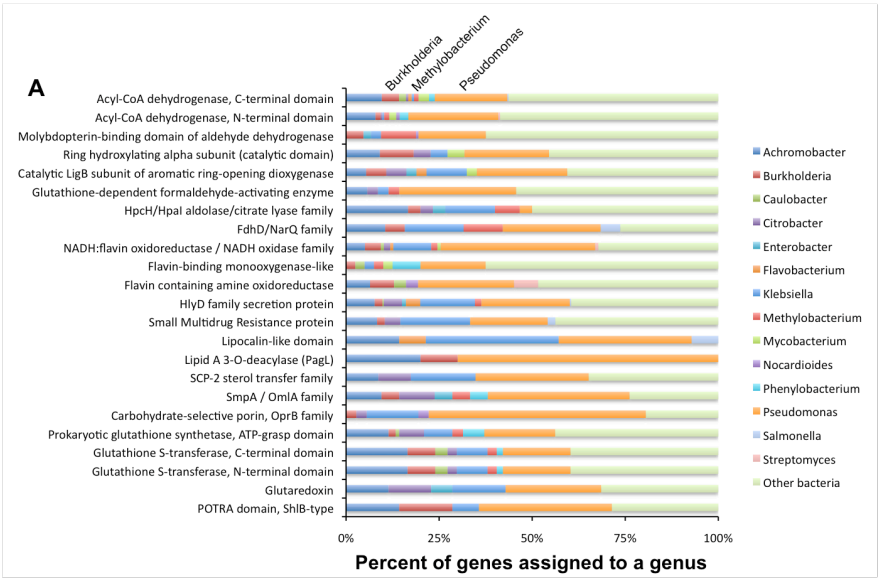
COG functional category analysis of genes recovered in the metagenomes revealed that the control DP community was characterized by a high abundance of carbohydrate transport and metabolism genes potentially related to the utilization of the dextrin/peptone substrate and functions involved in replication and translation (Table 2.3); the latter being presumably attributable to its smaller genome size relative to the BAC-fed communities (Table 2.2). The metabolic genes enriched in DPB and B communities relative to the control DP community, especially genes related to secondary metabolite catabolism (3.7% and 3.9% vs. 2.2%, respectively), reflected the organic substrates available and/or the gene complement of the species selected by these substrates. For example, BACs contain a benzyl group and this was reflected by the high abundance of aromatic compound degradation genes (secondary metabolism) in BAC-fed communities.

Table 2.3. Functional analysis of the protein sequences recovered in each metagenome based on the COG database. The table shows the percent of total genes in the metagenome assigned to each COG category (rows) for each of the three communities (columns).

COG categories	DP	DPB	B
Secondary metabolites biosynthesis, transport and catabolism	2.2	3.7	3.9
Nucleotide transport and metabolism	3.0	1.8	2.0
Lipid transport and metabolism	4.0	5.3	4.6
Inorganic ion transport and metabolism	6.9	7.9	7.2
Energy production and conversion	7.4	7.2	6.9
Coenzyme transport and metabolism	3.6	3.3	3.1
Carbohydrate transport and metabolism	9.0	6.6	5.5
Amino acid transport and metabolism	10.5	13.2	12.2
Signal transduction mechanisms	4.1	4.4	6.1
Posttranslational modification, protein turnover, chaperones	3.6	3.0	3.4
Intracellular trafficking and secretion	1.8	2.2	2.3
Defense mechanisms	2.4	1.8	2.1
Cell wall/membrane biogenesis	5.2	4.9	4.9
Cell motility	0.8	1.4	1.9
Cell cycle control, mitosis and meiosis	0.8	0.6	0.7
Translation, ribosomal structure and biogenesis	6.3	3.5	4.0
Transcription	5.9	6.9	6.7
Replication, recombination and repair	6.7	3.8	4.6
General function prediction only	10.7	11.9	11.8
Function unknown	5.0	6.6	6.0

2.3.4. Population structure of the BAC-selected *Pseudomonas*

To examine the phylogenetic affiliation of the genes enriched in the two BAC-fed communities, a best match Blastp search of the amino acid sequences of the proteins shown in Figure 2.2 against all available archaeal and bacterial genomes was performed. Many of the genes were phylogenetically affiliated with the dominant genus, *Pseudomonas* (Fig. 2.6).



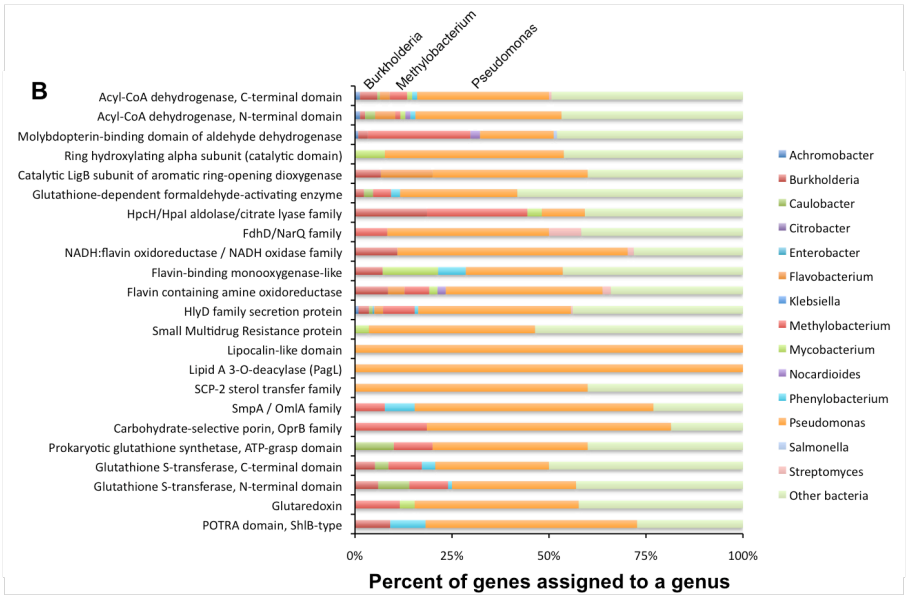


Figure 2.6. Phylogenetic affiliation of the protein functions enriched in the BAC-fed communities. The protein functions significantly enriched ($P < 10^{-4}$, Kolmogorov-Smirnov test) in DPB (**A**) and B (**B**) communities relative to the DP community were taxonomically classified at the genus level based on their best Blastp match against all available genome sequences in NCBI database. The graph shows the fraction of selected proteins assigned to each genus (figure key). The proteins shown are the same as those in Figure 2.5.

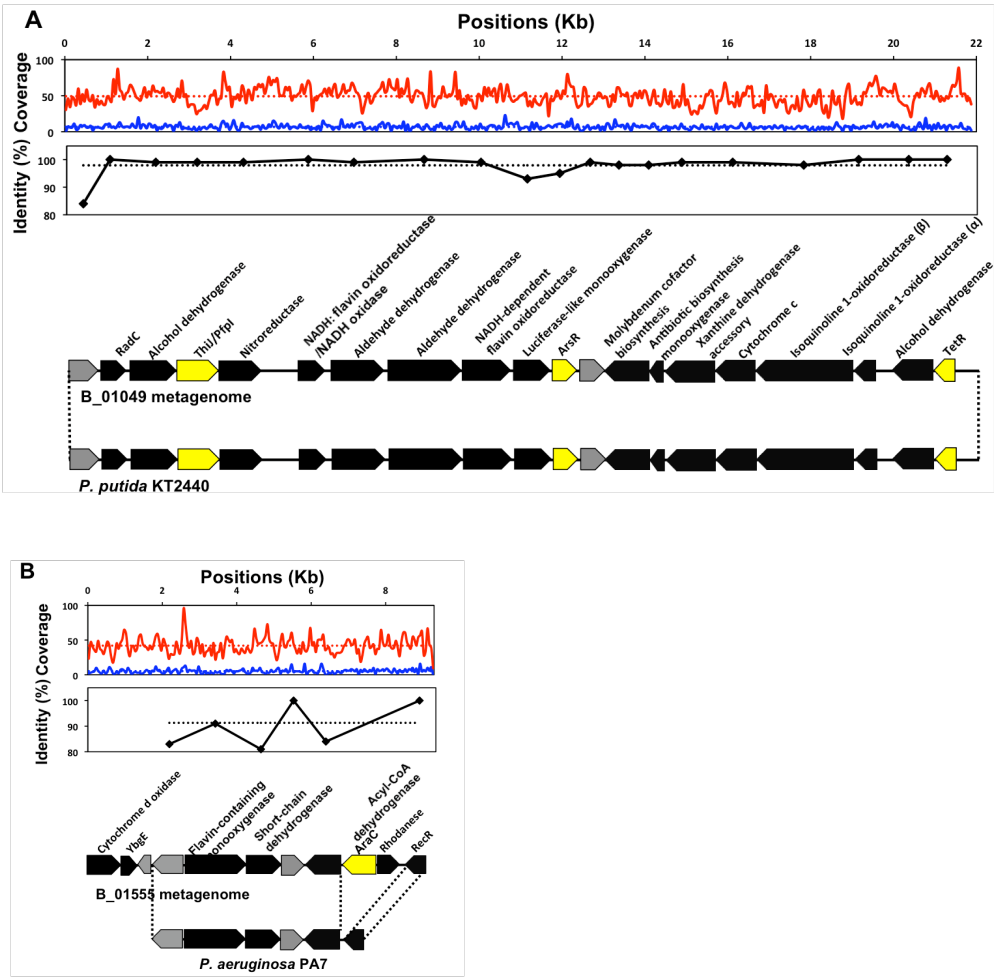


Figure 2.7. Comparison of representative contigs from the B metagenome against sequenced *Pseudomonas* genomes. The graph shows the alignment of contigs against their homologous regions in the genomes of *P. putida* KT2440 (**A**) and *P. aeruginosa* PA7 (**B**). Genes are color-coded based on their predicted function as follows: grey – unknown; yellow – transcription; black – other functions. The coverage of the contigs by the DPB (blue lines) and B (red lines) metagenomic reads is also shown on the top. Coverage was defined as the ratio of the total sum of the length of all reads mapping on the contig with at least 95% nucleotide sequence identity (only best matches were considered) divided by the total length of the contig. The identity plot shown represents the % nucleotide sequence identity between the genes encoded on the contigs vs. their homologs on the reference genomes. Dotted lines represent the average coverage and identity across the length of the contig. Note that the two contigs encoded genes potentially related to the degradation of BAC or its byproducts such as alcohol dehydrogenase, aldehyde dehydrogenase, amine oxidase, monooxygenase, and acyl-CoA dehydeorganse, and were mostly syntenic with the reference *Pseudomonas* genomes.

Moreover, two large contigs, recovered from the B metagenome, encoded genes potentially involved in BAC-transformation such as monooxygenase, alcohol/aldehyde dehydrogenase, amine oxidase, and acyl-CoA dehydrogenase. All genes in the two contigs shared 90% to 95% nucleotide sequence identities with sequenced *Pseudomonas* genomes (*P. putida* KT2440 and *P. aeruginosa* PA7), indicating that the contigs represent moderate relatives of sequenced *Pseudomonas* species (Fig. 2.7).

The population structure of the highly enriched *Pseudomonas* population(s) was examined further. A phylogenetic tree was built based on DPB and B metagenomic reads that were fully overlapping and encoded fragments of the 16S rRNA gene (positions 924 to 973; based on *P. aeruginosa* PAO1). The tree indicated that the total *Pseudomonas* population was composed of at least two distinct subpopulations: one abundant subpopulation (57% and 51% of the total DPB and B metagenome, respectively), showing 100% 16S rRNA gene sequence identity to *P. nitroreducens* and another population (29% and 40% for DPB and B communities, respectively) to *P. putida* and *P. entomophila* (Fig. 2.8A). The coverage of two other universal phylogenetic markers, DNA gyrase subunit B (*gyrB*) and DNA-directed RNA polymerase beta chain (*rpoB*), by B metagenomic reads was about 48X for the *P. nitroreducens*-like population and 4 to 10X for the other *Pseudomonas* population(s) (Fig. 2.8B), consistent with the 16S rRNA gene-based results. Thus, it appears that a *P. nitroreducens*-like population dominated the total *Pseudomonas* genus in the DPB and B communities.

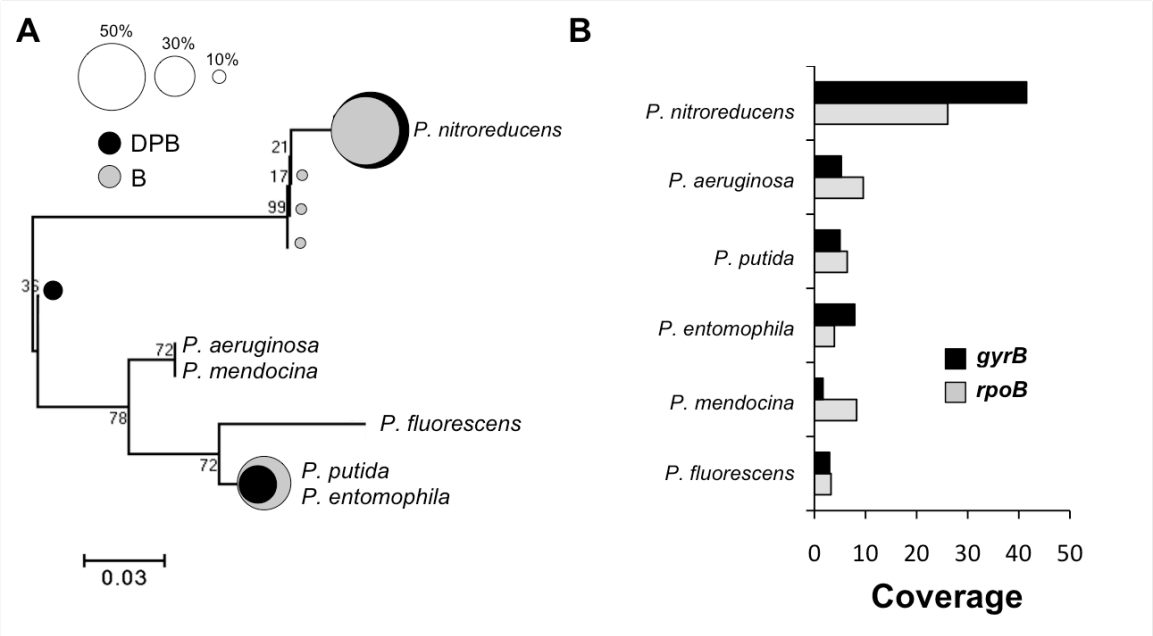


Figure 2.8. Population structure and relatedness to available *Pseudomonas* species of the abundant populations in BAC-fed communities. (**A**) Intra-population structure of the *Pseudomonas* population detected in BAC-fed communities based on fully overlapping, 16S rRNA gene-encoding reads. The size of the circles (DPB; black and B; grey) represents the relative abundance (see key for scale) of the corresponding organism based on all reads recovered in each metagenome (14 and 35 reads for DPB and B metagenomes, respectively). (**B**) Average coverage of available *Pseudomonas* spp genome sequences by B metagenomic reads (only the best match of a read was considered). The references sequences used were *P. nitroreducens* SPQ03 16S rRNA gene (accession number EU500825.1), *P. nitroreducens* ATCC 33634T *gyrB* and *rpoB* gene (FN554208.1 and FN568266.1, respectively), *P. aeruginosa* PA7 (NC_009656.1), *P. putida* KT2440 (NC_002947.3), *P. entomophila* L48 (NC_008027.1), *P. mendocina* ymp (NC_009439.1), and *P. fluorescens* Pf0-1 (NC_007492.1).

To obtain higher resolution than that provided by 50bp-long 16S rRNA gene fragments, coverage plots were built by mapping metagenomic reads against all long (>6 Kbp) contigs that were affiliated with *Pseudomonas* (Fig. 2.9), as described previously (66). Almost all reads mapped on the reference contigs at the >99% nucleotide identity level, indicating that the corresponding *Pseudomonas* populations were clonal. On average, contigs showed 56.0±5.9X coverage in the B metagenome, which was consistent with housekeeping gene-based coverage results reported above (Fig. 2.8B). When the B metagenomic reads were mapped against publicly available *Pseudomonas* genomes (best match), which do not include a sequenced *P. nitroreducens*, no genome provided higher coverage than 5X or average nucleotide identity higher than 90% (e.g. 88.9% for *P. aeruginosa*; 89.8% for *P. putida*; and 89.3% for *P. entomophila*; Fig. 2.9, inset). Collectively, these results showed that the *Pseudomonas* genus consisted of one clonal, dominant species (>99% identity within the population), which was close to *P. nitroreducens*, and a lesser abundant population(s) moderately related to other characterized *Pseudomonas* species, representing possibly a new species of the genus based on its genome-aggregate average nucleotide identity (<95%) to any available genome sequence of the genus (42).

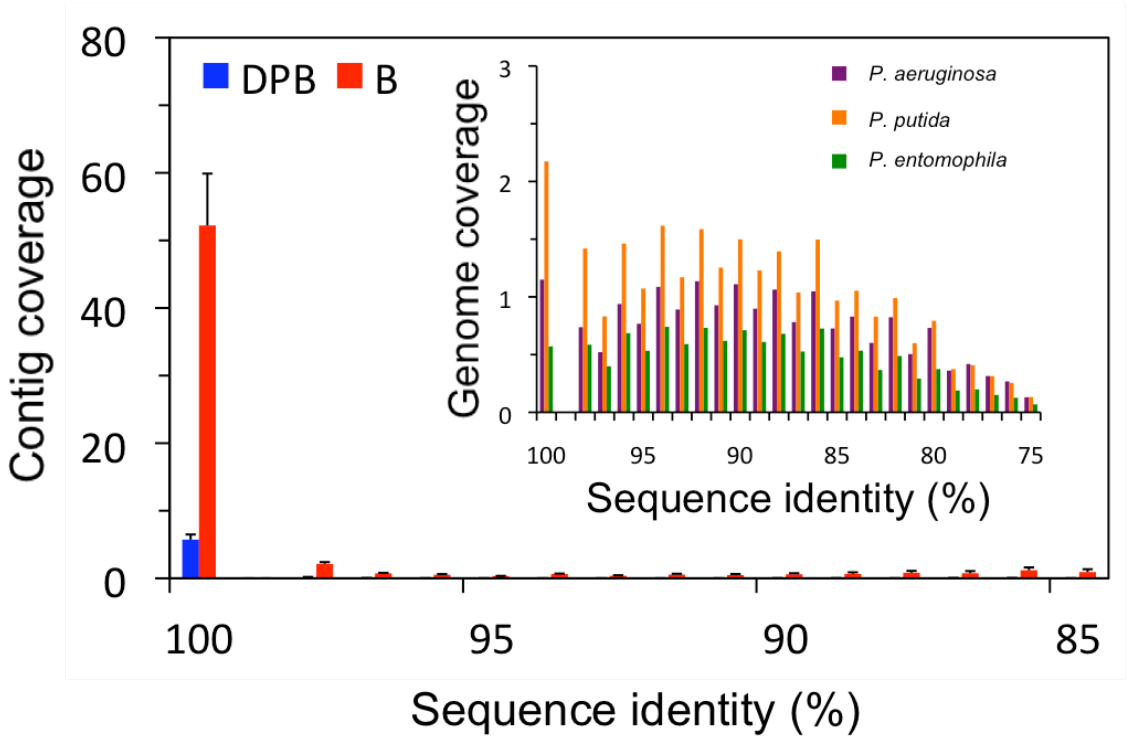


Figure 2.9. Population structure of *Pseudomonas* in DPB and B communities. The graph represents the coverage plots of the *Pseudomonas* contigs assembled from the DPB (blue) and the B (red) metagenomes by the corresponding metagenomic reads from the same metagenome. The coverage plot of representative *Pseudomonas* complete genomes available in NCBI database by B metagenomic reads is also shown for comparison (inset). Coverage (y-axes) was defined as the ratio of the total sum of the length of all reads mapping on the contig at a given unit of nucleotide sequence identity (x-axes) divided by the total length of the contig. The coverage plots revealed that the *Pseudomonas* population in our bioreactors was predominantly clonal (>99% average nucleotide identity), indistinguishable between B and DPB communities, and distinct (e.g., <95% average nucleotide identity) compared to the sequenced *Pseudomonas* species.

Two *P. nitroreducens* isolates, which were able to grow on solid agar and broth medium with BACs as the sole carbon and energy source, were obtained from the DPB and B communities. The partial 16S rRNA gene sequences (1,394 nt) of the two strains were identical (100% identity) and closely related (99.9% identity) to previously characterized *Pseudomonas nitroreducens* 16S rRNA gene sequences (Fig. 2.10). Accordingly, the two strains were designated as *P. nitroreducens*-DPB and *P. nitroreducens*-B, respectively.

