Corrinoid Cross-Feeding in the Microbial World

By

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Abstract

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The exchange of metabolites among members of microbial communities enables the catabolism of complex substrates and supports the growth of auxotrophic microbes. Many microbes depend on other organisms in their environment for the biosynthesis of essential metabolites such as corrinoids. Corrinoids are cobalt-containing tetrapyrroles that function as cofactors for enzymes that facilitate carbon skeleton rearrangements, methyl group transfers, and reductive dehalogenation. Members of all three domains of life use corrinoid cofactors, yet the complete biosynthesis of corrinoids, which requires approximately 30 enzymatic steps, is performed only by a subset of prokaryotes. Bioinformatic analyses show that while 76% of bacterial genomes contain corrinoid-dependent enzymes, only 39% of these contain the complete corrinoid biosynthesis pathway. As such, corrinoid cross-feeding is crucial for functionally integrated microbial communities.

Corrinoids are identified by the structure of their lower axial ligand, which can be a benzimidazole, purine, or phenolic compound. Corrinoids with different lower ligands do not function equivalently as cofactors. Given the diversity of corrinoid structures, microbes that require exogenous corrinoids must have mechanisms to acquire the specific corrinoids that function as cofactors for their corrinoid-dependent enzymes. However, the variety of corrinoids that can serve as cofactors for any one organism, the means by which microbes acquire specific corrinoids, and the diversity of corrinoids present in microbial communities have not been well studied.

In the first chapter, I summarize the strategies employed by corrinoid-dependent bacteria for fulfilling their corrinoid requirements and provide background on corrinoid structure, function, and biosynthesis.

Chapter 2 details my work examining the range of corrinoids that function as cofactors for the organohalide-respiring bacterium *Dehalococcoides mccartyi*, which has an obligate requirement for exogenously supplied corrinoids. *D. mccartyi* plays an important role in the bioremediation of chlorinated solvents in the environment, a process that relies on corrinoid-dependent enzymes. Together with my collaborators Shan Yi, Yujie Men, and Lisa Alvarez-Cohen, I showed that *D. mccartyi* can only use specific corrinoids containing benzimidazole lower ligands, but is capable of remodeling other corrinoids by lower ligand replacement when provided a functional benzimidazole base.

While Chapter 2 focuses on corrinoid metabolism in *D. mccartyi* in pure culture, my experiments presented in Chapter 3 focus on examining corrinoid cross-feeding in microbial communities containing *D. mccartyi*. Using a liquid chromatography-tandem mass spectroscopy method developed with my collaborators, I identified the specific corrinoids and free lower ligands present in microbial communities containing *D. mccartyi,* examined the contributions of different phylogenetic groups to the community corrinoid profile, and provided evidence that *D. mccartyi* engages in corrinoid remodeling in its natural environment. These findings provide novel insights into the roles played by different phylogenetic groups in corrinoid production and corrinoid cross-feeding within microbial communities, and may also have implications for optimizing chlorinated solvent bioremediation.

As a parallel to my work on how corrinoid cross-feeding supports the growth of *D. mccartyi* in a microbial community, and the strategies that *D. mccartyi* uses to obtain functional corrinoid cofactors, in Chapter 4, I explore how the cross-feeding of tetrapyrrole precursors allows microbes with an incomplete corrinoid biosynthesis pathway to fulfill their corrinoid requirements. Using a comparative genomics analysis, I identified 39 bacteria that lack genes required for the production of the universal tetrapyrrole precursors including 5-aminolevulinic acid (ALA), the first steps required for corrinoid biosynthesis, despite having otherwise complete corrinoid biosynthesis pathways. I tested the ability of three putative ALA auxotrophs to scavenge ALA from the growth medium and showed that in the presence of ALA, all three were capable of corrinoid production. My work showed that all 39 tetrapyrrole precursor auxotrophs are animal host-associated, raising the question of whether host-produced tetrapyrrole precursors might be scavenged by corrinoid-producing members of the microbiota.

The results of the experiments presented in this work provide insights into the array of strategies that microorganisms employ in acquiring essential nutrients from the environment. Better understanding of corrinoid production, modification, and utilization in microbial communities will aid in the exploration of how nutrient exchange shapes these communities, and the ecological roles played by individual community members.

Chapter 1

Corrinoid cross-feeding in the microbial world

Portions of this chapter are published in: Seth, Erica C., and Michiko E. Taga. "Nutrient cross-feeding in the microbial world." *Frontiers in microbiology* 5 (2014).

Introduction

Life in the microbial world exists as a dynamic network of interactions among microbes that fuels a complex web of interconnected metabolisms (1). Ignorance of what microbes gain via these interactions impedes our ability to cultivate the vast majority of microbes (2, 3). In addition, by failing to elicit a microbe's full range of metabolic responses to the presence of other organisms, the metabolic potential of microbes grown in isolation may not accurately reflect a microbe's ecological role (4, 5). Nutrient cross-feeding – the production of a molecule such as a vitamin or amino acid that is used by both the producing organism and other microbes in the environment – relaxes the metabolic burden on any one microbe in the community (6). Given the complexity of microbial communities, nutritional interactions between different species may have ripple effects on other community members.