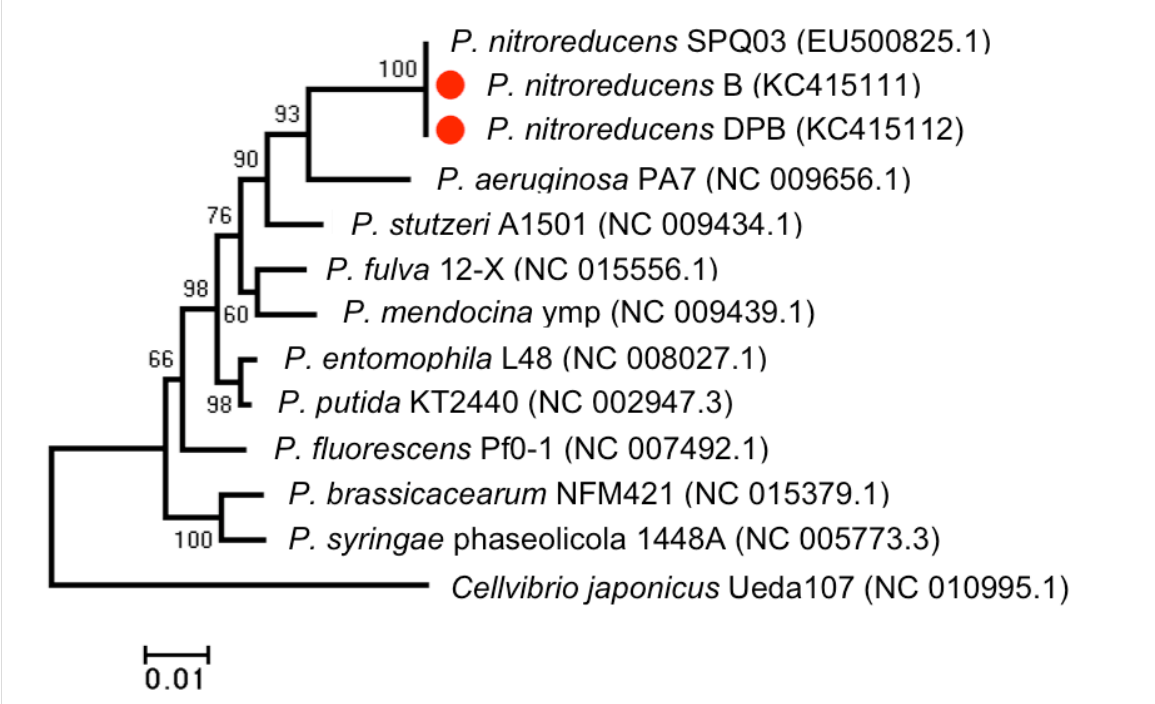


Figure 2.10. Phylogenetic tree of the *P. nitroreducens* strains isolated from the BAC-fed communities. The 16S rRNA gene-based tree was constructed using MEGA 4.0 (139) based on the maximum likelihood composite model and the neighbor-joining method. The values on the nodes represent bootstrap support from 100 replicates. Red circles represent the sequences determined in this study; the accession numbers of the remaining representative sequences from NCBI database are provided in parentheses.

HPLC analysis showed that the two strains almost completely transformed BACs to BDMA (~95% conversion) within two days (Fig. 2.11). After two days, the BDMA was not further transformed while the optical density of the cultures decreased (death phase). Other previously reported BAC intermediates such as BTMA, BMA, and BA were not detected. Collectively, our results indicate that the *P. nitroreducens* population was enriched in the BAC-fed communities due to its ability to transform BACs to a much less toxic product, BDMA, which presumably supported the growth of other community members, including other *Pseudomonas* species.

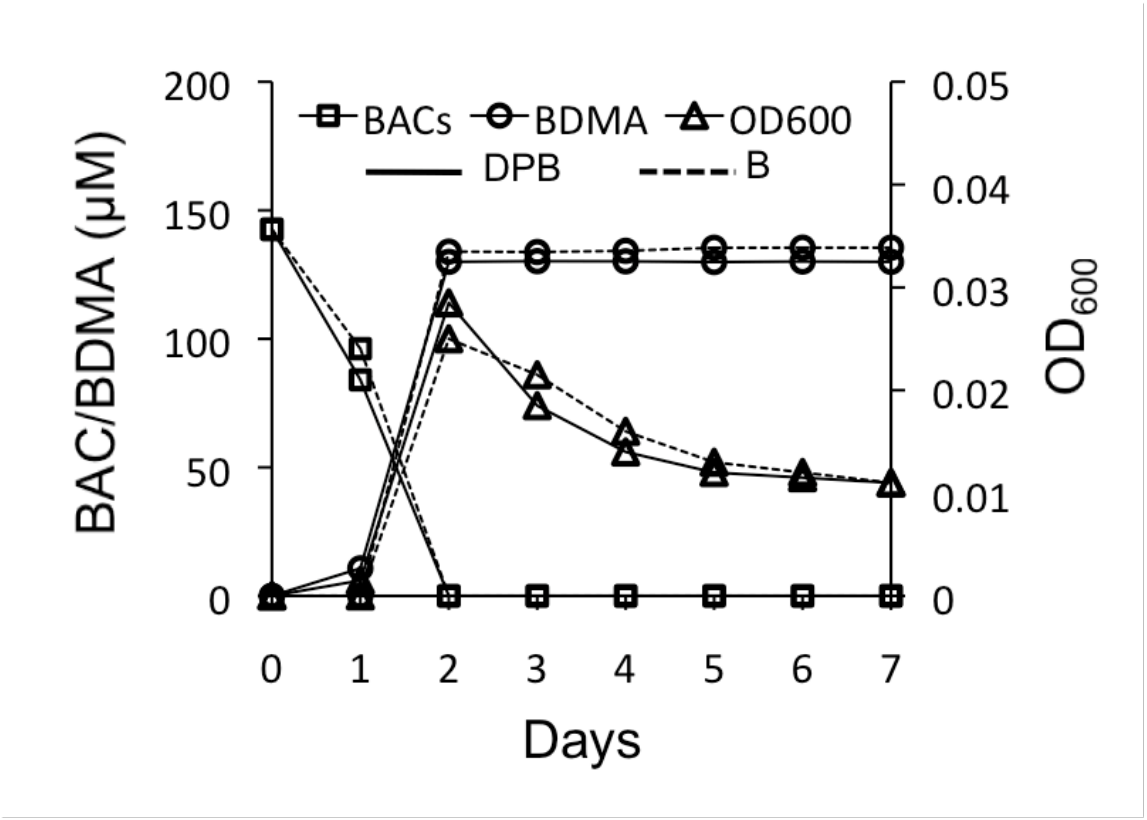


Figure 2.11. Batch growth of *P. nitroreducens*-B and *P. nitroreducens*-DPB isolates with BACs. 50mg/L (143 μM) of BACs was supplied in liquid broth medium as the sole carbon and energy source. Concentrations of BACs and byproducts of their degradation (y-axis) were followed over time (x-axis). The experiments were done in triplicate and the solid and the dotted lines represent mean values for *P. nitroreducens*-B and *P. nitroreducens*-DPB, respectively. While BACs were almost converted to BDMA (~95%) within two days, the slightly lower BDMA concentrations compared to the initial BACs were due to an experimental deviation associated with the quantification of these compounds.

2.3.5. How fast can communities adapt to BACs exposure?

To examine the tempo of community adaptation to BAC, the dextrin/peptone substrate was replaced with BACs as the sole carbon and energy source in the feed of a sub-community originated from the DP community and the BAC susceptibility of the resulting community was followed for 138 days. At day zero, the IC_{50}, IC_{90}, and MIC values against BACs were 47, 90, and 125 mg/L, respectively, and quickly increased to 85, 225, and 250 mg/L, respectively, at day 43 (Fig. 2.12). After 43 days, the BAC susceptibility began to level off, although it never reached that of the B community (i.e., MIC = 460 mg/L), which had adapted to BACs for over three years. HPLC analysis showed that BACs were completely removed within 48 hours during the first feeding cycle (cycle duration: 3.5 days) and within 10 hours during the second feeding cycle and thereafter. Concentrations of volatile suspended solids (VSS) significantly decreased during the time course of BAC exposure (2.5 g/L at day zero vs. 0.44 g/L at day 33 and 0.34 g/L at day 43), which presumably resulted from the lower amount of carbon source provided (2,200 mg/L COD of dextrin/peptone vs. 150 mg/L COD of BACs) and/or BAC toxicity. The metagenomes at day 33 (B_{33d}; when community BAC resistance was still increasing) and at day 43 (B_{43d}; when IC/MIC values leveled off) were analyzed to document the genetic mechanisms of community adaptation to BACs. These metagenomes revealed that the *Pseudomonas* genus (Fig. 2.12B) was dramatically enriched at day 33 (45.8% of the total community) and at day 43 (62.1%) compared to day zero (3.2%). The relative abundance of the *Pseudomonas* genus at day 43 was comparable to that in the B community (57%). In contrast to *Pseudomonas*, combined fraction of other minor genera (<1% of the total) dramatically decreased in relative

abundance at day 33 (making all together 40.3% of the total) and day 43 (16.0% of the total). An increase in abundance of *Stenotrophomonas* genus from day 33 (1.6%) to day 43 (12.7%) was also noted. Gene content (Fig. 2.12C) and phylogenetic sequence (Fig. 2.12D) comparisons of the metagenomes showed that the B_{33d} and B_{43d} communities clustered closely together with the B community, suggesting that the DP community quickly (<43 days) and reproducibly (e.g., a B-like community was obtained three years after the DP community was established) became phylogenetically and functionally similar to the B community.

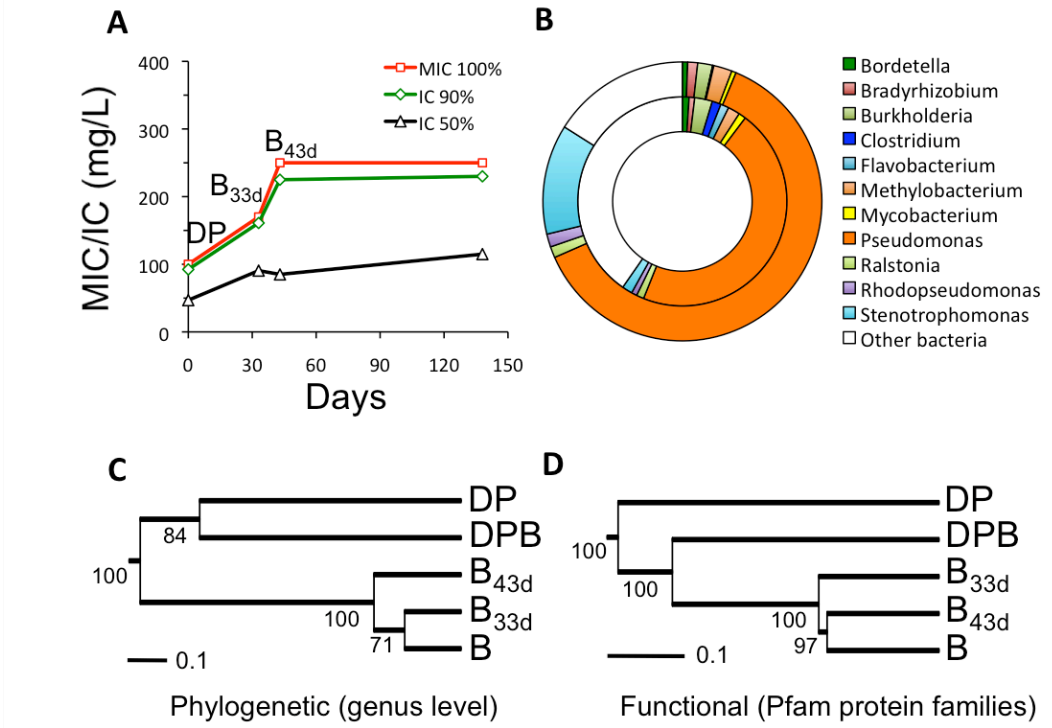


Figure 2.12. Phenotypic, phylogenetic, and metabolic relatedness of communities exposed to BACs. (**A**) Decrease of community-level BAC susceptibility (IC_{50}, IC_{90}, and MIC) of the DP inoculum after replacing dextrin/peptone substrate with BACs as the sole carbon source over a period of five months. (**B**) The composition of major genera (>1% of the total community) in the B_{33d} (inner circle) and the B_{43d} (outer circle) metagenome was based on the best match of metagenomic reads against all available archaeal and bacterial genomes. 'Other bacteria' represent the combined fraction of minor genera. The two cladograms represent the phylogenetic (**C**) and predicted functional gene content (**D**) relatedness among the communities sampled. The clustering was performed based on the relative abundance of genera (**C**) and Pfam protein families (**D**) recovered in the corresponding metagenomes, using Bray-Curtis distance. Numbers on the nodes indicate bootstrap support of 100 replicates.

2.3.6. Molecular mechanisms of community adaptation to BACs

Metagenomic read recruitment against the 316 long (>6Kb) *Pseudomonas* contigs recovered from the B_{43d} metagenome showed 275 contigs with about 70X coverage (dominant population) and 41 contigs with about 15X coverage (Fig. 2.13A). This *Pseudomonas* population structure resembled that of the B community, i.e., a dominant population sampled at 56X coverage and a less abundant population(s) at 14X coverage (Fig. 2.8B). The average gene sequence identity between the 275 *Pseudomonas* contigs (B_{43d} metagenome) and the *Pseudomonas* contigs (B metagenome) was >99.9%, indicating that the *Pseudomonas* populations enriched in both B_{43d} and B communities originated from the same *P. nitroreducens* population in the DP community. Recruitment of DP metagenomic reads against the 275 *Pseudomonas* contigs revealed that *P. nitroreducens* showed less than <0.1X in the latter metagenome (Fig. 2.13B). Consistent with these results, searching the 1,828 genes annotated in the 275 *P. nitroreducens* contigs against the B_{43d}, B, and DPB metagenomes yielded 1,819, 1,758, and 471 homologs with at least 97% nucleotide identity and 80% query length coverage, respectively, whereas no significant matches were obtained from the DP metagenome.

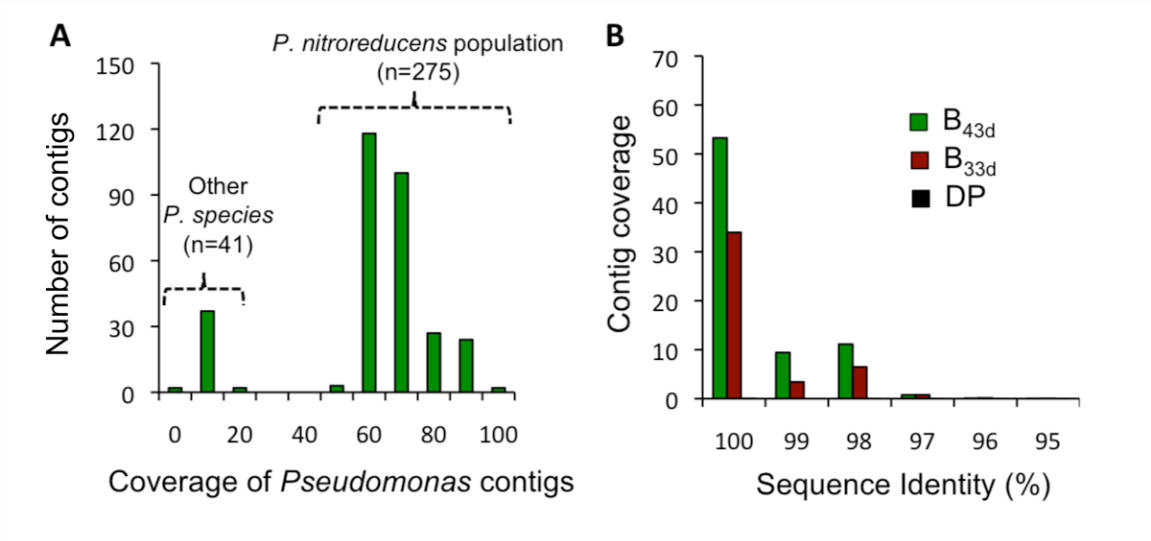


Figure. 2.13. *P. nitroreducens* population structure in the BAC-fed communities. (**A**) The number (y-axis) of long *Pseudomonas* contigs (316 contigs in total; >6 Kbp long each) assembled from the B_{43d} metagenome is plotted against their coverage (x-axis). One group of contigs (n=275) showed 50X to 100X coverage (dominant *P. nitroreducens* population) and another group (n=41) showed less than 30X coverage (less abundant *Pseudomonas* population). (**B**) The coverage of the 275 *P. nitroreducens* contigs from the B_{43d} metagenome by metagenomic reads from the three time-course metagenomes (DP, B_{33d}, and B_{43d}) is plotted against the average nucleotide sequence identity between the contigs and the reads. Note that the *P. nitroreducens* abundance in the DP metagenome was extremely low (<0.1X) and that the *P. nitroreducens* populations were indistinguishable among B_{33d}, and B_{43d} metagenomes based on these data.

Sequence comparison of *P. nitroreducens* homologous genes between the B_{33d} and the remaining BAC-fed community metagenomes identified mutations in four genes that occurred between day 33 and 44 and were also fixed, or almost fixed, in the population of the B_{43d} metagenome (Table 2.4). Among the four genes, two genes with nonsynonymous mutations were a PAS/PAC sensor gene and an anti-sigma factor, *mucA*. The PAS/PAC protein senses environmental stimuli such as oxygen, redox state, and light (143). *mucA* controls the alginate biosynthesis that results in extrapolysaccharide formation and mucoidy phenotype, which can confer resistance to oxidative stress, antimicrobials, and host immune systems (51). Mutations in *mucA* frequently occur in *P. aeruginosa* causing cystic fibrosis chronic infection and provide advantageous phenotypes that persist in the lung (36). Thus, it is likely that the latter mutations are related to enhanced BAC resistance, although this hypothesis awaits experimental verification.

Table 2.4. Mutations fixed in the *P. nitroreducens* population between day 33 and day 43. The mutations were observed in genes recovered from the B_{43d} and the B assembled contigs against those from the B_{33d} metagenome. Metagenomic reads (B_{33d}, B_{43d}, and B) that contain the normal or mutated nucleotide base were identified by searching the reads against a gene region of interest (10b-long region centered around the normal or mutated base) with a cut-off for a match of at least 99% identity and 40b-long match length. The mutant allele frequency (%) was defined as the number of reads with the mutant base divided by the total number of reads obtained. Only mutations for which the mutant allele frequency was <50% in the B_{33d} gene and >70% in the B_{43d} and the corresponding B gene showed at least 15X coverage were considered. The gene product was inferred based on its best match analysis against the non-redundant protein database (GenBank).

ORF	Gene product	Mutation	Amino acid change	Mutant allele frequency (%)		
				B_{33d}	B_{43d}	B
B_{33d} _04760_1	PAS/PAC sensor protein	G154C	V52L	42	96	94
B_{33d} _04760_4	Hypothetical protein	A213G	Silent	50	96	97
B_{33d} _69292_2	Anti-sigma factor MucA	A580C	S194R	5	93	79
B_{33d} _01476_2	Glucan biosynthesis protein D	T1035C	Silent	10	95	86

Finally, to examine whether community adaptation was attributable to horizontal transfer of ecologically important genes, the coverage of all long contigs assembled from the B_{43d} metagenome (Fig. 2.14A) was estimated. The coverage of the *P. nitroreducens* contigs ranged between 56 and 91X (95% confidence interval of the average 74X), whereas the contigs affiliated with other characterized (e.g., *Stenotrophomonas*) or uncharacterized genera typically showed less than 50X coverage. Notably, the coverage of three contigs, B_{43d}_01683 (239X), B_{43d}_03714 (200X), and B_{43d}_03399 (121X), were significantly higher ($P < 0.01$) than those of the *P. nitroreducens* contigs. The coverage of B_{43d}_01683, B_{43d}_03714, and B_{43d}_03399 were <0.1X, <0.1X, and 5.7X, respectively, at day 0 and increased more rapidly over time (4.9, 4.4, and 2.7 times per day, respectively) compared to the *P. nitroreducens* population (2.2 times per day) in the B_{43d} community (Fig. 2.14B). All three contigs encoded mobile genetic elements: contig B_{43d}_01683 encodes a transposase and an integrase; B_{43d}_03714 encodes a transposase and a resolvase; and contig B_{43d}_03399 encodes plasmid replication/partitioning proteins and conjugative transfer proteins. Collectively, these data imply that contigs B_{43d}_01683, B_{43d}_03714, and B_{43d}_03399 were subjected to horizontal transfer during community exposure to BACs or were acquired horizontally prior to our experiments and were subsequently duplicated (amplified) in our bioreactors. Several of the genes encoded on these contigs (e.g., ring-hydroxylating dioxygenase; see below) may be involved in the degradation of BDMA and other products of BAC degradation by *P. nitroreducens* and/or BAC-resistance. For instance, contig B_{43d}_01683 (10.6 Kb) encoded amine oxidase, ring-hydroxylating dioxygenase, SCP-2 sterol transfer, and major facilitator (MF) superfamily genes (Fig. 2.14C). Sterol carrier proteins transfer steroids between

cellular membranes and regulate membrane fluidity, permeability, and thickness (40), possibly enhancing the cell membrane integrity against BACs. Further, contig B_{43d}_03399 (14.0 Kb) encodes a resistance nodulation division (RND) family efflux pump gene operon. Whether or not the genes encoded in these contigs conferred any fitness advantage to BACs remains to be experimentally validated. However, the predicted functions of the genes related to BAC degradation and/or resistance, their high abundance in the B_{43d} metagenome, (Fig. 2.14A) and their much faster enrichment (Fig. 2.14B) compared to the most highly enriched *P. nitroreducens* population in the B_{43d} community indicate that these genes were strongly selected under the conditions of our study (i.e., BACs as the sole carbon and energy source).

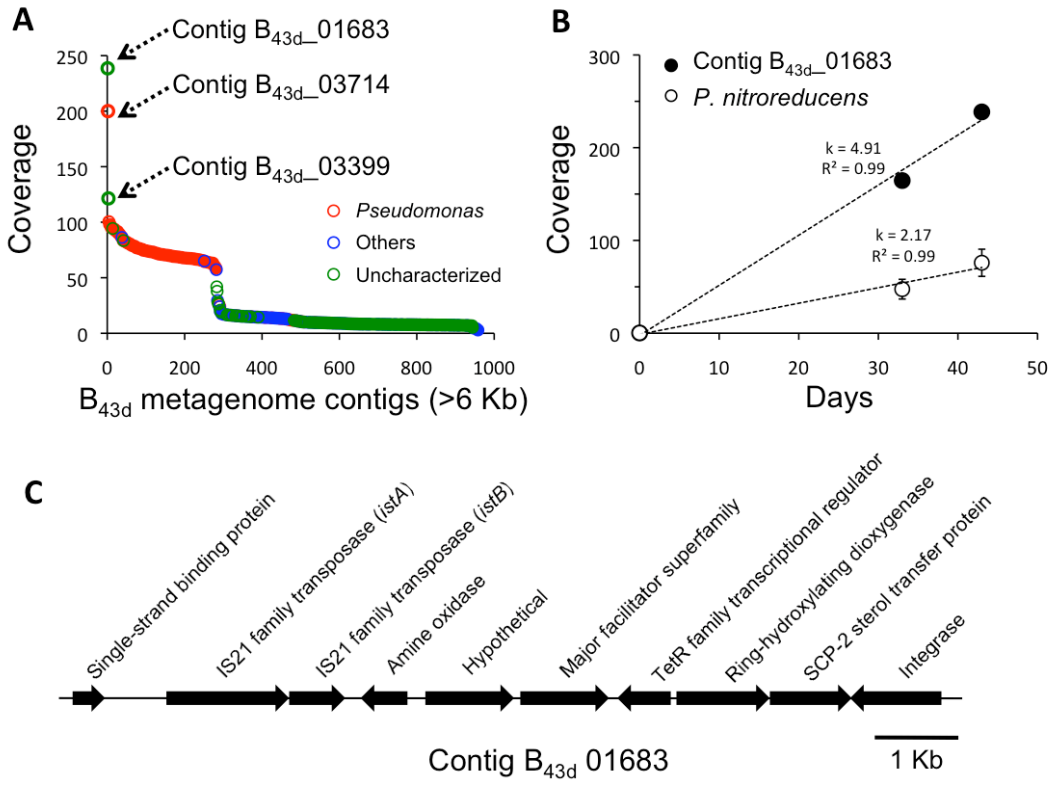


Figure 2.14. Gene abundance during community adaptation to BAC exposure. (A) Coverage of all large (>6 Kb long) contigs assembled from the B_{43d} metagenome (y-axis), ordered based on their coverage (x-axis). Contigs B_{43d}_01683, B_{43d}_03714, and B_{43d}_03399 showed significantly higher coverage ($P < 0.01$, Student's t-test) compared to contigs of abundant *P. nitroreducens* population (colored in red; average coverage 74X). (**B**) Comparison of the coverage for contig B_{43d}_01683 and the 275 *P. nitroreducens* contigs over time. The slope, k, was calculated based on least-squares linear regression and was significantly different by Student's t-test ($P < 0.01$). Error bars indicate one standard deviation from the mean coverage of the 275 *P. nitroreducens* contigs. (**C**) Organization and predicted function of the genes encoded on contig B_{43d}_01683. The gene product was based on best match Blastp analysis against the non-redundant protein database (GenBank). Note the occurrence of multiple mobile genes on the contig.

2.4. Discussion

Microbial community adaptation to synthetic toxic compounds may occur via several independent mechanisms, including -but not limited- to: (i) selective enrichment of a population (clonal amplification), (ii) advantageous mutations or gene rearrangements, and (iii) horizontal transfer of ecologically important genes. Our metagenomic data, in combination with phenotypic characterization, suggest that microbial community adaptation to BAC resistance and degradation took place primarily by clonal amplification of the BAC-degrading *P. nitroreducens* population and secondarily by horizontal gene transfer and nonsynonymous mutations in ecologically important genes primarily in the *P. nitroreducens* population. These metagenomic findings were also consistent with our previous study, which showed that 37% of the *Pseudomonas*-like 16S rRNA gene sequences recovered in a clone library from the B community were related to *P. nitroreducens* with 99% nucleotide sequence identity (145). Further, the same clonal *P. nitroreducens* population was highly enriched in two independent enrichment cultures with BACs as a sole carbon and energy source from the DP community (Fig. 2.8, 2.9, 2.12, 2.13, and 2.14). Interestingly, the second enrichment (B_{43d}) occurred after maintaining the DP community for three years with dextrin-peptone (no BACs) and thus, *P. nitroreducens* apparently survived as a rare community member during this period. Given also our physiological characterization (e.g., Fig. 2.10 and 2.11), *P. nitroreducens* appears to be a robust BAC-degrader.

P. nitroreducens was first identified as a hydrocarbon-utilizing organism from an oil-field (56), and is known for its versatility to metabolize a wide range of toxic compounds such as insecticides (hexachlorocyclohexane), antiseptics (eugenol),

herbicides (simazine), and surfactants (alkylphenol polyethoxylate) (21, 52, 148, 156). In addition, *P. nitroreducens* strains have been commonly isolated from microbial communities that were treated with antimicrobials (91) and antibiotics (94). Collectively, these findings suggest that *P. nitroreducens* may represent a useful inoculum for degrading various toxic compounds as well as quaternary ammonium biocides in biological engineered systems (e.g., wastewater treatment plants) or contaminated natural systems.

Several genes associated with mobile genetic elements were strongly selected under BAC exposure, which implied horizontal gene transfer-mediated community degradation of and resistance to BACs. Most importantly, an IS21-family transposase-encoded element (contig B_{43d}_01683) encoded genes predicted to function as amine oxidase, and ring-hydroxylating dioxygenase (Fig. 2.14C). Our previous study (145) and results presented here (Fig. 2.11) show that the B community transformed BACs to dimethylamine and benzoic acid through a benzyldimethylamine (BDMA) intermediate. Amine oxidase and ring-hydroxylating dioxygenase have been implicated in transforming BDMA and benzoic acid, respectively (111). Thus, it is likely that the genes found on this mobile element encode the enzymes responsible for these degradation steps, although direct experimental evidence is currently lacking. Based on mapping patterns of metagenomic reads (i.e., reads that simultaneously mapped on two distinct contigs), contig B_{43d}_01683 was frequently connected to those contigs phylogenetically affiliated with *Pseudomonas* species or phylogenetically uncharacterized contigs that encode mobile genes. Given also that the two *P. nitroreducens* isolates from the B and the DPB community could not transform BDMA, we hypothesize that this mobile genetic element

facilitates further degradation, by non-*P. nitroreducens* members of the community, of the products of BAC degradation by *P. nitroreducens* such as BDMA. Isolate genome and/or single-cell sequencing will allow to robustly test this hypothesis.

The metagenomic analysis of BAC-fed vs. control communities revealed the enrichment of genes that could be important for the degradation of BACs and their byproducts such as monooxygenase, various dehydrogenases, and aromatic-ring opening dioxygenases (Fig. 2.5 and Table 2.3). A monooxygenase is hypothesized to enable the first N-dealkylation step of QACs (78, 101, 150). The β–oxidation of the alkyl chain can be carried out by several dehydrogenases such as aldehyde, formate, and formaldehyde dehydrogenase, and citrate lyase (25, 111, 150), all found to be significantly enriched in BAC-fed communities. In addition to biodegradation genes, other highly enriched genes included functions potentially important for BAC resistance such as efflux pumps, cell envelope modification, chaperones, and oxidative stress defense systems (Fig. 2.5). The well-characterized QAC efflux pump systems, the RND-family and the small multidrug resistance (SMR) family (115), were also enriched in BAC-fed communities. Among the enriched cell envelope modification genes, lipid A 3-O-deacylase is an enzyme involved in aminoarabinose-modified lipid A, which decreases the negative charge at the membrane surface and reduces the ionic interaction of cationic antimicrobial peptides with the membrane core (63). In addition to this cell envelope modification, other genes associated with membrane biosynthesis, integrity, and selective permeability presumably reflected important adaptations to BACs (Fig. 2.5). Recently, the generation of intracellular superoxide and hydrogen peroxide was experimentally demonstrated during treatment with the QAC-family cationic surfactant, cetyltrimethylammonium bromide

(97), suggesting oxidative damage as a secondary (indirect) mode of action of QACs. Consistent with this previous study, the differentially abundant genes included glutathione S-transferase, glutathione synthetase and glutaredoxin, which all serve as antioxidants to defend against oxidative stress and detoxify the peroxidized lipids (20, 128). Chaperone proteins (e.g., POTRA domain) were also enriched in response to BAC, suggesting that such proteins can assist transport or assembly of unfolded polypeptides resulting from oxidative damage (64, 124). Therefore, functional adaptation of the whole community to BAC exposure appears to take place via an assortment of distinct and complementary cellular functions and enzymes. On the other hand, some of the genes enriched in the BAC-fed communities might not be actually related to BAC resistance or degradation but just encoded ("hitchhiked") on the genome of the dominant (selected) organism(s) in these communities (e.g., *P. nitroreducens*). However, the strong enrichment relative to housekeeping genes, predicted functions, and previous literature indicate that hitchhiked genes do not probably represent a major portion of the genes reported above.

Exposing microbial communities to BACs had a dramatic effect on the community diversity (Fig. 2.2, 2.3 and 2.12), resulting presumably from the toxicity of BACs and/or the competitive advantage of *P. nitroreducens*. The DPB community was highly enriched in *Citrobacter*, *Enterobacter*, *Salmonella*, and *Achromobacter* genera, in addition to *Pseudomonas*. The enrichment of the former genera was likely attributable to the high relative concentration of dextrin/peptone (2,200 mg COD/L) vs. BACs (150 mg COD/L) and their ability to tolerate BACs rather than to metabolize BACs as a primary carbon source. Consistent with these interpretations, the DPB and the B metagenome

contigs affiliated with *Citrobacter* and *Klebsiella* appear to encode the carbohydrate-selective porin genes and many antimicrobial resistance genes (Fig. 2.6).

Although for the most part shotgun metagenomic and 16S rRNA gene pyrosequencing-based results on the abundance of genera and populations within genera were in good agreement, we noted a few notable discrepancies. In particular, *Bacteroidetes* was overrepresented in the 16S rRNA gene pyrosequence data compared to metagenomic data: 25% to 50% vs. 1% to 3% depending on the community considered, respectively. The discrepancy could result from biases during PCR amplification of 16S rRNA genes or the underrepresentation of *Bacteroidetes* genomes among the publicly available sequences, which could limit the taxonomic identification of metagenomic sequences. To address this issue, we searched the metagenomic reads against the representative 16S rRNA gene pyrosequences with a 97% identity cutoff to detect and quantify reads that encoded fragments of *Bacteroidetes*-like 16S rRNA genes. The analysis showed that *Bacteroidetes* was more abundant in the three metagenomes (9% to 23%) relative to the best match analysis of genes encoded on assembled contigs (Fig. 2.15). In agreement with these findings, the most abundant *Bacteroidetes* 16S rRNA gene-based OTUs were affiliated with species that have few or no sequenced representative such as *Niabella aurantiaca* (NR_043862.1), *Chryseobacterium haifense* (NR_044167.1), and *Sediminibacterium salmoneum* (NR_044197.1). Therefore, about half of the overrepresentation of *Bacteroidetes* in 16S rRNA gene pyrosequence data might be attributed to PCR biases (23% vs. 50% for the metagenomic whole genome vs. 16S rRNA gene analysis) and the remaining half to lack of appropriate reference genome sequences.

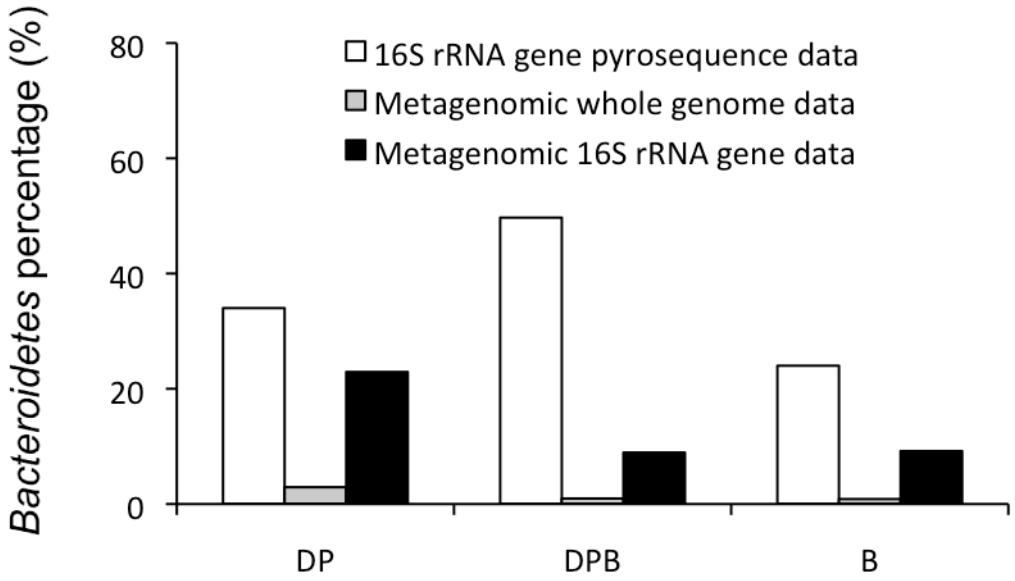


Figure 2.15. Comparison of *Bacteroidetes* abundance based on 16S rRNA gene amplicon pyrosequencing and metagenome data. 16S rRNA gene amplicon pyrosequences were assigned to the *Bacteroidetes* OTU as described in Table 2.1 ("16S rRNA gene pyrosequence data" on the graph). Metagenomic reads were assigned to the available *Bacteroidetes* genomes based on best-match Blastn analysis as described in the text ("Metagenomic whole genome data" on the graph). For "metagenomic 16S rRNA gene data", metagenomic reads from the three metagenomes (x-axis) were searched against the 168 OTUs recovered among all 16S rRNA gene amplicon sequences (Table 2.1), using a minimum cutoff for a match of 97% nucleotide sequence identity and 100% query length coverage. The number of reads assigned to an OTU was normalized by dividing by the total number of metagenomic reads assigned to all OTUs and the normalized values were compared to the abundance data based on the 16S rRNA gene pyrosequence and the Metagenomic whole genome data.

Our study provided, to the best of our knowledge, the first genomic view of the molecular mechanisms that facilitate community adaptation to QAC-family biocides and shows that communities employ a combination of evolutionary processes for successful adaptation. Further, biocide-exposed microbial communities were characterized by a versatile repertoire of antibiotic resistance genes such as efflux pumps (e.g., RND-family and SMR-family) and cell envelope modification systems (e.g., aminoarabinose lipid A modification). Therefore, the communities and data presented here represent a useful basis for future investigations on the role of biocides in promoting antibiotic resistance in microbial communities, which has important implications for both public and environmental health.

2.5. Material and methods

2.5.1. Bioreactor operation and antimicrobial susceptibility testing

A dextrin/peptone substrate, composed of 1 g/L of dextrin and 1 g/L of peptone, along with a salt medium, was fed to the DP community. The DP community was developed from an inoculum of a sediment sample collected at the Bayou d'Inde, a tributary of the Calcasieu River, near Lake Charles, LA, which has been contaminated with metals, polycyclic aromatic hydrocarbons, and chlorinated/halogenated organic compounds (82). Dextrin/peptone (1 g/L of dextrin and 1 g/L of peptone), salt medium, and 50 mg/L of a BAC mixture, composed of (w/w) 40% dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl) and 60% tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl), was fed to the DPB community, developed with inoculum from the DP community. Salt medium, BAC mixture (50 mg/L), and ammonium nitrate (NH_4NO_3) were fed to the B community, developed with inoculum from the DPB

community. The three communities were maintained in 2-L glass reactors at room temperature (22 to 23^{o}C), aerated, and fed twice a week by replacing one fourth of the mixed culture suspension with fresh medium that included the carbon substrate at appropriate concentration, with 14 days retention time. The salt medium consisted (per liter) of K_2HPO_4 (0.6g), KH_2PO_4 (0.34g), $CaCl_2.2H_2O$ (0.07g), $MgCl_2.6H_2O$ (0.14g), $FeCl_2.4H_2O$ (0.07g), and trace metal stock solution (0.7mL). The antimicrobial susceptibility test was performed as described previously (100). In brief, a sample from a target community was inoculated in Mueller-Hinton broth, supplemented with a range of BAC concentrations. Inocula were diluted to an optical density of 0.02 (equivalent to 0.5 McFarland turbidity standard) and incubated overnight in an orbital shaker at room temperature (22-23^{o}C), in triplicates. After incubation, the optical density (OD_{600}) of each sample was measured using a spectrophotometer and normalized to that of control samples (no BACs added) to provide relative cell growth. The 50% (IC_{50}), 90% (IC_{90}), and 100% minimum inhibitory concentrations (MIC) were determined as the concentrations at which 50%, 90%, and 100% of cell growth inhibition was observed.

2.5.2. Isolation and identification of BAC-degrading organisms

BAC-degrading organisms were isolated from the DPB and B communities. Briefly, 100 µl of mixed culture suspension was first diluted 10^5 to 10^7 times and then plated on BAC-containing agar medium (50 mg/L of BAC mixture as a sole carbon and energy source, salt medium, ammonium nitrate, and 1.5% agar). After several transfers to new agar plates, colonies were inoculated in 100 ml of liquid BAC broth medium. Cell growth was measured by optical density at 600 nm using a spectrophotometer. Samples were taken from the liquid cultures to measure C_{12}BDMA-Cl, C_{14}BDMA-Cl,

benzyldimethylamine (BDMA), benzylmethylamine (BMA), and benzylamine (BA) on a HP 1100 series HPLC system (Hewlett-Packard, Palo Alto, CA), as described previously (145). For taxonomic identification of the BAC-degrading isolates, DNA was extracted from 2 ml of pure culture suspension using the QIAamp DNA Mini kit and PCR-amplification of the 16S rRNA genes was performed using the universal bacterial primer set (8F/1492R). The resulting PCR products were sequenced using standard Sanger sequencing chemistry.

2.5.3. DNA extraction and metagenome sequencing

Samples of 50 ml of mixed culture suspension from the bioreactors were taken at the end of a feeding cycle and DNA was extracted as described previously (106). The obtained DNA concentrations were at least 0.8 μg/μl, with >1.8 absorbance ratios (A_{260}/A_{280}) for all samples. About 5 μg of a DNA aliquot was sequenced using the Illumina GA II sequencers at the Emory University Genomics Facility (DP, DPB, and B metagenomes; 50 bp single end reads) or Los Alamos National Laboratory (B_{33d} and B_{43d} metagenomes; 150 bp single end reads), according to established protocols by the manufacturer. Table 2.2 describes the statistics of all metagenomes used in this study.

2.5.4. Assembly and gene calling

Raw Illumina reads were trimmed using a Q=15 Phred quality score cutoff using SolexaQA (29). The hybrid protocol, which employs a combination of Velvet (155), SOAPdenovo (76), and Newbler 2.0 assemblers and was previously shown to provide longer contigs with higher sequencing accuracy than alternative approaches (80), was used to assemble each metagenome. Protein-coding genes were annotated on the assembled contigs using the Metagene pipeline (104).