Cofactor cross-feeding may serve as a model for nutritional interactions between microbes. While cofactor cross-feeding is not necessarily mutualistic, the availability of exogenously produced cofactors can have a profound impact on a microbe's mode of growth and metabolite production (7, 8). Examining cofactor cross-feeding can inform our understanding of the scope and relevance of metabolic interdependences in microbial communities. I focus on one group of cofactors, the corrinoids, as a model for understanding cross-feeding mechanisms. Corrinoid-dependent reactions function in diverse metabolic processes across all three domains of life, yet corrinoids are produced solely by a subset of prokaryotes (9). While the majority of bacteria (75%) are predicted to encode corrinoid-dependent enzymes, at least half of these lack the ability to produce corrinoids *de novo* (10, 11). As such, corrinoid cross-feeding is prevalent and may reflect the advantage of acquiring these complex cofactors from the environment rather than by *de novo* biosynthesis which requires approximately 30 enzymatic steps (9, 11, 12). The availability of corrinoids can have profound effects on a microbe's metabolism and its ability to occupy a specific niche. For instance, the ability to utilize ethanolamine as a sole carbon and nitrogen source relies on the corrinoid-dependent enzyme ethanolamine ammonia lyase, which converts ethanolamine into ammonia and acetaldehyde (13). In enterohemorrhagic *Escherichia coli,* ethanolamine utilization – enabled by corrinoid cross-feeding – provides a competitive advantage for colonization and persistence in the bovine intestine, a main reservoir for this pathogen (14, 15). Ethanolamine utilization is also important in other human pathogens that rely on exogenously produced corrinoids or corrinoid precursors including *Listeria monocytogenes* and *Enterococcus faecalis* (13, 16, 17). The mechanism by which corrinoids are released from corrinoid-producing microbes into the environment is unclear. As yet, no active means of corrinoid export has been identified. Obtaining functional corrinoid cofactors through cross-feeding is complicated by the structural diversity of corrinoids produced by different organisms (18).

Corrinoid structure and function

Corrinoids consist of a tetrapyrrolic corrin ring with a cobalt atom coordinated at the center by four of the ring's nitrogen atoms (Fig. 1) (9, 19). Two axial ligands are also coordinated to the cobalt. Interactions between the axial ligands and the cobalt drive the reactivity of corrinoid cofactors (20). The redox state of the cobalt, Co(I), (II), or (III) is determined by the number of ligands coordinated to it. With two axial ligands coordinated, the Co(III) state is achieved, with one axial ligand, Co(II), and with no axial ligands, Co(I) (21).The structural diversity of corrinoids exists in the identity of these axial ligands, which play important roles in determining cofactor function (21-23).

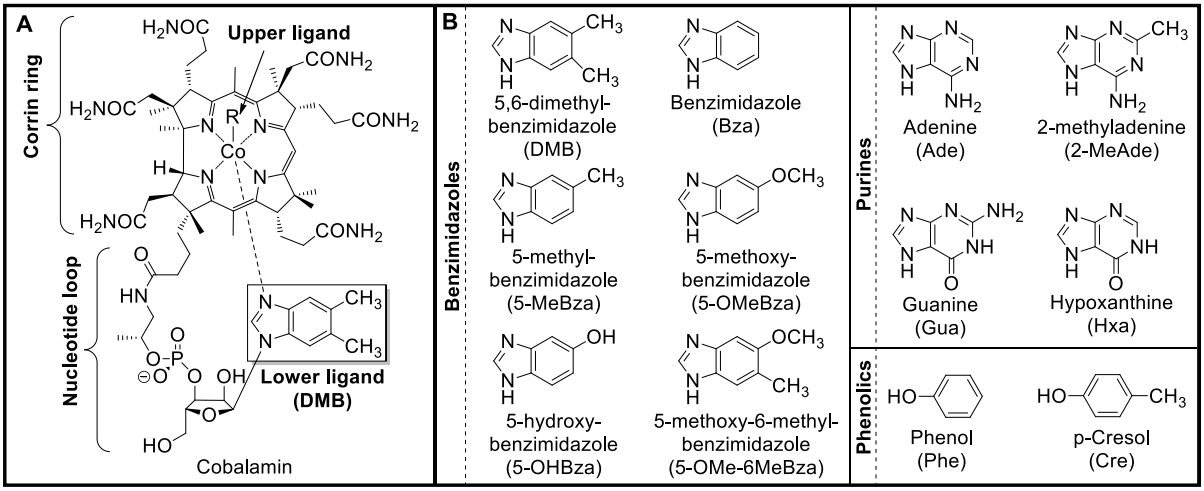
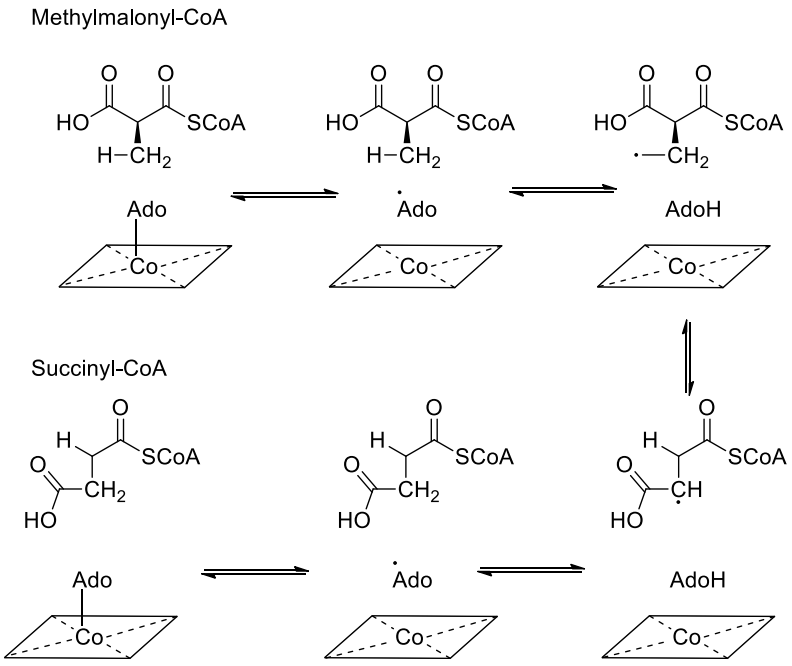