2.5.5. Phylogenetic affiliation of metagenomic sequences

The raw sequencing reads and protein sequences from assembled contigs were searched against all bacterial and archaeal genome sequences available in GenBank database (ftp://ftp.ncbi.nih.gov/, as of February 2012,), using Blastn and Blastp, respectively. Blastn was run with X = 150, q = -1, and F = F, remaining parameters at default settings, and a cutoff for a match of at least 80% identity and 50% of the length of the query sequence in the alignment. The cutoff for a Blastp match was at least 50% identity and 50% query sequence coverage. To estimate the relative abundance (expressed as coverage) of reference gene, contig, or genome sequences in a metagenome, all metagenomic reads mapping on the reference sequence with at least 95% identity and 50% query length coverage were considered. The length of all such metagenomic reads was summed and divided by the length of the corresponding reference sequence and the size of the metagenome (i.e., normalized to one Gb of metagenomic sequences) to provide a normalized estimate of abundance across metagenomes.

2.5.6. Gene functional annotation and abundance analysis

The amino acid sequences from the Metagene pipeline were functionally annotated using the Clusters of Orthologous Genes (COG) (142), Pfam (38), or non-redundant protein (GenBank) databases, using Blastp and the corresponding cutoff mentioned above. To provide a quantitative assessment of the differential abundance of genes and pathways between metagenomic datasets (e.g., DP vs. DPB and DP vs. B), the number of matches to a gene was normalized by dividing by the total number of genes recovered in each metagenome and the normalized values were compared in a pair-wise

mode. One hundred bootstraps, each based on a randomly drawn dataset of five thousand genes per metagenome, followed by a two-sided Kolmogorov-Smirnov test, were performed to examine the statistical support for the results obtained at the previous step. The Kolmogorov-Smirnov test was performed using Matlab version 7.9.0 software (MathWorks Inc., Natick, MA, USA).

2.5.7. PCR amplification and pyrosequencing of 16S rRNA genes

The same DNA aliquot used for metagenome sequencing was also used for 16S rRNA gene amplicon pyrosequencing. The PCR-amplification of 16S rRNA genes was carried out using the barcoded primers targeting the V1 to V3 region. 20μl PCR reaction volumes were used, containing 2 μl of undiluted template DNA, 1μl of 2μM "B" adaptor-labeled 8F primer, 1μl of 2μM "A" adaptor-labeled and barcoded 518R primer, 2μl of 10X AccuPrime PCR Buffer II, 0.15 μl of AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen), and 13.85μl of nuclease-free water. The PCR conditions were: initial denaturation step at 95°C for 2 min, followed by 25 cycles of 95°C for 20 sec, 50°C for 30 sec, and 72°C for 5 min. Three independent PCR amplifications were performed from the same DNA sample and the resulting aliquots were combined to represent a single sample for sequencing. The resulting PCR products were cleaned using the Agencourt AMPure beads (Beckman Genomics) and were sequenced on the GS-FLX 454 Titanium sequencer available at the Emory University Genomics Facility.

2.5.8. Pyrosequencing data analysis

The QIIME package was employed to process the 16S rRNA gene pyrosequencing data and perform phylogenetic analysis of the three microbial

communities (18). Briefly, the sequences were pre-filtered using the split_libraries.py script, based on the following parameters: minimum length = 200, maximum length = 1000, minimum average quality score = 20, and maximum length of homopolymer = 6. After denoising and chimera-checking of the pre-filtered sequences with the scripts denoise_wrapper.py and identify_chimeric_seqs.py (default settings), 911, 920, and 864 sequences were obtained from the DP, DPB, and B microbial communities, respectively. All sequences were clustered into 168 representative OTUs based on a 97% nucleotide identity threshold and rarefaction curves indicated that the OTU diversity within the communities was almost saturated by sequencing (Fig. 2.3). Representative sequences from each OTU were identified at the genus level, when possible, using the RDP classifier (152) based on the Greengenes 16S rRNA gene reference database (32). The number of denoised and chimera-checked sequences assigned to each OTU was taken as a proxy of OTU relative abundance.

2.5.9. Nucleotide sequence accession numbers

The metagenome and 16S rRNA gene pyrosequence data used in this study were deposited in GenBank under the accession numbers: SRR643889 (DP), SRR643891 (DPB), SRR643892 (B), SRR643893 (B_{33d}), and SRR643894 (B_{43d}) for metagenomes, SRR639747 (DP), SRR639749 (DPB), and SRR639751 (B) for 16S rRNA gene amplicon pyrosequences. The 16S rRNA gene sequences of two BAC-degrading isolates were deposited in GenBank under the accession numbers: KC415112 (*Pseudomonas nitroreducens*-DPB) and KC415111 (*Pseudomonas nitroreducens*-B).

2.6. Acknowledgements

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CHAPTER 3

TRANSCRIPTOMIC AND GENOMIC ANALYSIS OF A MICROBIAL COMMUNITY-DEGRADING BENZALKONIUM CHLORIDE DISINFECTANTS

This chapter is related to the manuscript:

Seungdae Oh, Despina Tsementzi, Madan Tandukar, Spyros G. Pavlostathis, and Konstantinos T. Konstantinidis. Transcriptomic and genomic analysis of a microbial community-degrading benzalkonium chloride disinfectants. *In preparation*.

3.1. Summary

Benzalkonium chlorides (BACs) represent widely used disinfectants and are frequently detected in the environment, even at toxic levels for life. Accordingly, microbial degradation of BACs in non-target environments is important in order to reduce the potential risks to environmental and public health. Our previous metagenomic analysis suggested that a microbial community selects several organisms and a variety of genes under long-term BAC exposure, some of which are potentially involved in BAC degradation. However, the in situ activities of the organisms and genes during community BAC degradation remain elusive. To address these issues, we employed an integrated approach that combines metatranscriptomics, genome sequencing of key community members, and characterization of time course BAC degradation. Significant increases in transcripts of *Pseudomonas* species, 60% of which was attributed to *Pseudomonas nitroreducens*, were detected during BAC degradation. Specific functions significantly expressed were related to i) dealkylation (the first step in a previously described BAC degradation pathway) and alkyl chain (a dealkylated product of BACs) metabolism by *P.*

nitroreducens and ii) benzyl compound (the other product of BACs) metabolism by other community members (e.g., *P. putida* and *P. entomophila*), suggesting cooperative BAC degradation among the community members. Multidrug resistance transporter genes horizontally transferred among community members were significantly upregulated in response to BACs. Collectively, our results advance current understanding of BAC degradation by a microbial community and have implications for treating BACs in biological engineering systems.

3.2. Introduction

Benzalkonium chloride (BAC) is a family of quaternary ammonium compounds (QACs), a widely used, broad-spectrum class of disinfectants. As a result of their extensive use in a variety of environmental and clinical settings, BACs are readily detected in natural environments (1.5 mg/kg in river sediment) (77) and wastewater systems (up to 6 mg/L) (70, 88). BACs cause cell membrane disruption by reacting with the cytoplasmic membrane and inhibit cell growth (0.4 to 1.8 mg/L in *Staphylococcus aureus* and 10 to 50 mg/L in *Pseudomonas* species) (15, 37, 57). Since the majority of QACs are typically collected into wastewater treatment plants (WWTPs) where QACs can potentially be detoxified by microbial consortia (103, 135), microbial degradation of BACs in WWTPs is a desirable process to reduce the potential risks to public and environmental health.

A few studies have identified organisms metabolizing BACs (*Aeromonas hydrophila* K., *Bacillus niabensis*, *Thalassospira* sp., and *Pseudomonas nitroreducens*) and described BAC degradation pathways (e.g., cleaving BACs by dealkylation) (6, 107, 111). Such pure culture-based studies have provided useful information about organisms

and pathways involved in BAC degradation. However, WWTPs are constantly loaded with diverse microorganisms, which often act in concert in degrading complex organic compounds. Accordingly, understanding how whole microbial communities degrade BACs is important for more reliable assessment, prediction, and optimization of BAC detoxification processes in biological engineering systems.

The microbial community analyzed in this study was developed with BACs as a sole carbon and energy source for three years in an aerobic fed-batch laboratory bioreactor, inoculated with a river sediment (107, 145). BACs are transformed into benzyldimethylamine (BDMA) and an alkyl chain by dealkylation based on work with the community (145) and pure-culture isolates from the community (107). Our recent metagenomics analysis of this community provides new insights into community adaptation to BACs (107). This community selectively enriched several species (mostly *Pseudomonas*) and a versatile repertoire of genes possibly related to BAC degradation (e.g., monooxygenase, dioxygenase, amine dehydrogenase, and amine oxidase).

Although our metagenomic findings indicate an array of organisms and genes potentially involved in community BAC degradation, their actual activities and temporal variation over the course of BAC degradation during a feeding cycle of the bioreactor remain elusive. In the present study, metatranscriptomics describes in situ activities of a few selected organisms and genes dynamically expressed during community BAC degradation. Newly sequenced genomes of key organisms were used to complement the metatranscriptomic data and reconstruct metabolic pathways of BACs in the organisms. Further, this study identifies significant upregulation of efflux pump genes upon BACs, which were encoded in a mobile genetic element subjected to horizontal gene transfer.

Taken together, our results provide novel insights into dynamic community responses to BAC exposure and advance current understanding of BAC degradation by a microbial community.

3.3. Results

3.3.1. BAC degradation performance by a microbial community

High-performance liquid chromatography (HPLC) analysis was used to determine BAC concentration during a representative feeding cycle (Fig. 3.1). While the feeding cycle duration was 84 hours, the majority of BACs was removed within the first twelve hours (detection limit: 1 mg/L). Soluble Chemical Oxygen Demand (sCOD) was measured to examine the complete degradation of BACs. Consistent with BAC disappearance, sCOD decreased from 301 mg sCOD/L at 0.5 hour to 99 mg sCOD/L at 12 hour. These results indicated that the majority (~80%) of BACs was degraded within half a day based on the residual sCOD value (45 mg sCOD/L at 0 hour), while some intermediates transformed from BACs (i.e., corresponding to the rest ~20% sCOD/L; carbon sources such as a benzyl group detected by sCOD but not HPLC analysis) were likely present at 12 hour. Other previously reported BAC intermediates such as BDMA, benzylmethylamine (BMA), and benzylamine (BA) were not detected during the feeding cycle based on HPLC analysis, presumably due to their fast transformation rates, as previously hypothesized (145).

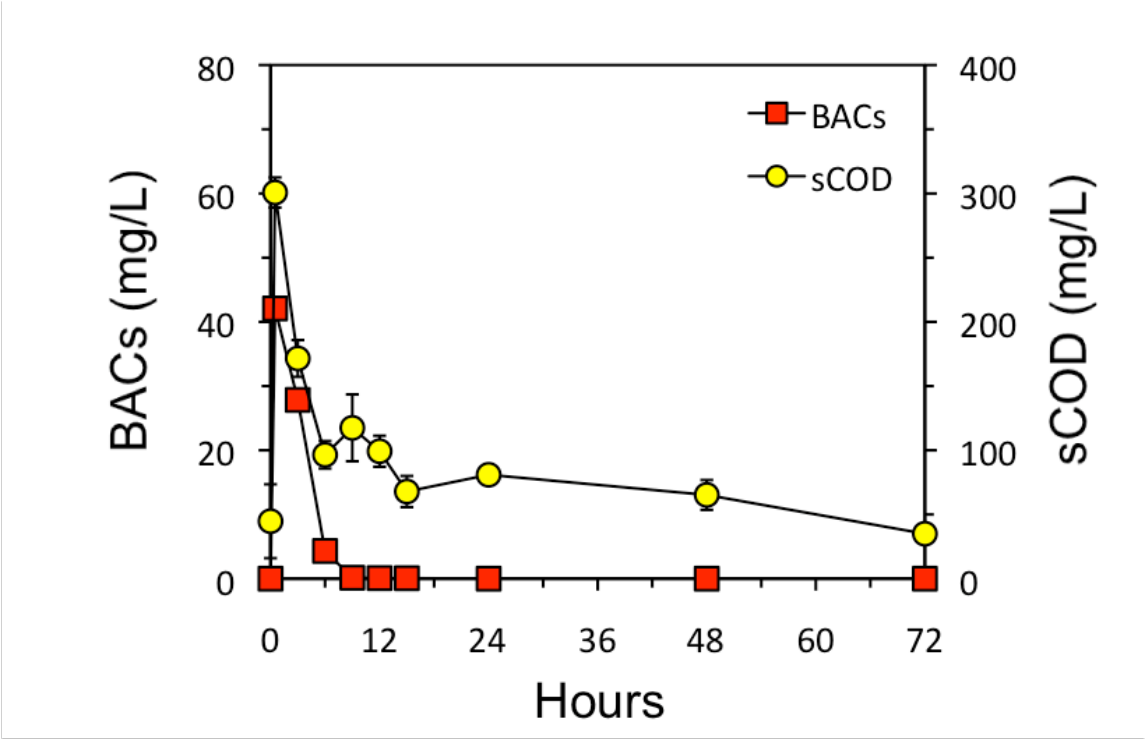

Figure 3.1. Time course of BAC concentration and sCOD during a feeding cycle. BACs and sCOD concentrations were measured at 0, 0.5, 3, 6, 9, 12, 15, 24, 48, and 72 hours in triplicates, during a representative feeding cycle. Similar BAC removal patterns were observed at 0, 0.5, 12, and 48 hours in additional feeding cycles (n=3; data not shown). The mean values and error bars (one standard deviation) of BACs and sCOD concentrations are shown.

3.3.2. Shifts in gene transcripts abundance during BAC degradation

The BAC concentration profile during a feeding cycle (Fig. 3.1) guided the sampling time points for metatranscriptomics. Metatranscriptomes at 0.5 hour, when BAC concentrations were the highest, and 0, 12, and 48 hours, when BAC concentrations were close to zero (i.e., below the limit of detection), were determined to analyze shifts in gene transcript abundance in response to BACs. Gene transcript (non-rRNA sequences) assignment was carried out based on best match Blastn analysis against all complete genome database at the genus level and all protein-coding genes assembled from the metagenome, which were subsequently clustered based on relative transcript abundance (Fig. 3.2). The analysis showed that the three biological replicates (from three independent feeding cycles) at 0.5 hour clustered closely together based on relative abundance of genera (Fig. 3.2A) and individual gene functions (3.2B) at the transcriptome level compared to datasets from other time points; hence the three metatranscriptomic replicates sampled at 0.5 hour were combined for further analysis.

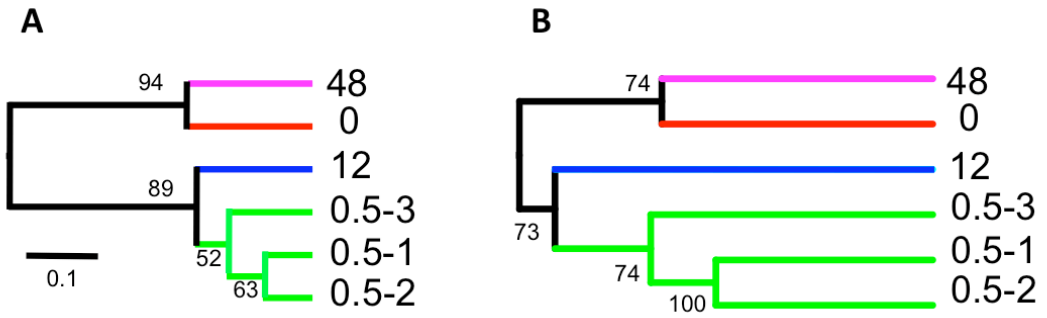

Figure 3.2. Clustering of metatranscriptomic datasets based on relative transcript abundance. Clustering was carried out using the Bray-Curtis metric, based on the relative transcript abundance of (A) ~500 genera recovered in the transcriprome and (B) all protein coding genes recovered in the metagenome. Numbers on the nodes represent bootstrap support from 100 replicates.

Profiling relative 16S rRNA gene transcript abundance revealed the overrepresentation of *Pseudomonas* genus (78±6.3% of total, average across the four sampling points), followed by *Stenotrophomonas* (1.9±2.1% of total), and *Flavobacterium* (1.2±0.5% of total) (Fig. 3.3). Our previous metagenomic survey shows the dominance of *Pseudomonas* (57%), followed by *Methylobacterium* (5.6%), *Burkholderia* (2.9%), *Mycobacterium* (2.0%), and *Stenotrophomonas* (1.3%) in this community (107). Metatranscriptomic data confirmed the significant activity of *Pseudomonas* genus, as consistent with the previous DNA-based results, but *Methylobacterium*, *Burkholderia*, and *Mycobacterium* turned out to be much less active (<0.5% of the total community 16S rRNA gene transcript abundance throughout the feeding cycle). These transcriptomic results suggest that the previous metagenome-based results do not necessarily reflect accurately the activity of the major taxa present in the community.

Analysis of non-rRNA gene transcripts revealed substantial shifts in the activity of the major genera (>3% of the total) during the feeding cycle. The most pronounced difference was observed during BAC degradation (i.e., from 0 to 12 hours) for *Pseudomonas*, whose relative gene transcripts made up 29.7%, 75.5%, and 86.4% of the total community at 0, 0.5, and 12 hour, respectively. The genomes of two *P. nitroreducens* isolates (*P. nitroreducens*-B and *P. nitroreducens*-DBP) were newly sequenced; this species was highly enriched in the BAC-degrading community based on our previous metagenomic survey and is able to grow with BACs as a sole carbon and energy source (107). Mapping non-rRNA sequences on the whole genome sequences at high stringency revealed significant increase of *P. nitroreducens* gene transcripts during

the feeding cycle from 12.5% to 42.1%, and 59.7% at 0, 0.5, and 12 hour, respectively, comprising more than three quarters of the total *Pseudomonas* gene transcripts at 12 hour. Collectively, these results suggested that *P. nitroreducens* played a key role in community BAC degradation, consistent with our previous results (107).

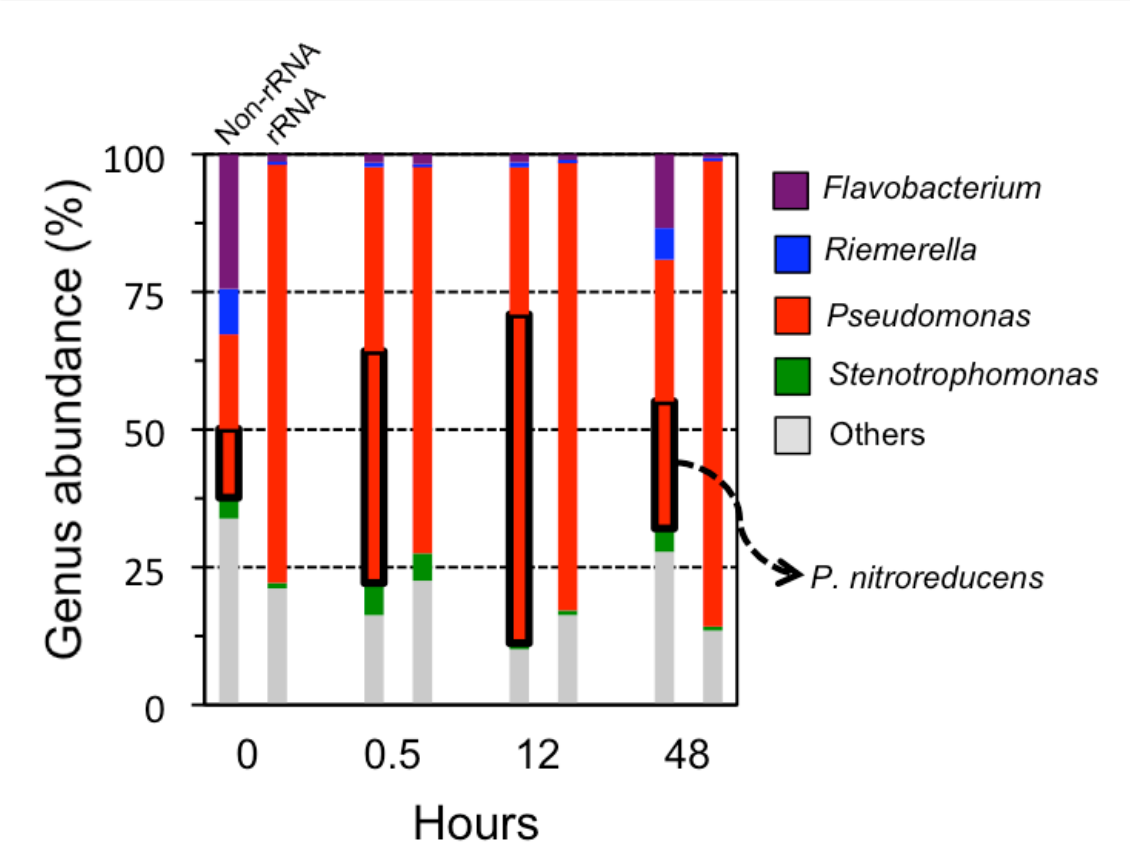

Figure 3.3. Phylogenetic affiliation of gene transcripts during BAC degradation. Colors represent relative transcript abundance of the major genera (>3% of the total) based on 16S rRNA (right) and non-rRNA (left) genes. 'Others' represents the combined fraction of the remaining minor genera (see figure key). The fraction of non-rRNA sequences phylogenetically affiliated to *P. nitroreducens* is marked.

3.3.3. Overrepresented gene transcripts of *P. nitroreducens* and the other community members

Clustering relative transcript abundance of 6,621 *P. nitroreducens* protein-coding genes, annotated based on the SEED subsystems (109), identified metabolic functions significantly responded at 0.5 hour compared to those at 0, 12, and 48 hours (Fig. 3.4A). Functional categories significantly upregulated ($P < 0.01$ by Student's t-test) at 0.5 hour were associated with i) energy generation (e.g., TCA cycle, serine-glyoxylate cycle, and ubiquinone menaquinone-cytochrome C reductase), ii) cell wall biosynthesis/maintenance (KDO2-lipid A biosynthesis, UDP-N-acetylmuramate, and peptidyl-prolyl cis-trans isomerase), and iii) cell division/cycle (ribosomal proteins).

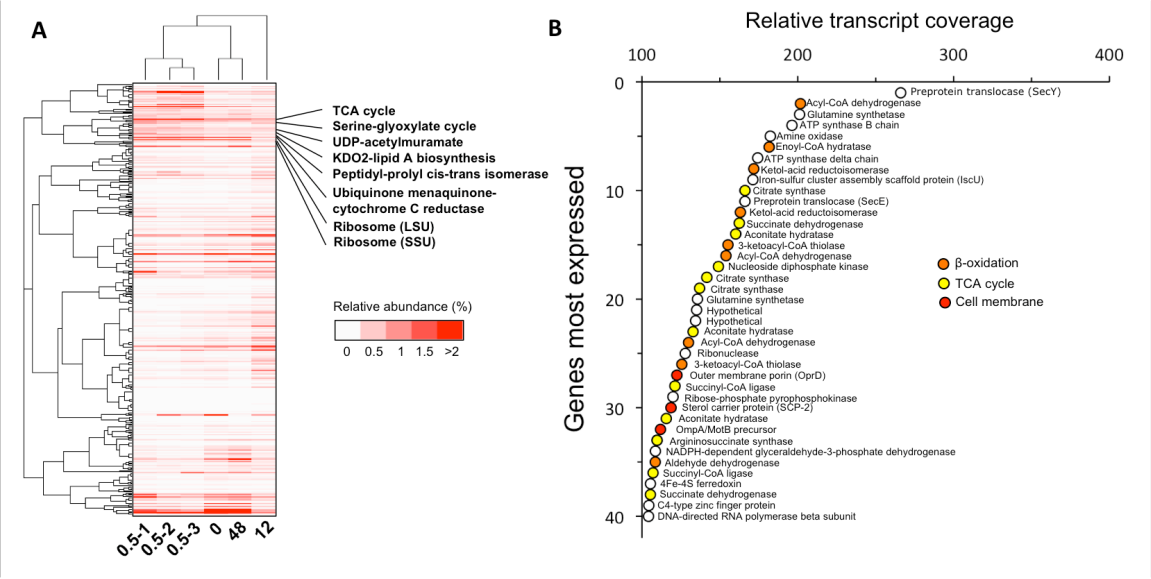

Figure 3.4. Metabolic profiling of *P. nitroreducens* gene transcripts during BAC degradation. (A) Clustering of the relative abundance of gene transcripts; gene transcripts are annotated based on the SEED subsystems. Hierarchical clustering was carried out with Spearman rank correlation metric and complete linkage method. Selected SEED subsystem categories significantly differentially expressed ($P < 0.01$ by Student's t-test) and clustered together at 0.5 hour are shown. (**B**) Relative transcript coverage of *P. nitroreducens* genes significantly upregulated ($P < 10^{-4}$ by Chi-squared test) at 0.5 hour compared to 0, 12, and 48 hours, excluding ribosomal protein genes.

The most significantly upregulated *P. nitroreducens* genes (n = 40 with $P < 10^{-4}$ by Chi-squared test) at 0.5 hour compared to at 0, 12, and 48 hours were determined to describe the metabolic functions (Fig. 3.4B). Analyzing the average transcript coverage of randomly sampled 40 protein-coding genes showed that the 40 genes (>100X) listed in Fig. 3.4B had statistical significance ($P < 10^{-4}$ by Student's t-test) compared to those of the other *P. nitroreducens* genes (11.9±7.5X) at 0.5 hour (Fig. 3.5).

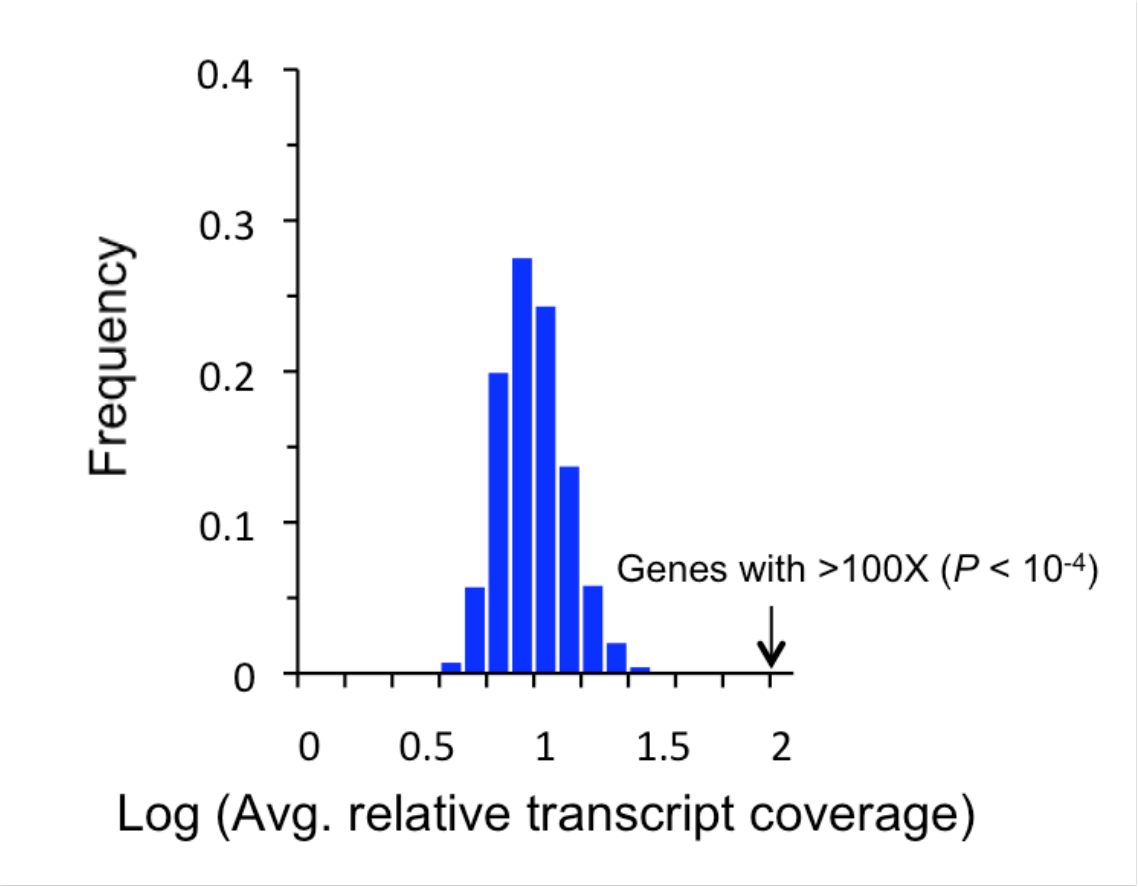

Figure 3.5. Expected transcript coverage of randomly sampled genes. The null distribution of the average transcript coverage of randomly sampled genes (n = 40) at 0.5 hour was plotted with 100 permutations. The distribution was normally distributed ($P < 0.05$) by Jarque-Bera test (58). Forty genes that were most significantly expressed (>100X, $P < 10^{-4}$ by Student t-test) compared to the expected transcript coverage (11.9±7.5) of randomly sampled genes at 0.5 hour were selected to describe their metabolic functions (Fig. 3.4B).

Foremost, the highly expressed genes at 0.5 hour were related to fatty acid metabolism (e.g., acyl-CoA dehydrogenase, aldehyde dehydrogenase, enoyl-CoA hydratase, ketol-acid reductoisomerase, and 3-ketoacyl-CoA thiolase) and energy production through TCA cycle (e.g., succinate dehydrogenase, aconitate hydratase, enoyl-Coa hydratase, ketol-acid reductoisomerase, nucleoside diphosphate kinase, citrate synthase, and succinyl-CoA ligase) (Fig. 3.4B). In addition, cell membrane-associated genes such as outer membrane porin/lipoprotein and SCP-2 sterol transfer protein genes were also significantly responded at 0.5 hour. Although the definitive function of the outer membrane lipoprotein remains unclear, a previous study reported the significant overexpression of this gene in response to QACs (138). This study suggested that it might function as an efflux pump or enhance mechanical cell membrane integrity against QACs. Since sterol carrier proteins transfer steroids between cellular membranes and regulate membrane fluidity, permeability, and thickness (40), the SCP-2 sterol transfer protein may play a role in alleviating BAC toxicity by enhancing cell membrane integrity. Notably, the SCP-2 sterol transfer genes are largely enriched in *Pseudomonas* members of the BAC-degrading community (107). A significant expression of an amine oxidase gene was also noticed (174X at 0.5 hour), showing about 15 times higher relative transcript coverage compared to those 0 and 48 hours. This gene is bioinformatically inferred to carry out the oxidative deamination of a wide range of amines (119) and thus, might catalyze the first dealkylation step (i.e., cleaving the C_{alkyl}-N bond) in the previously known BAC degradation pathway (6, 107, 111, 145).

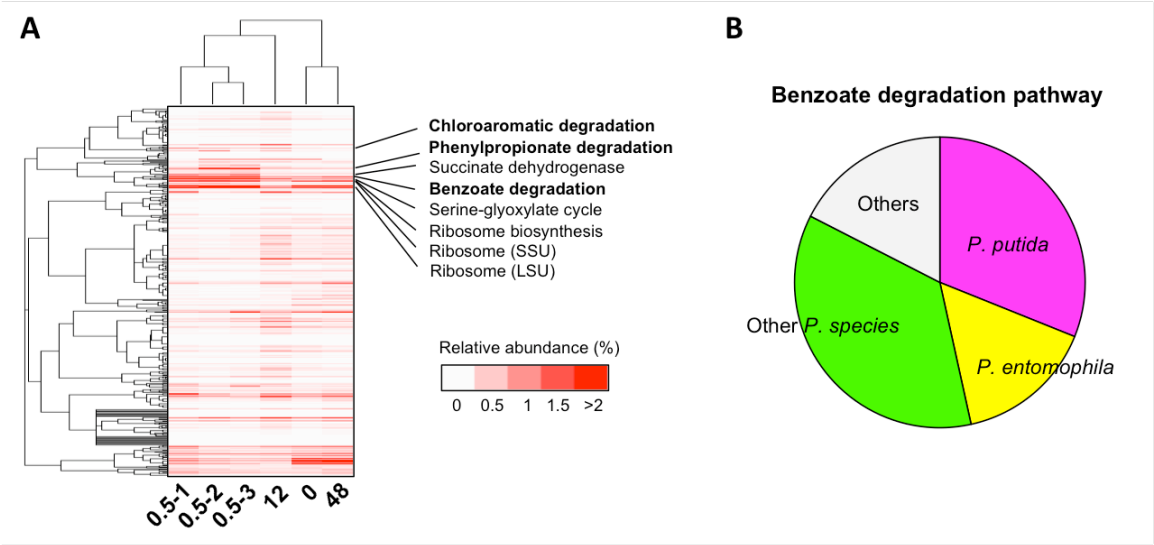

Figure 3.6. Metabolic profiling of non-*P. nitroreducens* gene transcripts during BAC degradation. (A) Clustering of the relative abundance of gene transcripts; gene transcripts that are not affiliated to *P. nitroreducens* are annotated based on the SEED subsystem. Hierarchical clustering was carried out as performed in Fig. 3.5. Selected SEED subsystem categories differentially expressed ($P < 0.01$ by Student's t-test) and clustered together at 0.5 hour are shown. (**B**) Phylogenetic affiliation of gene transcripts related to the benzoate degradation pathway. 'Others' represents the combined community transcripts that are not taxonomically assignable (i.e., they show <80% nucleotide sequence identity and <50% query length coverage) at the genus level. Similar results (i.e., phylogenetic affiliation of gene transcripts) were observed for a benzoate 1.2-dioxygenase gene, representative of the benzoate degradation pathway genes, and also for genes of the other two pathways (chloroaromatic and phenylpropionate) encoded by the major community members. (data not shown). These results suggested that the picture of the contribution by the major community members to the community transcriptome associated with benzyl group metabolism is robust.

Metabolic responses of the other (non-*P. nitroreducens*) community members were also characterized during the feeding cycle. The metatranscriptomic profiles at 0.5 hour were distinguished from the other time points (Fig. 3.6A) and showed overexpression of gene transcripts associated with i) benzyl-group compound metabolism (benzoate, chloroaromatic, and phenylpropionate), ii) energy production (serine-glyoxylate cycle), and iii) cell division/cycle (ribosome protein and biosynthesis). The majority of the community transcripts related to benzyl-group compound metabolism were phylogenetically affiliated to *Psedomonas* species (83%), particularly *P. putida* (31%) and *P. entomophila* (15%) (Fig. 3.6B). Therefore, these results suggested that the benzyl group, a major product dealkylated from BACs by the *P. nitroreducens* (107, 145), was predominantly metabolized by *P. putida* and *P. entomophila*.

3.3.4. Overexpression of horizontally transferred efflux pump systems upon BAC exposure

Our previous metagenomic study revealed three mobile genetic elements encoding genes associated with antimicrobial resistance and/or BAC degradation, all of which had been rapidly and reproducibly selected (e.g., their relative gene abundance in metagenomes increased >2.7 times per day for 43 days of BAC exposure) (107). Among these mobile genetic elements, the assembled metagenomic contig B_00388 (14 kb long) was found to be conserved in the *P. nitroreducens* isolate genomes and encoded a part of conjugative transfer operon (*trbBCDE*), type IV secretion protein, and transposase genes, indicating that it may be a part of a conjugative plasmid (Fig. 3.7A). The genetic element also encoded four efflux pumps system genes, including two small multi drug resistance (SMR) family systems (*sugE*), one resistance nodulation division (RND) family system,

and one ABC transporter system, all of which can potentially export a wide range of toxic compounds outside cells. Metatranscriptomics revealed significant upregulatation ($P <$ 0.01 by Chi-squared test) of the four efflux pump system genes at 0.5 hour compared to 0, 12, and 48 hours (Fig. 3.7A). This genetic element was also conserved, with similar gene content and organization, in the genomes of other organisms such as *P. aeruginosa*-39016, *P. aeruginosa*-NCGM2.S1, and *Salmonella enterica* Montevideo S-403 isolated from clinical settings (31, 95, 133) (Fig. 3.7A). Phylogenetic analysis of *sugE*-A, which was encoded on the genetic element and previously shown to confer BAC resistance (48), was carried out to examine whether this genetic element was subjected to horizontal gene transfer. All *sugE*-A genes conserved in the phylogenetically diverse organisms (Fig. 3.7B) isolated from our bioreactor and clinical settings were identical (100% nucleotide identity; see Fig. 3.7C) with one another. Although *sugE*–family genes are also encoded on the chromosome of *Pseudomonas* species, including *P. nitroreducens*, the *sugE*-A genes found on the genetic element was not closely related (<70% nucleotide identity) to the chromosomally encoded *sugE* genes (Fig. 3.7C). Accordingly, these results strongly supported horizontal transfer of the efflux pump genes via a conjugative plasmid, which may function to detoxify BACs by regulating intra-cellular BAC concentration.

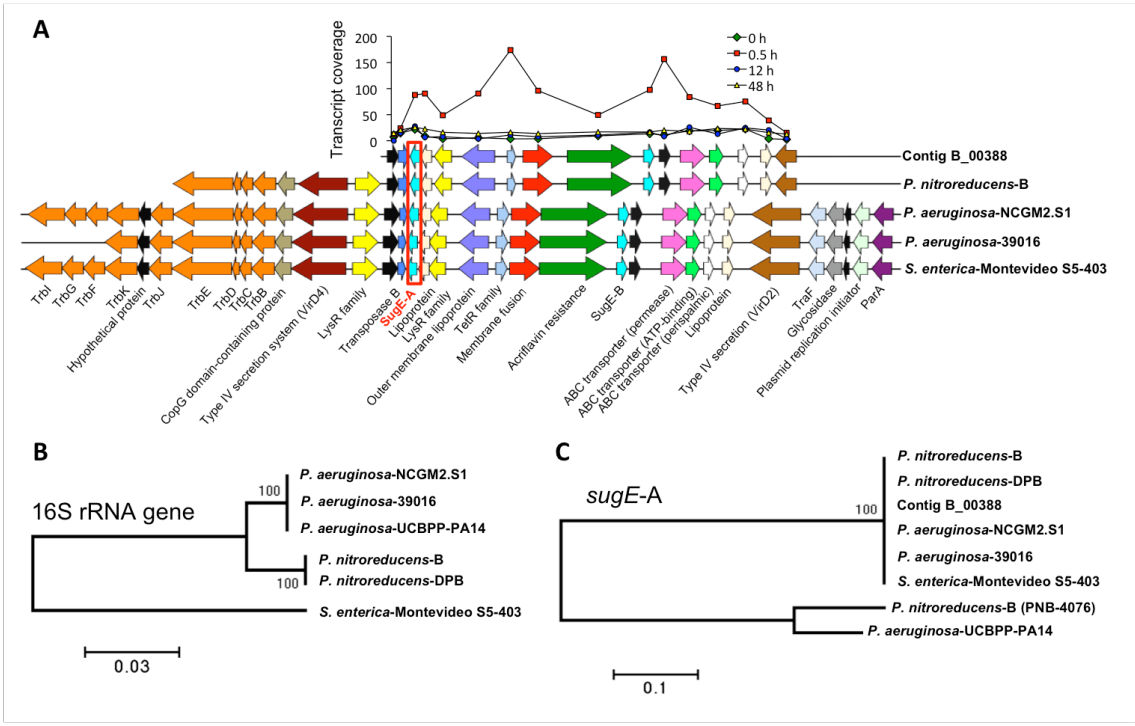

Figure 3.7. Significant upregulation of efflux pump genes in response to BACs. (A) Organization, predicted function, and relative transcript coverage of genes encoded on contig B_00388. The corresponding genomic region of *P. nitroreducens*, *P. aeruginosa*-39016 (accession no. NZ_CM001020.1), *P. aeruginosa*-NCGM2.S1 (NC_017549.1), and *S. enterica*-Montevideo S5-403 (AFCS01000001.1) are shown for comparison. Note the significant ($P < 0.01$ by Chi-squared test) expression of the four efflux pump system genes at 0.5 hour. Phylogenetic relationship of 16S rRNA genes (B) and *sugE*-A (highlighted in Fig. 3.7A) genes (C) encoded on contig B_00388 and chromosomes of *P. aeruginosa*-UCBPP-PA14 (NC 008463.1) and *P. nitroreducens*-B (ORF no. PNB_4076). The phylogenetic tree was built based on maximum likelihood composite model and the neighbor-joining algorithm. The values on the nodes represent bootstrap support from 100 replicates. Similar phylogenetic relationship was observed in other efflux pump genes (ABC and RND families; data not shown), as shown in *sugE*-A.

3.3.5. Functional versatility in fatty acid metabolism in *P. nitroreducens*

Our previous 16S rRNA gene-based study revealed that *P. nitroreducens* isolated from this community showed 92 to 96% identity to other representative *Pseudomonas* species (107). Whole genome sequence analysis of *P. nitroreducens* showed a high fraction of species-specific genes (2,360 genes among 6,621 protein-coding genes in the genome of *P. nitroreducens*-B based on a sequence homology search using >70% nucleotide identity and >70% query length coverage). The 22 *Pseudomonas* species (complete genomes as of February 2012, available at ftp://ftp.ncbi.nih.gov/) and *P. nitroreducens* genomes determined here were functionally characterized using the RAST server (4). The most differentially enriched SEED subsystem in the *P. nitroreducens* genome compared to other *Pseudomonas* genomes was associated with the fatty acids, lipids, and isoprenoids category (Fig. 3.8). The species-specific genes that were associated with the category included aldehyde dehydrogenase, alcohol dehydrogenase, and 3-oxoacy-(acyl-carrier-protein) reductase genes. Aldehyde dehydrogenase and alcohol dehydrogenase are potentially related to alkyl chain metabolism of BACs (e.g., β-oxidation and fatty acid biosynthesis pathway). 3-oxoacyl-(acyl-carrier-protein) reductase is a key component in fatty acid biosynthesis and enoyl-acyl carrier reductase is involved in fatty acid elongation cycle (89). Cell membrane biosynthesis represents an important cellular response to BACs (19, 73), due to the mode of action on the cell membrane; hence, the genes identified here may be associated with the maintenance of the cell membrane integrity in presence of BAC toxicity.