Figure 1. Corrinoid and lower ligand structures. A). The structure of cobalamin is shown, with the major components (corrin ring, nucleotide loop, and upper and lower ligand) indicated. In cobalamin, the lower ligand is 5, 6-dimethylbenzimidazole (DMB). The upper ligand, shown here as R, is variable and may be a methyl or adenosyl group. B). The structures of lower ligands of other corrinoids are shown. These are grouped into three different classes; the benzimidazoles, purines, and phenolics.

Complete corrinoids, i.e. those with a nucleotide loop and lower ligand attached, are called cobamides. Cobamides are distinguished from one another by the structure of the lower ligand, which is attached to a substituent of the corrin ring via a nucleotide loop and forms the nucleotide's base (Fig. 1A). A variety of different lower ligand bases have been identified, and fall into three categories: benzimidazoles, purines, and phenolics (Fig. 1B) (24). Even within one structural class, corrinoids with different lower ligands are not necessarily functionally equivalent as cofactors (18).The range of corrinoid that organisms can use is variable. For instance, *Salmonella enterica* naturally produces the purinyl corrinoids adeninylcobamide and 2-methyladeninylcobamide, but is also capable of using benzimidazolyl corrinoids (Anderson, 2008; Keck, 2000). My work on *Dehalococcoides mccartyi* revealed that this organism, which is unable to produce corrinoids *de novo* is limited to just a subset of the benzimidazolyl corrinoids (18, 20). In humans, cobalamin, which contains 5,6-dimethylbenzimidazole (DMB) as the lower ligand, appears to be the only corrinoid that is capable of serving as a cofactor (25). The reasons for corrinoid specificity are not well understood. However, the structure of the lower ligand has been shown to be important in cofactor binding and catalysis (23, 26). Corrinoid-dependent

enzymes bind their corrinoid cofactors in one of three different conformations: with the lower ligand coordinated to the cobalt, or "base-on"; without the lower ligand or a histidine residue from the protein coordinated, "base-off/his-off", or with a histidine residue of the protein coordinated in place of the lower ligand, "base-off, his-on" (27, 28). The lower ligands of phenolic corrinoids cannot be coordinated to the cobalt, and as such, these corrinoids do not function as cofactors for base-on enzymes (29).

A



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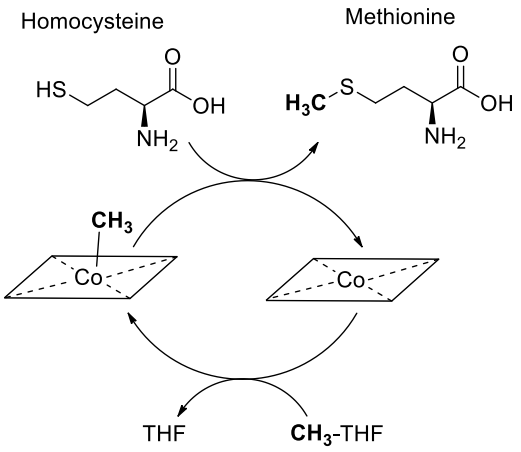

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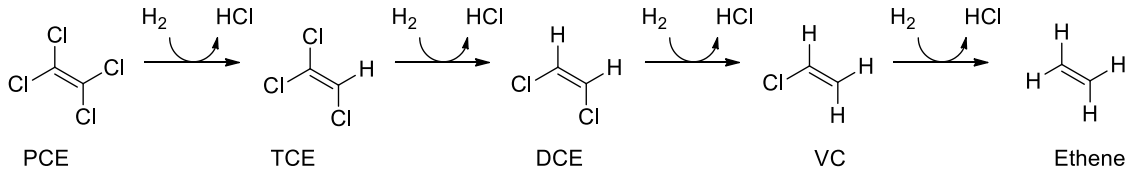

Figure 2. Examples of reactions catalyzed by corrinoid-dependent enzymes A). The reaction catalyzed by methylmalonyl-CoA mutase, one of the two corrinoid-dependent enzymes in humans is illustrated. The corrinoid cofactor used by this enzyme (shown here with a parallelogram representing the corrin ring) possesses an adenosyl upper ligand. B. The reaction catalyzed by methionine synthase, which uses a methylcorrinoid cofactor is illustrated. The methyl group, which is transferred from the donor, 5-methyltetrahydrofolate (THF), to the acceptor, homocysteine, is shown in bold. C. The corrinoid-dependent reductive dehalogenation of tetrachloroethene (PCE) to trichlorothene (TCE), 1, 2-*cis* dichloroethene (DCE), vinyl-chloride (VC), and finally to the non-toxic end-product ethene is shown.

The majority of corrinoid-dependent enzymes use corrinoids with either a methyl or adenosyl group as the upper ligand which forms an organometallic bond with the cobalt. The bond between the upper ligand and the cobalt is labile, and the cleavage and reformation of this bond is central to a corrinoid's catalytic activity (21). Corrinoid cofactors with an adenosyl group as the upper ligand are used in radical-mediated intramolecular rearrangement reactions. Homolytic cleavage of the Co-C bond generates an adenosyl radical, which abstracts a hydrogen atom from the substrate, creating a substrate radical. The substrate radical then undergoes an intramolecular rearrangement. Following the rearrangement, the radical is regenerated on the adenosyl group, and the Co-C bond is reformed (21). Adenosylcorrinoid-dependent enzymes play a role in a wide variety of processes including nucleotide biosynthesis, fermentation of substrates such as glycerol and ethanolamine, and amino acid and fatty acid metabolism (9). The reaction carried out by the adenosylcobalamin-dependent enzyme methylmalonyl CoA mutase, one of the two cobamide-dependent enzymes present in humans, is illustrated in Figure 2.

Corrinoid cofactors with a methyl group as the upper ligand function in methyl transfer reactions. Here, a methyl group is passed from a donor molecule to an acceptor molecule via a corrinoid. Corrinoid-dependent methyltransferases typically consist of at least three modules; one to bind the corrinoid, another to bind the methyl donor and catalyze transfer of a methyl group from the donor to the corrinoid, and the third to bind the acceptor molecule and catalyze transfer of the methyl group from the methylcorrinoid to the acceptor (20). The model corrinoid-dependent methyltransferase, methionine synthase, MetH, transfers a methyl group from methyltetrahydrofolate (the methyl donor) to homocysteine (the methyl acceptor),via the corrinoid cofactor, thus producing methionine (Fig. 2B). Corrinoid-dependent methyltransferases play an important role in a variety of different forms of anaerobic metabolism, including CO_2-reductive acetogenesis, and methanogenesis (30). These enzymes bind corrinoids in the base-off conformation, which may allow for a wider range of corrinoids to function as cofactors.

Cobamide-dependent enzymes also function in the anaerobic respiration of halogenated compounds as terminal electron acceptors, termed halorespiration. In reductive dehalogenation reactions, halogen substituents of an organohalide such as tetrachloroethene are removed, and replaced by hydrogen atoms (31). Unlike other corrinoid-dependent enzymes, reductive dehalogenases use corrinoids without a methyl or adenosyl upper ligand, and with the lower ligand in the base-off conformation (32, 33). One proposed reaction mechanism for reductive dehalogenation reactions involves the formation of a cobalt-halogen bond, leading to cleavage of the C-halogen bond, and concomitant protonation of the leaving group (32). Another suggests a

dissociative one-electron transfer from Co(I) onto the halogenated substrate via a radical mechanism (33). The stepwise dechlorination of tetrachloroethene carried out by corrinoid-dependent reductive dehalogenases in *D. mccartyi* is illustrated in Figure 2C (34).

In addition to serving as cofactors, corrinoids also play an important role in gene regulation as the metabolite bound by corrinoid riboswitches (35). Corrinoid riboswitches can allow organisms to sense intracellular concentrations of corrinoids and appropriately control gene expression, for example, turning off corrinoid transporter genes when sufficient corrinoid levels are reached (36, 37). Genes encoding corrinoid biosynthesis and alternatives to corrinoid-dependent enzymes are also controlled by corrinoid riboswitches (38, 39).