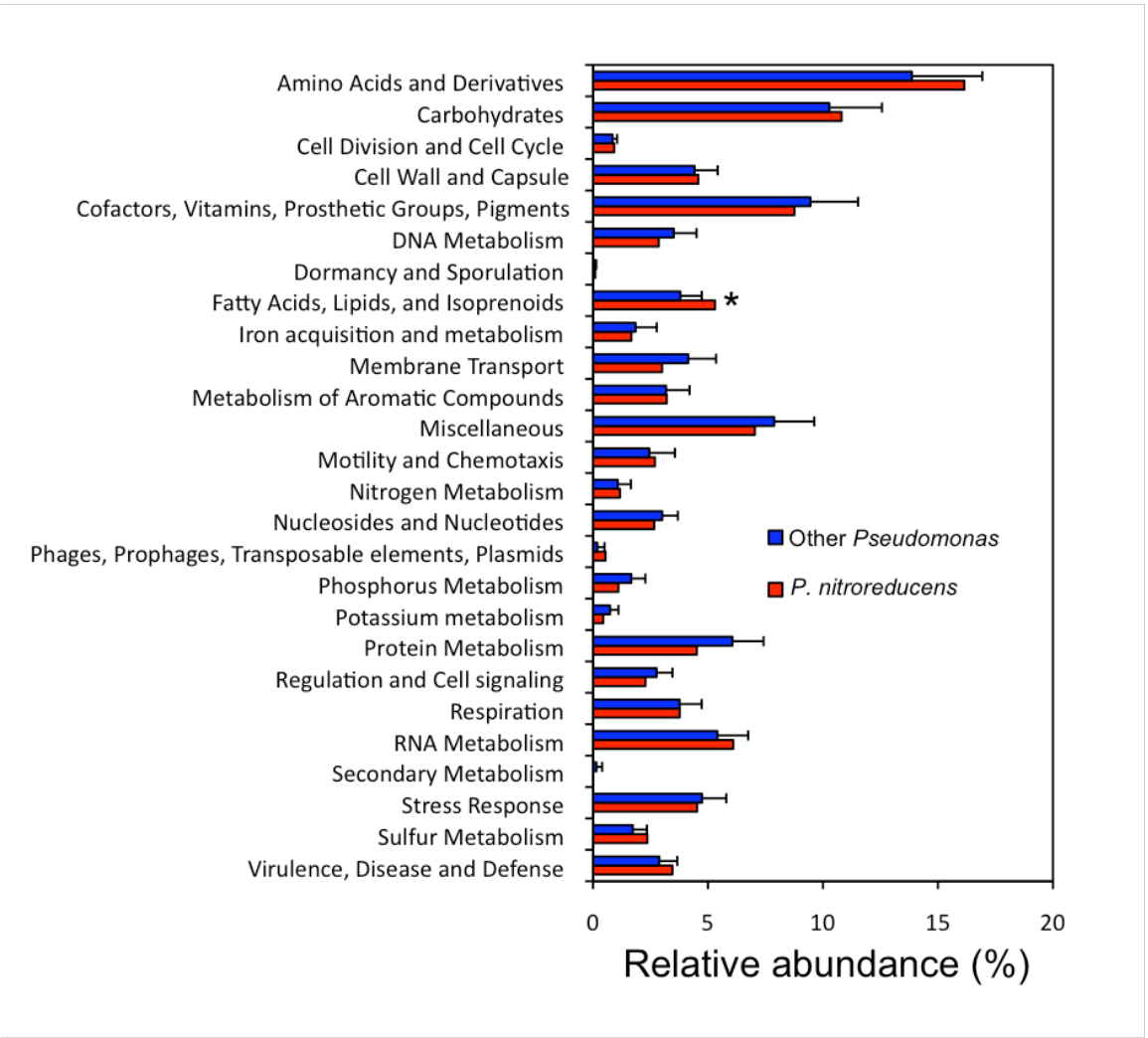

Figure 3.8. Gene content comparison of *P. nitroreducens* compared to other *Pseudomonas* species. The relative abundance of each SEED subsystem in a *Pseudomonas* species genome is shown. The mean values of 22 *Pseudomonas* species are shown with error bars (one standard deviation). The most enriched ($P = 0.12$ by Student's t-test) SEED subsystem in *P. nitroreducens* compared to other *Pseudomonas* species is marked (asterisk).

3.4. Discussion

Microorganisms can cooperate to degrade complex organic compounds. One may partially metabolize the substrate and excrete secondary product(s), which may be subsequently used by other organisms. Such cross-feeding among microorganisms is an important process for nutrient cycling in diverse ecosystems and degradation of synthetic toxic compounds (114). Our metatranscriptomic data suggested cooperative interactions (Fig. 3.3, 3.4, and 3.6) among key members of the whole microbial community that successfully degraded BACs (Fig. 3.1), which was not achieved by certain single organisms such as *P. nitroreducens* (107).

The microbial community carried out rapid BAC degradation within half a day (Fig. 3.1), while volatile suspended solids concentrations (about 140 mg/L) of the mixed liquor suspension were more or less stable across the entire feeding cycle (145). *P. nitroreducens* played a key role in degrading BACs based on gene transcript activities (Fig. 3.3 and 3.4) and previous metabolite analysis of the pure culture showing transformation of BACs by dealkylation (107). Our transcriptomic data revealed significant upregulation of an amine oxidase gene (Fig. 3.4B) of *P. nitroreducens*. Amine oxidase and dehydrogenase catalyze the oxidative deamination of amines by cleaving the C-N bond (119). Both amine dehydrogenase and amine oxidase genes are enriched in the BAC-degrading community and the dominant *Pseudomonas* species (107). Although previous enzymatic assays identified amine dehydrogenase involved in the dealkylation of QACs (68, 150), the present RNA-based study suggested the amine oxidase gene of *P. nitroreducens* as a candidate for the catalysis of the dealkylation step in the BAC degradation pathway. Further work is necessary, however, to test this hypothesis. Since

the dealkylated BDMA is about 500 times less toxic than BACs (145), the dealkylation of BACs is also an important detoxification process, which presumably provide significant benefits to other BAC-sensitive community members. After dealkylation of BACs, *P. nitroreducens* is hypothesized to generate energy for cell growth by successive β-oxidation of the alkyl chain (i.e., a dealkylated part of BACs) and subsequent TCA cycle based on the overexpression of the corresponding genes (Fig. 3.4).

Although *P. nitroreducens* dominated the microbial community, our previous isolate-based work suggested that this species can not completely degrade BACs but release BDMA as a secondary product (107). Our metatranscriptomic data indicated that the benzyl group was predominantly metabolized by other *Pseudomonas* species such as *P. putida* and *P. entomophila*, reflected by the phylogenetic affiliation of transcripts associated with benzyl group (i.e., BDMA intermediates) metabolism (Fig. 3.5). Collectively, the metatranscriptomic data suggested cross-feeding among key member of the microbial community successfully degrading BACs (Fig. 3.9). Further studies employing isotope fractionation analyses or metabolite analysis with defined mixture of the species identified here will robustly validate these findings.

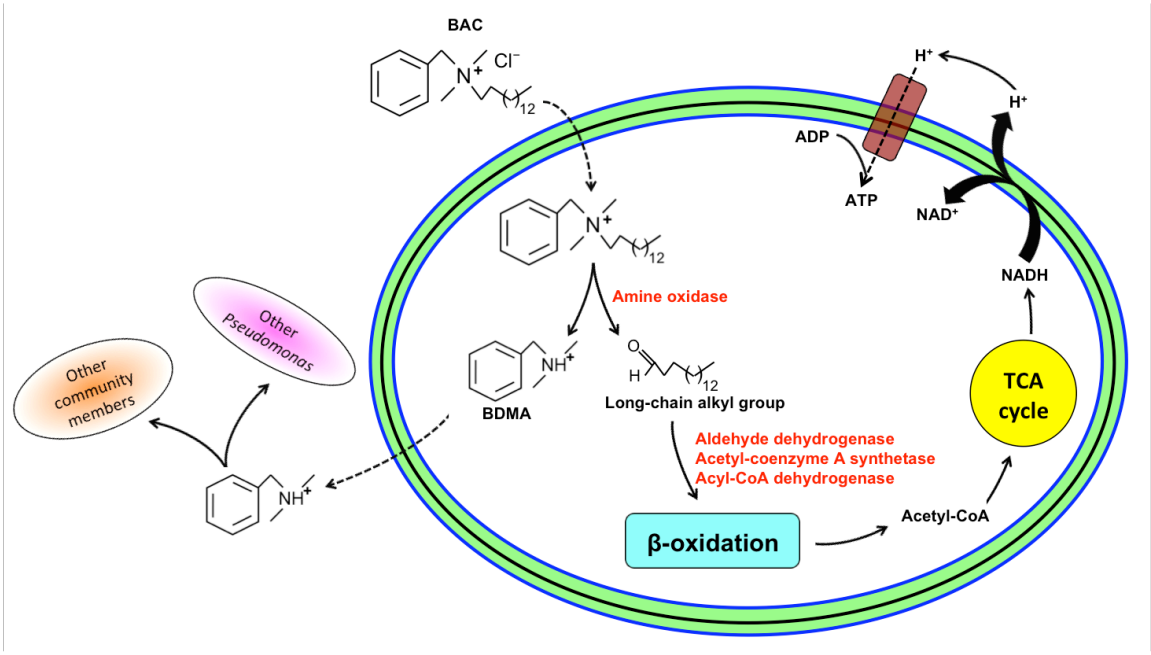

Figure 3.9. Metabolism of BACs (C_{14}BDMA-Cl) by microbial consortia. The big ellipse represents *P. nitroreducens.* The genes involved in dealkylation and β-oxidation inferred based on transcript activities are denoted beside arrows.

Several additional *Pseudomonas* species present in the community can possibly metabolize intact BACs, as they have been previously shown to be the case of *P. putida* for QAC degradation (68, 78, 150). Nevertheless, the *P. nitroreducens* was selectively and reproducibly enriched in this community fed by BACs (107) and showed much higher transcriptional activity than other *Pseudomonas* members during BAC degradation. *P. nitroreducens* particularly enriched genes associated with fatty acid metabolism (e.g., aldehyde and alcohol dehydrogenase) in the genome, compared to other *Pseudomonas* species (Fig. 3.8). Since aldehyde dehydrogenase catalyzes the β-oxidation processes of the alkyl group of BACs (Fig. 3.9), the significant expression and enrichment (Fig. 3.4 and 3.8) of fatty acid metabolism genes likely underlies high fitness of *P. nitroreducens* species in metabolizing BACs (particularly the alkyl chain) compared to other *Pseudomonas* species present in the community (e.g., *P. putida*). In addition to the gene expression data, analyzing specific activities of the *P. nitroreducens* proteins associated with dealkylation and alkyl chain metabolism compared to those of other *Pseudomonas* species will also help understanding high fitness of *P. nitroreducens* upon BAC exposure (107).

Microorganisms can resist toxic compounds such as QACs by exporting out of cells through efflux pump system. Some SMR-family efflux pumps (*qac* gene homologues) are often found in mobile genetic elements such as plasmid and integron, (50, 113), which can be horizontally transferred across phylogenetically diverse organisms. Although our previous metagenomic study identified significant selection of three mobile genetic elements upon BACs (i.e., encoding antimicrobial resistance genes), subjected to horizontal gene transfer, the present study revealed that genes encoded only

in the contig B_00388 (i.e., representing a conjugative plasmid) showed significant gene expression upon BACs (Fig. 3.7). Since efflux pump systems encoded in the plasmid, *sugE* and a RND-family efflux pump confers resistance to BACs (48, 134), the conjugative plasmid that were shared among members this community (107), including *P. nitroreducens* (Fig. 3.7), likely represent a community-wide defense mechanism to BACs.

In this study, we provided the first metatranscriptomic view of a whole microbial community degrading BACs under an aerobic fed batch condition and provide insights into the key community organisms, their cooperative interactions, and genes involved in BAC detoxification. The microbial consortia studied here have relevance and implications for biological engineering system such as activated sludge in WWTPs. Further, our study identified significant activities of efflux pump genes encoded in a conjugative plasmid. It should be noticed that the efflux pump systems (SMR, ABC, and RND families encoded in the plasmid subjected to horizontal gene transfer) can potentially extrude a wide range of toxic compounds such as disinfectants (e.g., QACs and triclosan) and antibiotics. Our results, therefore, represent a useful basis for future investigations on the role of such disinfectants in propagating (e.g., co-selecting) antibiotic resistance in microbial communities, which has important implications for both public and environmental health.

3.5. Materials and methods

3.5.1. Characterization of BAC degradation performance by a microbial community

The microbial community analyzed in this study was described previously (107, 145). In brief, the inoculum originated from a river sediment contaminated with metals

and numerous organic pollutants (82) and the community was developed in an aerobic fed batch bioreactor fed with BACs as a sole carbon and energy source for three years. The media and operating parameters were described in details previously (145). To characterize community BAC degradation, triplicate samples were taken from the liquid cultures at 0, 0, 0.5, 3, 6, 9, 12, 15, 24, 48, and 72 hours, respectively, during a typical feeding cycle (cycle duration: 84 hours). C_{12}BDMA-Cl and C_{14}BDMA-Cl were measured using a HP 1100 series HPLC system (Hewlett-Packard, Palo Alto, CA) and soluble chemical oxygen demand (COD) was measured as described previously (145).

3.5.2. RNA/DNA extraction and sequencing

50 ml of mixed culture suspension was sampled to extract RNA with an organic nucleic acid extraction method (106) with little modification for RNA. A lysis buffer was prepared with 50 mM Tris-HCl, 40 mM EDTA, and 0.75 M sucrose, and was added to the filters with 1 mg/ml lysozyme, with subsequent incubation for 30 min at 37°C. A second 2 h incubation at 55°C was performed with the addition of 1% SDS and 10 mg/ml proteinase K. Acid phenol and chloroform was added to the lysates for the extraction of RNA. RNA was isolated using filter columns from the mirVANA RNA isolation KIT (Ambion), washed twice following the manufacturer's instructions and eluted in TE buffer. DNase treatment was performed using the TURBO DNA-*free* kit (Ambion, Austin, TX), followed by rRNA depletion by subtractive hybridation of rRNA (MICROB*Express*, Ambion). Enriched mRNA samples were amplified with the MessageAmp II-Bacteria Kit (Ambion) and the resulting antisense RNA (aRNA) was reverse transcribed with random hexamer primers and the SuperScript™ II Reverse Transcriptase Kit (Invitrogen) and purified with MinElute DNA clean-up kit (Qiagen).

Quality and quantity of nucleic acids during the cDNA preparation protocols were motored using the Agilent RNA 6000 Pico Kit (Agilent), and Qubit RNA Assay Kit (Invitrogen). The resulting cDNA libraries were sequenced (150 bp single end reads) using the Illumina GA II sequencer available at the Los Alamos National Laboratory sequencing facilities. The two *P. nitroreducens* (*P. nitroreducens*-B and *P. nitroreducens*-DPB) isolated from the community were able to grow with BACs as a sole carbon and energy source, as described previously (107). DNA was extracted from pure culture suspension of the species as described previously (106) and the whole genome was sequenced by the Ion Torrent platform (Life Technologies) using the 316 chip.

3.5.3. Sequence data analysis

The raw sequencing reads were trimmed using a Q=15 Phred quality score cutoff using SolexaQA (29) and the homopolymer sequences with consecutive nucleotides ($n \geq 9$) were also trimmed. All the trimmed reads were first searched against 5S (137) and 16S and 23S ribosomal RNA gene databases (116) using Blastn with a cutoff of 40 bit-score. The remaining reads (non-rRNA sequences) that were not identified as rRNA sequences with the cutoff used above were then searched against all bacterial and archaeal genome sequences available in NCBI database (as of February 2012, ftp://ftp.ncbi.nih.gov/), using Blastn with a cutoff of >80% identity and 50% query length coverage at the genus level. Blastn was run with X = 150, q = -1, and F = F, remaining parameters at default settings. To provide a quantitative assessment of relative abundance of gene transcripts across metatranscriptomic datasets, the number of matches (i.e., rRNA or non-rRNA sequence reads) to each genus was divided by the total number of matches assigned to all genera in the database (i.e., normalization across metatranscriptome datasets with different sizes).

Protein-coding genes were recovered in the community metagenome (accession no. SRR639751), as described previously (107). The amino acid sequences of the protein-coding genes were functionally annotated based on SEED protein and subsystem database using sequence homology search (Blastp) with a 30% amino acid sequence identity and 50% query length coverage cut-off for a match. Unassembled metatranscriptomic reads (non-rRNA sequences) were mapped on the protein-coding genes with at least 95% identity and 50% query length coverage to bioinformatically infer the functional roles of the individual reads based on their best matching protein-coding genes. To estimate relative transcript coverage, the length of the non-rRNA sequence reads assigned on a protein-coding gene were summed and divided by the length of the corresponding gene sequence. The transcript coverage was normalized by the size of all non-rRNA sequence reads assigned to all protein-coding genes, which provided relative transcript coverage (expressed as X per 100 Mb non-rRNA sequences) of a protein-coding gene comparable across datasets with different sizes.

For genome analysis of *P. nitroreducens* species, raw sequences were trimmed and assembled as performed for metatranscriptomic reads above. Protein-coding genes (>300 b) were obtained by selecting the longer ones predicted by both Prodigal (V. 2.60) (55) and GeneMarkS (V. 4.6b) (9). The gene product of a protein-coding gene was predicted as performed for those recovered in the metagenome.

3.5.4. Nucleotide sequence accession numbers

The metatranscriptome data used in this study were deposited in GenBank under the accession numbers: XXX (B_{0h}), XXX ($B_{0.5h-1}$), XXX ($B_{0.5h-2}$), XXX ($B_{0.5h-3}$), XXX (B_{12h}), and XXX (B_{48h}). The whole genome sequences of *Pseudomonas nitroreducens*

isolates were deposited in GenBank under the accession numbers: XXX (*Pseudomonas nitroreducens*-DPB) and XXX (*Pseudomonas nitroreducens*-B).

3.6 Acknowledgements

This work was supported by the U.S. National Science Foundation (NSF), awards no. 0967130 (to SGP and KTK) and no. 1241046 (to KTK). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of NSF. We thank Ulas Tezel for helpful discussions and suggestions regarding this study and the personnel of the Los Alamos National Laboratory for sequencing of the samples.

CHAPTER 4

WIDELY USED DISINFECTANTS CO-SELECT FOR BACTERIAL ANTIBIOTIC RESISTANCE

This chapter is related to the manuscript:

Seungdae Oh, Michael R. Weigand, Madan Tandukar, Spyros G. Pavlostathis, and Konstantinos T. Konstantinidis. Widely used disinfectants co-select for bacterial antibiotic resistance. *In preparation*.

4.1. Summary

Whether or not disinfectant exposure promotes antibiotic resistance has been a long-standing debate. An integrative approach that combines community genomics and isolate characterization with genetic manipulations showed that widely used, broad-spectrum benzalkonium chloride (BAC) disinfectants induce antibiotic resistance by co-selecting for horizontally transferred plasmids that encode a BAC-specific efflux pump together with antibiotic resistance genes. These findings advance our understanding of the molecular mechanisms that proliferate antibiotic resistance upon disinfectant exposure and have implications for controlling the spread of antibiotic resistance in clinical or environmental settings.

4.2. Introduction

Antibiotic resistance is one of the most serious issues for public health in that it makes antibiotics ineffective to common bacterial infections. While inappropriate prescription to humans and misuse of antibiotics in the animal feed are thought to be the primary causes worsening the antibiotic resistance problem in recent years, there has also

been an increasing concern that disinfectants such as quaternary ammonium compounds (QACs) may fuel the emergence of antibiotic resistance in the environment (16). A substantial body of literature reports that exposure to QACs promotes antibiotic resistance (62, 79, 90, 118, 121, 140, 141). Yet, other studies have argued that the resistance linkage is rare, unsystematic, and/or clinically insignificant (3, 26, 28, 69, 71, 131). Therefore, whether or not disinfectant exposure indeed mediates antibiotic resistance and, if so, what molecular mechanisms enable the resistance link remain to be clearly elucidated.