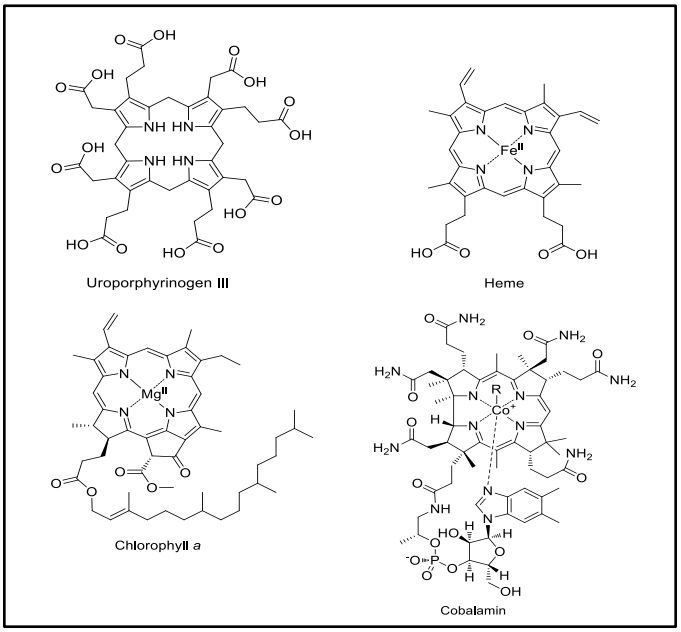

Figure 3. Cyclic tetrapyrroles. Corrinoids, heme, chlorophyll and other cyclic tetrapyrroles are all produced from the same common precursor, uroporphyrinogen III.

Corrinoid biosynthesis

Corrinoids are structurally and biosynthetically related to other cyclic tetrapyrroles, such as heme and chlorophyll, all of which are produced from the same common precursor, uroporphyrinogen III (Uro'gen III) (Fig. 3) (9, 40). Separate aerobic and anaerobic corrinoid biosynthesis pathways exist, and diverge after the production of Uro'gen III(Fig.4)(41). Corrinoid production can be divided into two main parts: corrin ring production, and lower ligand production and attachment. The production of the corrin ring differs in aerobic and anaerobic pathways in the timing of cobalt insertion and oxygen requirement(Fig.4)(42). Cobalt insertion is performed early in the anaerobic pathway, before ring contraction and amidation (12). In the aerobic pathway, cobalt insertion is one of the final steps in ring modification (43). While oxygen is required in the aerobic pathway, several reactions in the anaerobic pathway are oxygen sensitive (44-46)After production of the corrin ring, the aerobic and anaerobic pathways converge and the remaining steps of corrinoid biosynthesis (upper and lower ligand attachment, and nucleotide loop formation) are the same in each, with the exception of lower ligand

formation (Fig.4)(47-52). In the aerobic pathway, production of DMB, the lower ligand of cobalamin, is carried out by the flavin destructase enzyme BluB, which uses reduced flavin mononucleotide as its substrate (53, 54). The genes involved in the anaerobic production of benzimidazoles have not yet been reported, though labeling studies showed that purines and benzimidazoles likely share a common precursor (24). Recently, using a combination of bioinformatic, genetic and biochemical techniques, our lab has identified the set of genes, *bzaABCDE*, responsible for the production of the benzimidazoles such as 5-hydroxybenzimidazole (OHBza), methoxybenzimidazole (OMeBza), and DMB (Hazra, Mok, Han, and Taga, unpublished).

Once the lower ligand has been formed, it must be activated and attached to the corrin ring. The enzymes responsible for these steps have been found not only in organisms that produce corrinoids *de novo*, but also in many that require exogenous corrinoids, which enables them to salvage cobinamide, a cobamide precursor, from the environment (11, 55).