Benzalkonium chlorides (BACs) are QAC-family disinfectants that are widely used in a variety of clinical and environmental settings. We previously reported that exposing microbial communities to BACs significantly reduces (2.5 to 4.6 fold) their susceptibility to BACs, determined as minimum inhibitory concentration (MIC), and also three clinically relevant antibiotics such as penicillin G (>2 fold), tetracycline (>95 fold), and ciprofloxacin (>8 fold) (140). Further, our previous comparative metagenomics of the BAC-exposed communities compared to the unexposed (control) revealed that the former communities are enriched in a variety of genes associated with broad resistance capabilities such as efflux pumps, cell envelope modification, and oxidative stress defense systems (107). In the present study, isolates of the same species were recovered from BAC-exposed and unexposed communities and were examined to determine the molecular underpinnings of BAC-induced antibiotic resistance. These results suggest the definitive role of BAC disinfectants in proliferating antibiotic resistance.

4.3. Results

4.3.1. Metagenomics reveals species enriched by BAC exposure.

Three years of long-term BAC exposure led to significant changes in the composition of microbial communities compared to the same community maintained with dextrin/peptone (DP; control community) under the same conditions (Fig. 4.1). While *Klebsiella oxytoca* (3.0%) and *Achromobacter xylosoxidans* (1.4%) were major species in the control DP community, dextrin/peptone plus BACs (DPB) and BACs only (B)-fed communities were enriched in several different species. *Pseudomonas nitroreducens* (3.2% and 27.3% for DPB and B, respectively) and *Pseudomonas putida* (4.6% and 1.0%) were commonly selected in both BAC-exposed communities. In addition, the DPB community was highly enriched in *Citrobacter freundii* (30.7%), *K. oxytoca* (8.6%), *A. xylosoxidans* (2.8%), and *Pseudomonas aeruginosa* (1.4%), and the B community in *Methylobacterium extorquens* (1.4%). The metagenomic data allowed testing of current hypotheses related to the acquired or intrinsic resistance capabilities of BAC-selected organisms against antibiotics and BACs.

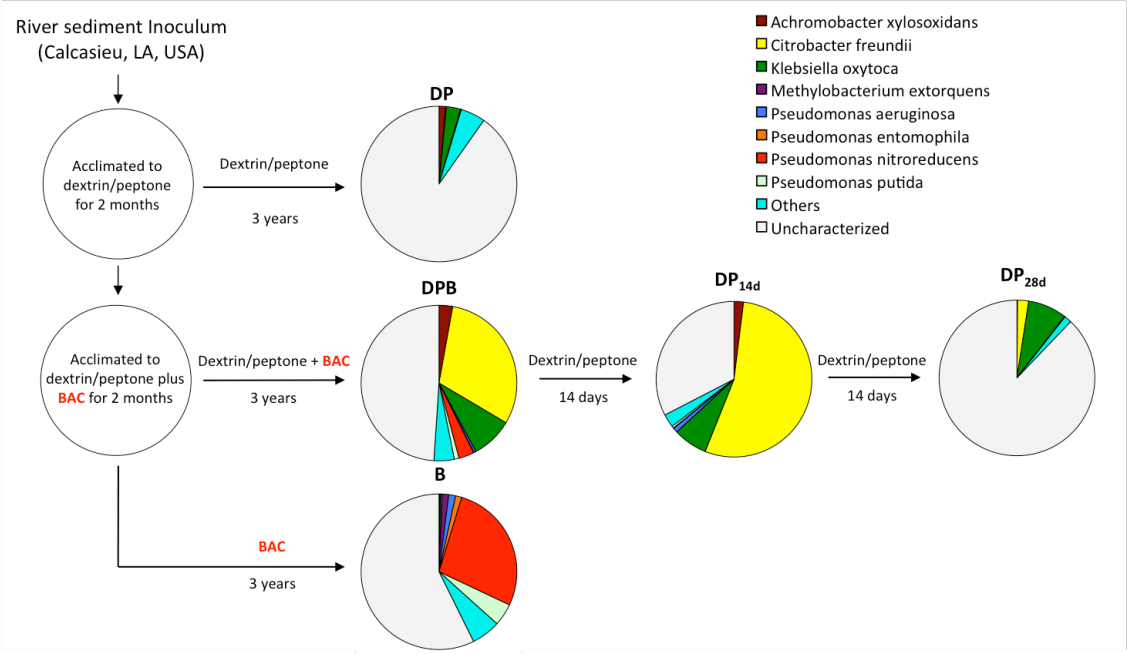

Figure 4.1. A schematic representation of the development history and relative species abundance of microbial communities in the presence or absence of BACs. The substrates/community ages for each bioreactor are shown above/below arrows, respectively. Colors represent the major species (>1% of the total community; see key) based on best match Blastn analysis against available all genome database. 'Other bacteria' represents the combined fraction of minor taxa. 'Uncharacterized' represents the fraction of metagenomic reads that were not assignable to the species level, presumably due to the lack of appropriate reference genome sequences. Note that the majority (90.2%) of the DP community were 'Uncharacterized', reflecting the large diversity of uncharacterized species in the original inoculum (river sediment) (107).

4.3.2. BAC exposure induces antibiotic resistance.

To examine whether or not BAC exposure induced antibiotic resistance, antibiotic susceptibility of five pairs of isolates, one from DPB and/or B communities, representing mostly abundant community members, and the other from the DP community were characterized for their resistance to five major classes of antibiotics (Table 4.1). These isolates also represented five phylogenetically diverse species (13 isolates in total), covering a substantial part of the diversity within the target communities. *P. aeruginosa*-DPB showed higher IC_{80} values for BACs (4 fold), rifampicin (2 fold), and tetracycline (2 fold) compared to its unexposed counterpart, *P. aeruginosa*-DP. Similar results were observed in *A. xylosoxidans* for four antibiotics (2 to 32 fold increase), except for rifampicin, compared to the control isolate from DP culture. *C. freundi*-DPB and *K. oxytoca*-DPB did not exhibit significant changes in IC_{80} values compared to their unexposed counterparts. It should be noted, however, that the latter isolates showed higher IC_{80} values than *C. freundii* and *K. oxytoca* isolates from culture collections (type strains) and the human gastrointestinal tract (>4 fold for BACs and >2 fold for most of the antibiotics), indicating that the *C. freundii*-DP and *K. oxytoca*-DP might be intrinsically multi-drug resistant (e.g., 100 mg/L BAC IC_{80}). The intrinsic resistance may underlie their dominance in the DPB community upon BAC exposure. There was no significant difference in antibiotic susceptibility for the *P. aquatica* isolate pair. Overall, although BAC exposure did not necessarily result in higher antibiotic resistance in all isolates tested, at least *P. aerugionsa* and *A. xylosoxidans* showed reduced susceptibility to BACs and several antibiotics, suggesting that there might be a resistance link between

BACs and antibiotics. These results called for further investigations to reveal the molecular mechanisms responsible for the phenotypic data obtained.

Table 4.1. 80% inhibitory concentrations (IC_{80}) of BACs and antibiotics[a] for isolates of same species. A range of antibiotic and BAC concentrations (0.4 to 200 mg/L) were tested and the table shows the determined MIC values. [a] RF, rifampicin; TC, tetracycline; CF, ciprofloxacin; CP, chloramphenicol; PB, polymyxin B. [b] 16S rRNA gene identity against the control isolate (-). [c] Reference strain (4_7_47_CFAA; accession no. ADLG00000000.1) of the Human Microbiome Project (http://www.hmpdacc.org/). [d] Reference strain (KCTC 1686; CP003218.1) of Korean Collection for Type Cultures. [e] Four independent isolates tested. *P. nitroreducens*-DPB and -B showed high IC_{80} values against BAC and antibiotics, but no *P. nitroreducens* isolate was recoverable from the DP community, presumably due to the extremely low abundance of the species in the community based on our previous metagenomic survey (107).

Species	Source	Identity[b] (%)	IC_{80} (mg/L)[a]					
			BAC	RF	TC	CF	CP	PB
Pseudomonas aeruginosa	DP	-	50	50	25	1.6	>200	6.3
	DPB	100	200	100	50	1.6	>200	6.3
Achromobacter xylosoxidans	DP	-	50	25	6.3	12.5	25	1.6
	DPB	97.8	200	25	200	25	100	3.1
	B	97.7	>200	25	>200	50	50	25
	Human[c]	-	25	50	0.8	<0.4	6.3	3.1
Citrobacter freundii	DP	98.5	100[e]	100	3.1	1.6	12.5	12.5
	DPB	99.5	100	100	3.1	1.6	6.3	6.3
	Type[d]	-	25	50	1.6	<0.4	12.5	6.3
Klebsiella oxytoca	DP	99.1	100[e]	50	6.3	3.1	50	6.3
	DPB	99.9	100	50	6.3	3.1	50	6.3
Pelomonas aquatica	DP	-	6.3	3.1	<0.4	<0.4	3.1	25
	DPB	100	6.3	3.1	<0.4	<0.4	3.1	25
Pseudomonas nitroreducens	DPB	-	200	50	6.3	1.6	>200	>200
	B	100	200	50	6.3	3.1	>200	>200

4.3.3. Horizontal transfer of efflux pump genes mediates antibiotic resistance.

Alignment of the *P. aeruginosa*-DPB genome against the *P. aeruginosa*-DP one identified a few genetic modifications. More specifically, three single nucleotide mutations in three genes and two inserted genomic islands (GIs) that encoded a conjugative plasmid (GI-I) and several integrases and transposases (GI-II) in the *P. aeruginosa*-DPB genome (Fig. 4.2A). Importantly, the number (n=3) of fixed mutations identified in the genomes was comparable to the number of mutations predicted (n ~ 9) based on the spontaneous mutation rate for a bacterial genome (5.4×10^{-10} per base per generation) (33), the genome size compared (5.6 Mb), and the estimated number of generations, since the establishment of the DPB and DP communities was three years ago (n ~ 3,000 generations). These results strongly supported that the two isolates track back to the same recent ancestor in the original DP inoculum and that the genetic modifications identified took place during the period of BAC exposure.

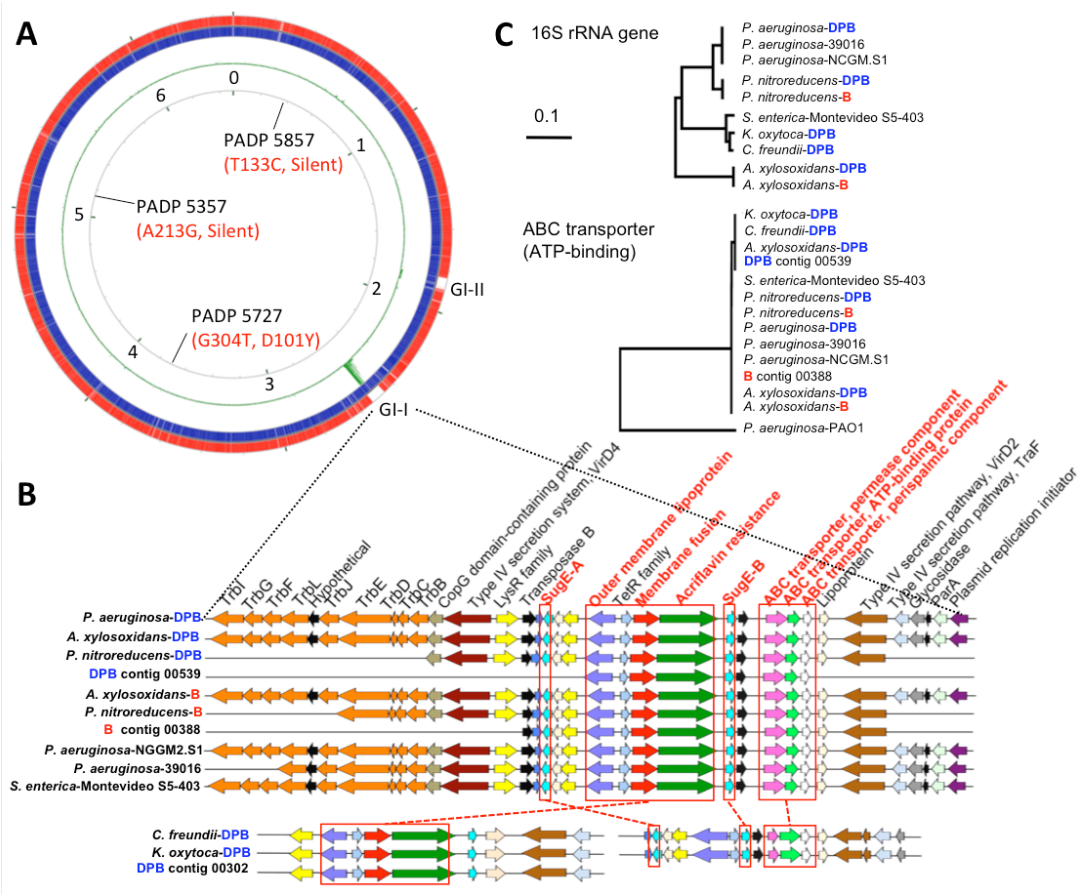

Figure 4.2. Horizontal transfer of efflux pump genes via a conjugative plasmid. (**A**) Comparison of draft genomes of *P. aeruginosa*-DPB vs. *P. aeruginosa*-DP. From outermost to inwards: genes (red) of *P. aeruginosa*-DP, genes (blue) of *P. aeruginosa*-DPB, coverage plot using 1 Kb long windows (green; 0 to 100X) of *P. aeruginosa*-DPB genes from DPB metagenomic reads. Genes with mutations found between two genomes are denoted on the graph. Note the two genomic islands (GI-I and GI-II) conserved in the *P. aeruginosa*-DPB genome but not the *P. aeruginosa*-DP one, amongst which genes of GI-I showed high coverage (Avg. 52X) compared to those (Avg. 1X) of the other genes ($P < 0.01$, Student's t-test). **(B)** Graphic representation of the organization of genes encoded on the conjugative plasmid recovered from isolate genomes and metagenomes. Note that the conjugative plasmids were found in species isolated only from BAC-exposed communities but not from their unexposed counterparts. Four efflux pump systems were highlighted in red boxes. **(C)** Phylogenetic tree of 16S rRNA gene sequences and ATP-binding gene sequences (ABC transporter) based on the maximum likelihood composite model and the neighbor-joining method. Note that the phylogenetically diverse isolates conserved almost identical (>99% identity) ATP-binding gene sequences. The phylogenetic tree of *sugE*-A and *sugE*-B genes showed similar patterns as those observed in the ATP-binding protein gene (>99% identity) (data not shown). The reference sequences used were *P. aeruginosa*-39016 (accession no. NZ_CM001020.1), *P. aeruginosa*-NCGM.S1 (NC_017549.1), *P. aeruginosa*-PAO1 (NC_002516.2), and *Salmonella enterica*-Montevideo-S5-403 (AFCS01000001.1).

Among the mutations identified, those in gene locus PADP_5357 and PADP_5857 were synonymous and the one in locus PADP_5727, which was predicted to encode an aldehyde dehydrogenase, was nonsynonymous (Fig. 4.2A). Gene sequence comparison to other available *P. aerugionsa* genome sequences suggested that the latter mutation aroused on the *P. aeruginosa*-DP gene and not on the *P. aeruginosa*-DPB one, suggesting that the nonsynonymous mutation was not selected by BAC exposure. Further, the predicted gene product (i.e., aldehyde dehydrogenase) is unlikely relevant for antibiotic resistance; hence, the different phenotypes of antibiotic resistance observed between the *P. aerugionsa* isolates were presumably attributable to the GIs rather than the single nucleotide mutations observed.

Read recruitment analysis revealed that the coverage of the GI-I (Avg. 52X) by DPB metagenomic reads was significantly higher ($P < 0.01$, Student's t-test) than the rest of the *P. aeruginosa*-DPB genome (Avg. 1X), indicating that the GI-I was present in additional members of the DPB community (Fig. 4.2A). Consistent with this interpretation, genome sequence analysis of *A. xylosoxidans* and *P. nitroreducens* isolates from DPB and/or B communities revealed an almost identical (>99% identity) genetic element to that of *P. aeruginosa*, despite the significant evolutionary divergence of the organisms (Fig. 4.2C). The GI-I harbored four efflux pump systems: two small multi-drug resistant (SMR) family systems, (*sugE*-A and *sugE*-B), an ATP-binding cassette (ABC) family, and a resistance nodulation division (RND) family (Fig. 4.2B). The GI-I also encoded genes associated with conjugative transfer (*trbBCDEJLFGI*), plasmid partitioning (*parA*), and plasmid replication initiator, suggesting that the GI-I is, at least in some members of the community, a conjugative plasmid. Genome analysis identified

the gene content of the GI-I (*P. aeruginosa*-DPB) in all other BAC-exposed isolate genomes: *A. xylosoxidans*-DPB, *P. nitroreducens*-DPB and -B, *C. freundii*-DPB, and *K. oxytoca*-DPB (Fig. 4.2B).

Phylogenetic analysis of the concatenated alignment of the RND-family efflux pump genes distinguished *C. freundii*-DPB and *K. oxytoca*-DPB from the other isolates recovered, indicating that the former genomes encoded another version of the RND-family efflux pump genes (i.e., 70% average nucleotide identity), with genetic rearrangement caused possibly by the transposase encoded on the conjugative plasmid (Fig. 4.2B). Nevertheless, the conjugative plasmids identified in the BAC-exposed strains resembled one another in terms of gene content, organization, and sequence similarity (Fig. 4.2B and 4.2C) and were not found in the genomes of the unexposed isolates. These results suggest that horizontal gene transfer had mobilized the plasmids among the members of the community, which could possibly mediate the resistance phenotypes to BACs and antibiotics observed in the BAC-exposed isolates.

To test this hypothesis, the *sugE*-A, *sugE*-B, and ABC transporter system genes were cloned into a broad-host range vector (pBBRMCS-4) and transformed into the *P. aeruginosa*-PAO509 host (93). PAO509 lacks several efflux pumps systems present in wildtype *P. aeruginosa* and hence is easier for testing efflux pump-mediated antibiotic resistance. Plasmids pBBRsugE-A and pBBRABC conferred increased resistance to BACs (2X MIC) and rifampicin (4X MIC) compared to an empty control vector (pBBRMCS-4) (Table 4.2). The BAC-resistance phenotype of the transformant carrying *sugE*-A (PAO509/pBBRsugE-A) is in agreement with the previous results that *sugE* confers resistance to BACs (48). Rifampicin is known to act on the RNA polymerase β

subunit (*rpoB*) and interfere transcription. Rifampicin-resistant bacteria frequently emerge with single point mutations in the *rpoB* gene (27). To exclude the possibility that the rifampicin-resistant phenotype was due to spontaneous mutation (as opposed to the ABC transporter system cloned), *rpoB* gene sequences of Rif clusters I, II, and III were PCR-amplified from four independent colonies of PAO509 and PAO509/pBBRABC using previously determined primers PAO1rpoB1 (59) and PAOrpoB3 (153) and subsequently sequenced using Sanger sequencing chemistry. No mutation was identified between PAO509 and PAO509/pBBRABC; hence, the rifampicin resistance phenotype is likely not due to spontaneous mutation but the ABC transporter system cloned. No difference in tetracycline susceptibility was observed in the three recombinants compared to the control. Therefore, these results demonstrated that the conjugative plasmid confers resistance to both antibiotics (e.g., rifampicin), mediated by the ABC family efflux pump, as well as BACs, mediated by the *sugE*-A gene.

Table 4.2. IC_{80} values of transformants carrying efflux pump genes.

Strain	IC_{80} (mg/L)		
	BACs	Rifampicin	Tetracycline
PAO509/pBBRMCS-4	25	25	0.4
PAO509/pBBRsugE-A	50	25	0.4
PAO509/pBBRsugE-B	25	25	0.4
PAO509/pBBRABC	25	100	0.4

4.3.4. Antimicrobial resistance is reversible when BAC exposure is interrupted.

To examine the stability of community BAC resistance, BAC was removed from the feed of the DPB community. While the community evolved using only dextrin/peptone, the BAC susceptibility of the community was followed for 165 days. The 50% (IC_{50}), 90% (IC_{90}), and 100% minimum inhibitory concentrations (MIC) values quickly decreased to 46, 83, and 92 mg/L at day 14 and reached 25, 62, and 100 mg/L at day 28 (Fig. 4.3A), compared to 125, 220, and 250 mg/L at day zero (140). After day 28, the MIC values leveled off until 165 days and were comparable to those of the DP community (50, 90, and 100 mg/L), which was not exposed to BACs (140). The metagenomes at day 14 (DP_{14d}; when community susceptibility was still increasing) and at day 28 (DP_{28d}; when IC/MIC values began to level off) were determined to document the dynamics of the community members and the conjugative plasmids.