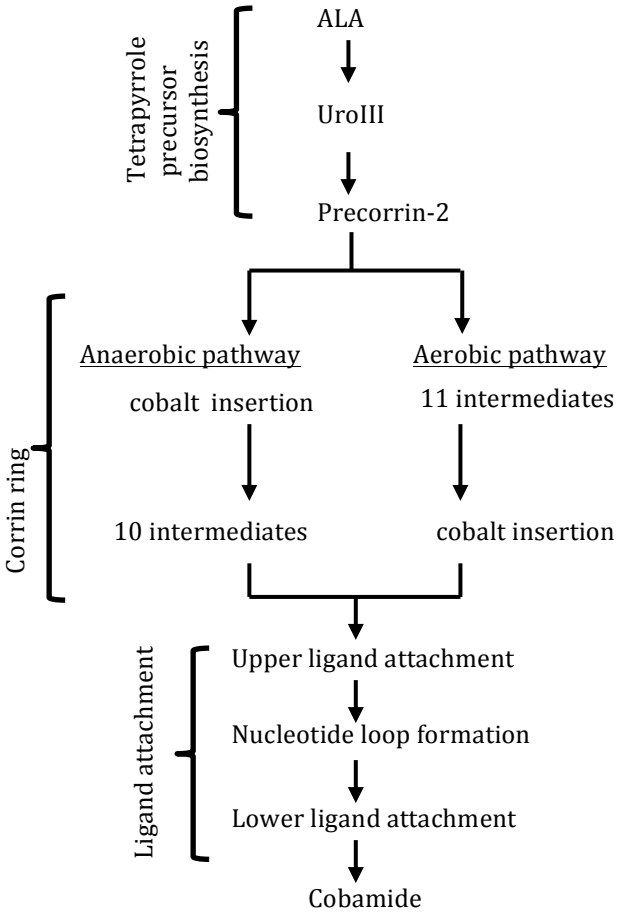

Figure 4. The corrinoid biosynthesis pathway. Corrinoid biosynthesis is complex and requires more than 20 enzymatic steps. Corrinoids, as well as other tetrapyrroles such as heme and chlorophyll, are produced using 5-aminolevulinic acid (ALA) as the first dedicated precursor. Uroporphyrinogen III (Uro'III) is the final common precursor before tetrapyrrole biosynthesis pathways diverge. In the anaerobic corrinoid biosynthesis pathway, cobalt insertion is the first step in corrin ring production, while it is the final step in the aerobic pathway. After corrin ring production, the pathways converge, and the upper and lower ligands are attached to produce a complete cobamide.

Examples of specific mutualistic interactions involving corrinoids

Phytoplankton are of great ecological importance in their roles in the global carbon cycle, as primary producers in the marine food web, and occasionally as producers of toxins in harmful algal blooms (HABs). Half of eukaryotic algal species are predicted to require exogenously produced cobamides for growth (56, 57). Algal corrinoid requirements can be fulfilled via symbiotic relationships with corrinoid-producing bacteria that colonize the algal surface (56, 58). The availability of corrinoids may play an important role in the occurrence of HABs, as cobamide auxotrophy is especially prevalent among HAB species (59).

Symbiotic relationships can also be fueled by cross-feeding of products of corrinoid-dependent enzymes. Genomic studies of the cicada endosymbiotic bacteria, *Hodgkinia cicadicola* and *Sulcia muelleri*, indicate that the ability to produce a corrinoid is vital for maintaining this tripartite symbiosis (60). *H. cicadicola* and *S. muelleri* together produce essential amino acids that are cross-fed between the co-symbionts and provided to the host. The burden of methionine production rests on *H. cicadicola*, which devotes 7% of its 144 kb genome to corrinoid biosynthesis because the methionine synthase it encodes requires a corrinoid cofactor. Interestingly, the corrinoid biosynthesis pathway in *H. cicadicola* is incomplete, and lacks genes involved in the production of precursors common to the biosynthesis of corrinoids as well as other tetrapyrroles such as heme (60). We speculate that *H. cicadicola* may acquire tetrapyrrole precursors from the host, completing a loop between organisms which allows maintenance of the symbiotic relationship.

Strategies used by bacteria that scavenge corrinoids and their precursors from environmental sources

Because multiple corrinoids are present in microbial communities, corrinoid-dependent microbes employ a variety of strategies to acquire the specific corrinoids that function in their metabolism (Fig. 5) (61-63). The ability of bacteria to selectively transport specific corrinoids has been largely unexplored, yet may play an important role in this process. The corrinoid transporter - BtuBFCD in gram negative bacteria, and BtuFCD in gram positive bacteria - allows bacteria to import corrinoids from the environment (Fig. 5A) and is present in an estimated 76% of bacterial genomes (11). A recent study of the human gut microbiome found that many bacteria encode multiple copies of the BtuBFCD corrinoid transporter, and that the three homologs of the outer membrane transporter BtuB encoded by the model gut bacterium *Bacteroides thetaiotaomicron*, though apparently redundant, have distinct preferences for different corrinoids (64). The presence of multiple corrinoid transport systems with different affinities for specific corrinoids may allow bacteria to fine-tune their responses to the array of corrinoids present in the environment.

In contrast to obtaining specific corrinoids through selective transport, the ability to remodel corrinoids (that is, to remove the lower ligand of an imported corrinoid and attach a preferred lower ligand in its place) can enable microbes to make use of many different corrinoids (65). For instance, the work described in Chapter 2 shows that *D. mccartyi,* which has an obligate requirement for exogenously produced corrinoids as cofactors in the reductive dehalogenation of the common groundwater pollutants tetrachloroethene (PCE) and trichloroethene (TCE), is restricted to the use of just three benzimidazolyl corrinoids (18, 66, 67). However, if an appropriate benzimidazole lower ligand base is supplied, *D. mccartyi* can fulfill its corrinoid requirements by remodeling other corrinoids(Fig. 5C) (18). The ability to remodel corrinoids may be essential to *D. mccartyi*'s survival in the environment. My work described in

Chapter 3 identifying the corrinoids and free lower ligands present in a microbial community containing *D. mccartyi* shows that the most abundant corrinoid is *p*-cresolylcobamide ([Cre]Cba), a cobamide that *D. mccartyi* cannot use without remodeling. Moreover, I show that free DMB is present in the community at levels sufficient for corrinoid remodeling, and that the amount of [Cre]Cba in the supernatant increases, while the amount of cobalamin decreases when *D. mccartyi* is eliminated (63). These results support the hypothesis that *D. mccartyi* carries out corrinoid remodeling within its community. Corrinoid remodeling is also performed by some bacteria capable of producing a corrinoid *de novo* (65), and has been observed in the human gut, where examination of fecal corrinoid profiles before and after ingestion of cobalamin suggests that at least some members of the gut microbiota are engaged in active modification of exogenous corrinoids (61).

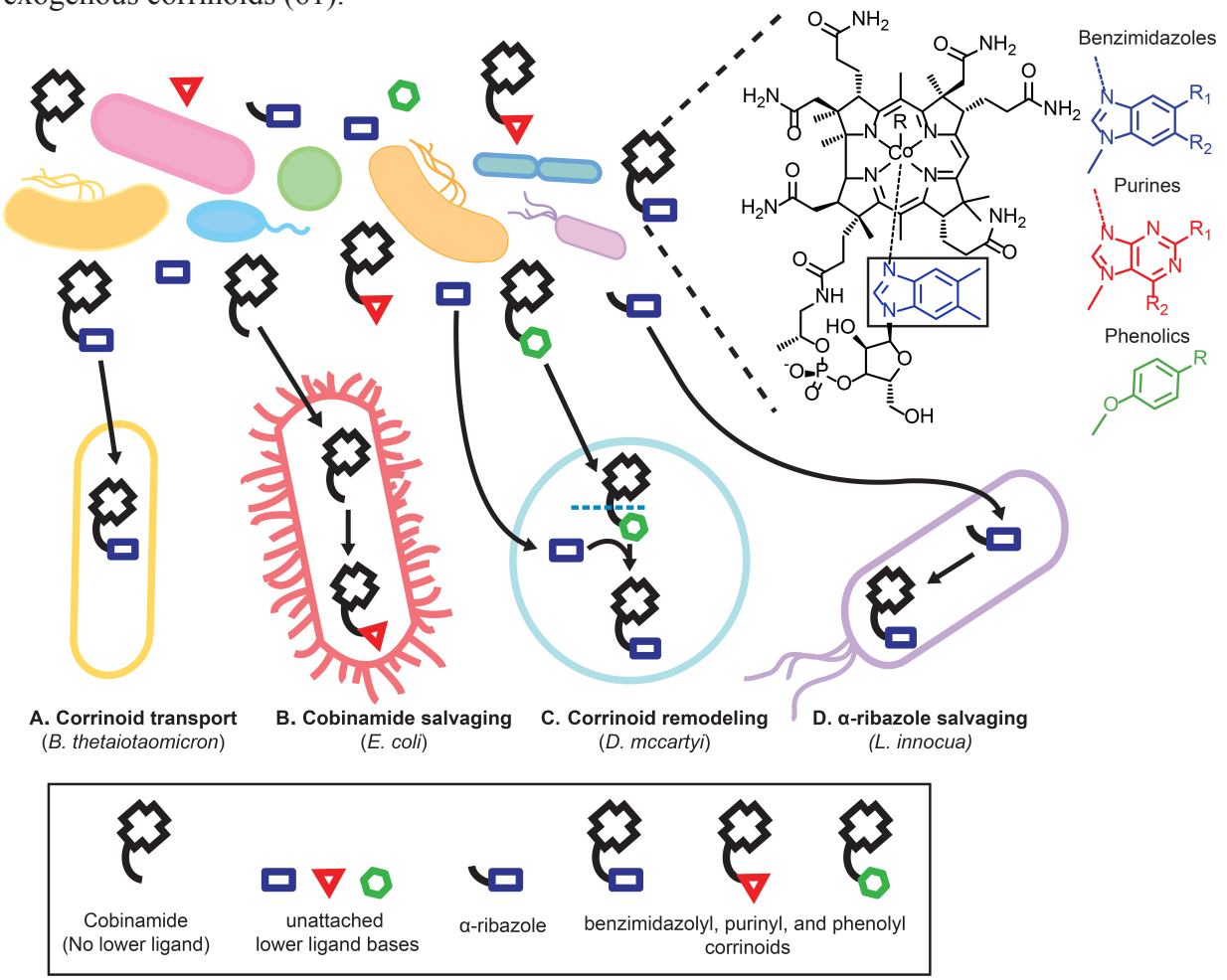

Figure 5. Microbial strategies for fulfilling corrinoid requirements
Microbes employ various strategies to obtain specific corrinoids from environments that contain a variety of corrinoids and corrinoid precursors. The structure of cobalamin, a corrinoid with the lower ligand 5,6-dimethylbenzimidazole (boxed) is shown, as are the structures of the three classes of lower ligand bases of other corrinoids. Specific strategies for obtaining corrinoids are illustrated with representative bacteria listed in parentheses. (A). Corrinoid transport in *Bacteroides thetaiotaomicron*. (B). Cobinamide salvaging in *Escherichia coli*. (C). Corrinoid remodeling in *Dehalococcoides mccartyi*. (D). α-ribazole salvaging in *Listeria innocua*.