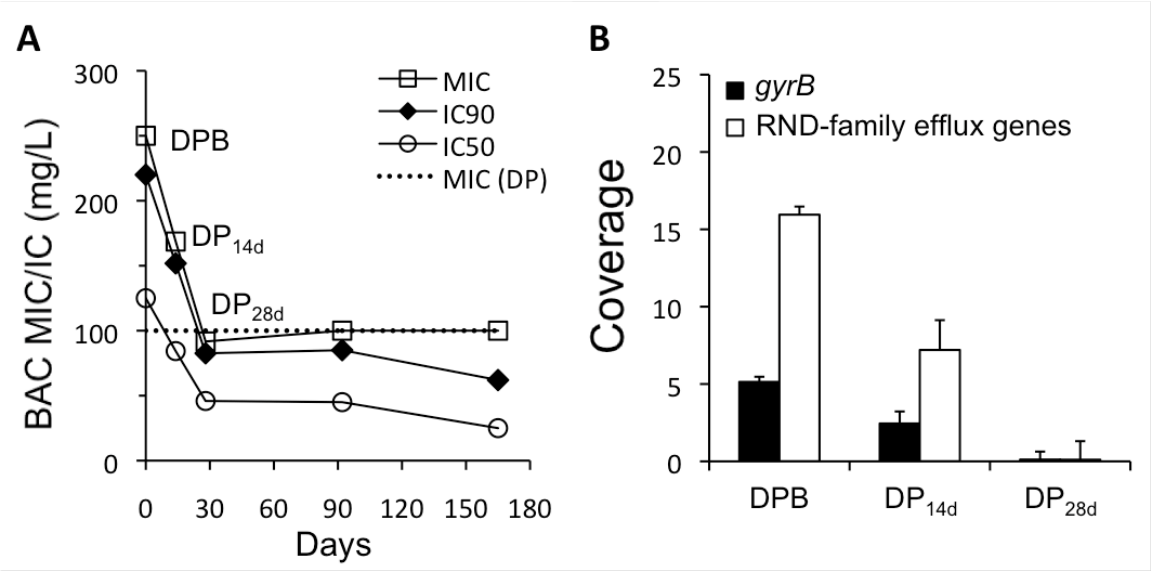

Figure 4.3. Stability of antimicrobial resistance. (A) Increase of community-level BAC susceptibility (IC_{50}, IC_{90}, and MIC; figure key) of the DPB community after removing BACs from the feed over a period of five months. **(B)** Relative coverage of *gyrB* (*A. xylosoxidans*, *P. aeruginosa*, and *P. nitroreducens*) and the RND-family efflux pump operon present in the three species (distinguishable from those in *C. freundii*-DPB and *K. oxytoca*-DPB) in the DPB, DP_{14d}, and DP_{28d} metagenomes. 10,000 metagenomic reads per metagenome were randomly sampled and searched (>95% identity) against the corresponding genes to estimate the relative coverage (per one Gb metagenome) with 100 permutations.

Relative coverage of the single copy informational *gyrB* gene (a proxy of species abundance) and the RND-family efflux pump operon of *P. aeruginosa, A. xylosoxidans,* and *P. nitroreducens* (a proxy of the conjugative plasmid that can distinguish the version encoded in *C. freundii*-DPB and *K. oxytoca*-DPB; see Fig. 4.4) was calculated to track relative abundance of the three species and the plasmid in the DPB, DP_{14d}, and DP_{28d} metagenomes (Fig. 4.3B). Recruitment of genomic reads against the genome sequences of *A. xylosoxidans* (-DPB and -B), *P. aeruginosa* (-DPB), and *P. nitroreducens* (-DPB and -B) showed similar coverage between the genome and the plasmid (about 1:1 ratio), suggesting that this genetic element likely existed as a single copy plasmid in its hosts. The relative coverage of the species and the conjugative plasmid was 5.2±0.8X and 15.9±1.9X, respectively, in the DPB metagenome. After removing BACs from the feed, the relative coverage of the three species and the plasmid quickly decreased at day 14 (2.4±0.5X and 7.2±1.2X, respectively) and at day 28 (0.3±0.1X and 0.5±0.1X, respectively). The relative coverage of the three species and the plasmid at day 28 was even lower than those in the DP metagenome (1.3±0.3X and 1.4±0.5X, respectively). In addition, *C. freundii*, which had been strongly selected by BAC exposure, dramatically decreased from day zero to day 28 (30.7% to 2.3%) while *K. oxytoca* maintained its relative abundance for 28 days (8.6% to 8.1%; see Fig. 4.1). Collectively, these results suggested that most populations that had been selected by BACs due to their acquired (e.g., *P. aeruginosa*-DPB) or (presumably) intrinsic resistance phenotypes (e.g., *C. freundii*) quickly lost selective advantages and were outcompeted by other community members when BAC was removed from the feed.

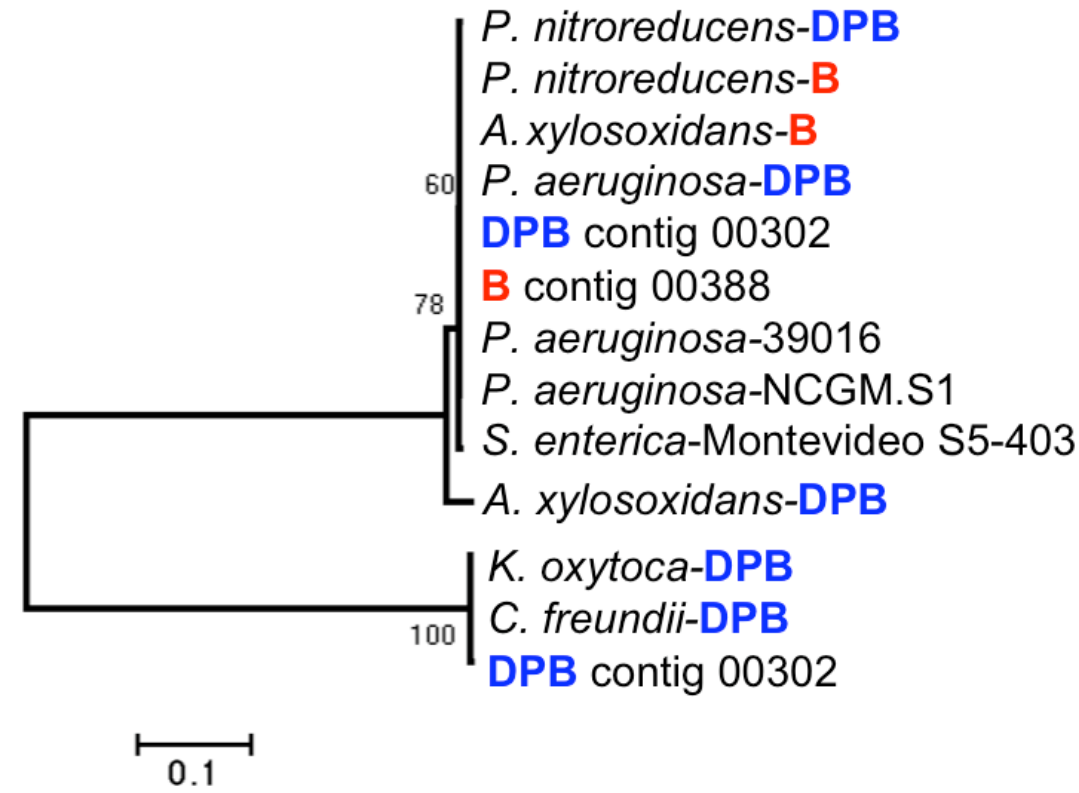

Figure 4.4. Phylogenetic tree of the RND-family efflux pump operon. The phylogenetic analysis was based on the concatenated alignment of the three genes comprising the RND-family efflux pump operon using the maximum likelihood composite model and the neighbor-joining method (139). The values on the nodes represent bootstrap support from 100 replicates.

4.4. Discussion

Antibiotics typically have a specific mode of action, on a single target, whereas disinfectants such as QACs act non-specifically and have multiple target sites. For this reason, a resistance linkage between QACs and antibiotics is thought to be rare. Disinfectant-induced antibiotic resistance may occur via at least two evolutionary processes within complex microbial communities. First, microorganisms can acquire resistance phenotypes through adaptive evolution (e.g., advantageous mutations and/or horizontal transfer of resistance genes). Second, disinfectants may select organisms that are intrinsically multi-drug resistant (selection of multi-drug resistant species). For the former, although a similar multi-drug resistant plasmid conferring resistance to both BACs and antibiotics was previously reported (154), the underlying genetic determinants responsible for antibiotic resistance and the horizontal transfer (i.e., whether or not BAC exposure indeed co-select) of the plasmid were not elucidated. In this study, BAC disinfectants indeed induced antibiotic resistance (Table 4.1 and 4.2) via primarily horizontal transfer of a conjugative plasmid carrying antibiotic resistance genes (e.g., ABC transporter) (Fig. 4.2). The ABC transporter genes were co-selected (hitchhiked) upon BACs with the *sugE*-A gene, which was encoded on the same plasmid and conferred BAC resistance.

In addition to *sugE*-A, *sugE*-B, and ABC transporter systems, the plasmid also encoded a RND-family efflux pump operon, which is known to mediate export (detoxification) of a wide range of antimicrobials and was previously shown to confer resistance to not only tetracycline but also BACs (22, 46, 102, 134). It was, however, not possible to produce transformants carrying the RND family efflux pump operon of the

conjugative plasmid, despite repeated attempts; hence, its role associated with antibiotic resistance remains to be confirmed. Similar technical difficulties in cloning RND-family efflux pump genes were previously reported, resulting presumably from instability and/or toxic effects on the host from the large piece of DNA encoding the RND system (44). However, it is highly likely based on previous literature that this efflux system is involved in tetracycline resistance.

A question of important practical consequences for assessing the risks to public and environmental health is whether or not induced antibiotic resistance persists in the absence of the BAC agent. Our metagenomic analysis showed that, although several multi-drug resistant species were selected by BAC exposure, most of these quickly disappeared within a month of incubation time, when BACs were removed (Fig. 4.1 and 4.3B). The disappearance of the species was accompanied by increased antimicrobial susceptibility of the community up to a level that was comparable to that of the control community (Fig. 4.3A). Consistent with our results, bacteria that acquire antibiotic resistance through plasmids and/or mutations frequently show a competitive disadvantage (trade-off) in non-selective environments (75, 126). Accordingly, removal of BACs in non-target environments (e.g., employing microbial degradation of BACs in wastewater treatment plants) and appropriate usage of BAC in target environments (e.g., the clinic) can provide significant benefits for restricting the spread of antibiotic resistance.

It is also important to consider whether or not the conjugative plasmid selected by BAC exposure can be transferred to clinically relevant pathogenic or opportunistic pathogenic bacteria. Our results showed that the plasmid was horizontally transferred across phylogenetically diverse organisms (e.g., *Gammaproteobacteria* and

Betaproteobacteria) (Fig. 4.2B & 4.2C), including species that are known as opportunistic pathogens such as *P. aeruginosa*. Hence, the results reported here are relevant for infectious disease control. Consistent with these interpretations, we found that two *P. aeruginosa* strains isolated from a corneal (39016) and urinary tract (NCGM2.S1) infection (95, 133), both of which were highly antibiotic resistant, and one potential pathogen, *Salmonella enterica* (31), share almost identical conjugative plasmids (>99% identity) to the one reported here (Fig. 4.2B & 4.2C). Collectively, these findings indicate that horizontal transmission of antibiotic resistance genes between environmental and clinical microorganisms may be more widespread than frequently assumed and possibly enable pathogenic bacteria to survive at high disinfectant concentrations (50 mg/L BACs as used in our study). The BAC concentrations commonly applied in practice greatly exceed that used in our study. Nevertheless, since most QAC applications in practice do not require immediate rinsing or removal, it is possible that a gradient of QAC concentrations exists in clinical or municipal settings, and hence, the concentrations used in our study are relevant for various settings.

This study revealed that although disinfectants did not induce antibiotic resistance in all organisms surveyed (e.g., *P. aquatica*), they did cause antibiotic resistance in at least a few of the organisms (e.g., *P. aeruginosa*). Further, the underlying genetic determinants of the BAC-induced antibiotic resistance were spread across diverse environmental (river sediment) and clinical settings. Our results, therefore, have important implications for monitoring and regulating environmental pollutants that can induce antibiotic resistance, relevant to human and environmental health, and should be helpful for the development of guidelines for appropriate use of disinfectants.

4.5. Materials and methods

4.5.1. Microbial community development

All microbial communities (DP, DPB, and B) analyzed in this study originated from the same inoculum, from a contaminated sediment sample collected at the Bayou d'Inde, a tributary of the Calcasieu River, near Lake Charles, LA. Community development, bioreactor operation (e.g., substrates, feeding cycle, temperature), and community susceptibility testing were described previously in detail (107, 140, 145).

4.5.2. Species isolation, identification, and antibiotic susceptibility test

Mixed community suspension from each bioreactor (DP, DPB, and B) was first diluted 10^5 to 10^7 times and then plated on agar medium containing dextrin/peptone, salt medium, and 1.5% agar. Taxonomic identification of isolates was carried out based on the analysis of their 16S rRNA gene sequences. The antibiotic susceptibility test for isolates was performed as described previously (107, 140) and determined as the concentrations at which 80% of cell growth inhibition (IC_{80}) compared to control was observed.

4.5.3. DNA extraction and sequencing

Mixed community suspension from bioreactors and pure culture suspensions were taken for DNA extraction. DNA was extracted as previously described (106). All DNA samples showed >0.4 μg/μl and >1.8 absorbance ratios (A_{260}/A_{280}) and were sequenced using the Illumina HighSeq 2000 sequencers at the Los Alamos National Laboratory Genomics Facility.

4.5.4. Community metagenome and isolate genome sequence analysis

Raw Illumina reads were trimmed using a Q=15 Phred quality score cutoff using SolexaQA (29) for further analysis. Community and isolate genomes were assembled using the hybrid protocol previously described (81). For metagenome analysis, protein-coding genes encoded on assembled contigs were annotated with the Metagene pipeline (104). The phylogenetic affiliation of assembled contigs or unassembled metagenomic reads were determined based on best match searches against all bacterial and archaeal genome sequences available in GenBank database (as of February 2012, ftp://ftp.ncbi.nih.gov/), using Blastn (X = 150, q = -1, and F = F, remaining parameters at default settings) with a cutoff of for a match >95% identity and 50% query length coverage. The functional annotation of protein-coding genes were performed using Blastp search against the SEED protein database (109), with a cutoff of for a match >40% amino acid sequence identity and >50% query length coverage. For analysis of isolate genomes, gene prediction and functional annotation were performed using the RAST server (4). To estimate the relative abundance (coverage) of reference gene, contig, or genome sequences in a metagenome, all metagenomic reads mapping on the reference sequence with at least 95% identity and 50% query length coverage were considered. The length of all mapped reads was summed and divided by the length of the corresponding reference sequence and subsequently by the size of the metagenome to provide a normalized estimate of abundance per one Gb of metagenomic sequences across metagenomic datasets with different sizes.

4.5.5. Construction of plasmids carrying efflux pump genes

Efflux pumps genes encoded on the conjugative plasmid were cloned from *Achromobacter xylosoxidans*-B. The genes, with their native promoters, were individually amplified by PCR using primers containing enzyme restriction sites (EcoRV-BamHI for *sugE*-A and *sugE*-B and KpnI-EcoRI for ABC transporter operon). Digested amplicons were ligated into the multiple cloning site of pBBRMCS-4 (67). The resulting constructs were transformed into *P. aeruginosa*-PAO509, which was deficient for several RND-family efflux pump genes (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexJK*, and *mexXY*) (93), by electroporation as described previously (24). Transformants were selected on LB agar medium supplemented with 100 mg/L of ampicillin. Recombinant plasmids extracted from transformants were confirmed by PCR-amplification of inserts and enzyme restriction digestion.

4.5.6. Nucleotide sequence accession numbers

The metagenome and genome data used in this study were deposited in GenBank under the accession numbers: XXX (DP_{14d}) and XXX (DP_{28d}) and XXX (*Achromobacter xylosoxidancs*-DP), XXX (*Achromobacter xylosoxidancs*-DPB), XXX (*Achromobacter xylosoxidancs*-B), XXX (*Psedomonas aerugionsa*-DP), XXX (*Psedomonas aerugionsa*-DPB), XXX (*Citrobacter freundii*-DPB), and XXX (*Klebsiella oxytoca*-DPB).

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CHAPTER 5

CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

5.1. Conclusions and implications

The research conducted as part of this thesis resulted in the following major findings.

1. Widely used BAC disinfectants contribute to the proliferation of antibiotic resistance in a microbial community through at least two distinct mechanisms: i) horizontal transfer (co-selection) of multi-drug resistant and mobile genetic elements among community members and ii) selection of intrinsically multi-drug resistant organisms. These findings reconciled previous conflicting results relative to whether or not a resistance linkage exists between disinfectants and antibiotics and thus, resolved a long-standing debate in the literature.
2. The gene cloning approach revealed that the previously uncharacterized SugE (SMR-family) and ABC family efflux pumps encoded on the conjugative plasmid conferred resistance to BACs and rifampicin, respectively. The plasmid has been horizontally transferred among phylogenetically diverse organisms, including potential human pathogens, within various environments, favored (selected) by BAC exposure. These findings highlight the adverse effects of the inappropriate use of disinfectants in clinical or environmental settings.
3. Antibiotic resistant bacteria and the multi-drug resistant conjugative plasmids that are selected by disinfectants quickly disappear under non-selective conditions (e.g., when disinfectants are removed). This finding has important practical implications for restricting the spread of disinfectant-induced antibiotic resistance. This finding also suggests that appropriate removal (e.g., bioremediation) of disinfectants in non-target

environments such as wastewater treatment plants will have significant benefits for controlling disinfectant-induced antibiotic resistance. The results of this thesis should be useful in developing appropriate guidelines for regulating the use of disinfectants in health care facilities, households, food processing lines and storage tanks, and dairy and agricultural settings.

4. Microbial communities employ a combination of adaptive mechanisms to cope with BAC exposure. More specifically, this study shows the selective enrichment of BAC-degrading *Pseudomonas* species and the horizontal transfer and amino acid substitutions of genes associated with BAC degradation and resistance. These findings provide the first genomic view of microbial community adaptation to BACs (compared to previous, mostly isolate-based studies) and highlight the importance of horizontal gene transfer in community adaptation, which can be relevant to other environmental pollutants and synthetic toxic compounds.
5. Metatranscriptomics suggest that the microbial community studied here degraded toxic BACs by syntrophic interactions among *Pseudomonas* species. *P. nitroreducens* cleaved BACs by dealkylation, thereby reducing toxicity and presumably providing significant benefits to other BAC-sensitive organisms. Subsequently, while *P. nitroreducens* further metabolized the long alkyl chain (a dealkylated part of BACs), other community members, mostly *P. putida*, metabolized the BDMA (the other part of BACs). These organisms are robust key BAC degraders, e.g., they were recovered reproducibly after prolonged starvation and/or lack of BACs in the feed of our bioreactors. Thus, these organisms represent useful seed inocula that can be used in a biological engineering system for treating BAC-bearing waste streams. Such microbial

consortia will work not only to reduce BAC toxicity but also limit the possible occurrence of antibiotic resistance.

5.2. Future studies

The research presented here also identifies several areas that should be the subject of future research. In particular:

1. It will be important to determine whether or not the RND-family efflux pump operon encoded on the conjugative plasmid confers resistance to BACs and antibiotics such as tetracycline. This efflux pump showed significant gene activities upon BACs and previous literature indicates that the RND efflux systems are related to antimicrobial/antibiotic resistance. However, the exact antimicrobial substrate(s) of the RND efflux system encoded on the plasmid remain to be elucidated.
2. The transcriptomic and genomic data indicate that the amine oxidase enzyme (bioinformatic functional annotation) of *P. nitroreducens* potentially performs the first step in the BAC degradation pathway, i.e., the cleavage of the C_{alkyl}-N bond of BACs. Future genetic manipulations should confirm the functionality (dealkylation) of this predicted enzyme. Identifying the enzymatic activity of the gene product will have important practical implications, e.g., designing molecular assays to assess gene presence in natural and engineered environments and thus to assess the potential for BAC biodegradation.
3. Future studies should also include functional and genetic experiments to determine whether or not the non-synonymous mutation in a *mucA* gene affects the regulatory transcriptional network associated with mucoidy phenotypes that can result in resistance to BACs and/or antibiotics.

4. It is important to realize that although our laboratory set up provided important new insights into disinfectant-induced antibiotic resistance, it did not closely simulate natural or clinical environments. Therefore, it will be necessary to validate our findings under real-life situations such as disinfection in health care facilities (e.g., hospitals).

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