Some microbes are capable of importing corrinoid precursors from the environment and carrying out the remaining biosynthetic steps to produce a complete corrinoid. For example, though *E. coli* is incapable of *de novo* corrinoid biosynthesis, it possesses the ability to salvage cobinamide(Fig. 5B) (68, 69). In contrast, *Listeria spp.* encode a nearly complete corrinoid biosynthesis pathway, but apparently lack the genes necessary for lower ligand activation, a step that must be completed before the lower ligand can be attached. *L. innocua* was shown instead to rely on the *cblT* encoded transporter to take up activated lower ligands such as alpha-ribazole from the environment, which it then phosphorylates and attaches to produce a complete corrinoid(70) (Fig. 5D). CblT homologs have been identified in a variety of human pathogens including *L. monocytogenes* and *Clostridium botulinum* (70). While organisms carrying out cobinamide salvaging or activated lower ligand transport rely on molecules produced by other microbes, I identified a group of microbes that may scavenge tetrapyrrole precursors produced by an animal host. In these microbes, some of the genes involved the biosynthesis of the universal tetrapyrrole precursors are absent, though the remainder of the corrinoid biosynthesis pathway is intact. Each of the three microbes tested showed the ability to scavenge 5-aminolevulinic acid, the first universal tetrapyrrole precursor, from the growth medium and produce corrinoids. Of the 39 bacteria identified, all are animal host associated (Chapter 4).

Microbes that produce corrinoids *de novo* can also be affected by the presence of corrinoid precursors. Guided biosynthesis, the process of controlling which corrinoid a microbe produces by providing an excess of a particular lower ligand base, can be a useful tool for determining the specific corrinoids required in different metabolic pathways (71, 72). For example, in *Sporomusa ovata*, which produces phenolyl corrinoids, the addition of benzimidazoles and their subsequent incorporation into benzimidazolyl corrinoids inhibits growth to different degrees on substrates that require a corrinoid-dependent methyltransferase for utilization (73, 74). We have detected free lower ligand bases in a variety of environmental samples, which raises the question of whether the production of a free lower ligand base by one organism is capable of affecting the corrinoid production of another; that is, whether guided biosynthesis occurs in nature.

Corrinoids as lynchpins of microbial community dynamics?

Given that the majority of bacteria depend on corrinoids for their metabolism, and that only a fraction of available corrinoids may be suitable for use by a particular organism, could it be possible to manipulate microbial communities by targeting corrinoid-dependent metabolism? The work described in Chapter 3 shows that manipulation of the composition of a TCE-dechlorinating community can result in a shift in corrinoid composition. Conversely, the addition of cobalamin was shown to cause a dramatic shift in marine algal community composition (63, 75). With growing interest in developing methods for targeted manipulation of microbial communities to benefit human health and the environment, the utility of altering the composition and/or metabolism occurring in microbial communities via corrinoid supplementation or guided biosynthesis deserves further investigation.

Conclusion

Corrinoid cross-feeding impacts individual species as well as entire communities. The following chapters detail my work on newly emerging aspects of corrinoid cross-feeding, from *D. mccaryti*'s strategies for obtaining a functional corrinoid cofactor from a variety of forms, to the impact of changes in community composition on corrinoid content.

Chapter 2

Versatility in Corrinoid Salvaging and Remodeling Pathways Supports the Corrinoid-Dependent Metabolism of *Dehalococcoides mccartyi*

Published: Yujie Men[1], Erica C. Seth[1], Shan Yi, Terence S. Crofts, Robert H. Allen, Michiko E. Taga, and Lisa Alvarez-Cohen. "Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities." *Environmental microbiology* (2014).

1. These authors contributed equally to this paper

Abstract
Corrinoids are cobalt-containing molecules that function as enzyme cofactors in a wide variety of organisms, but are produced solely by a subset of prokaryotes. Specific corrinoids are identified by the structure of their axial ligands. The lower axial ligand of a corrinoid can be a benzimidazole, purine, or phenolic compound. Though it is known that many organisms obtain corrinoids from the environment, the variety of corrinoids that can serve as cofactors for any one organism is largely unstudied. Here, we examine the range of corrinoids that function as cofactors for corrinoid-dependent metabolism in *Dehalococcoides mccartyi* strain 195. *Dehalococcoides* bacteria play an important role in the bioremediation of chlorinated solvents in the environment because of their unique ability to convert the common groundwater contaminants perchloroethene and trichloroethene to the innocuous end product ethene. All isolated *D. mccartyi* strains require exogenous corrinoids such as vitamin B_{12} for growth. However, like many other corrinoid-dependent bacteria, none of the well-characterized *D. mccartyi* strains has been shown to be capable of synthesizing corrinoids *de novo*. In this study, we investigate the ability of *D. mccartyi* strain 195 to use specific corrinoids, as well as its ability to modify imported corrinoids to a functional form. We show that strain 195 can only use specific corrinoids containing benzimidazole lower ligands, but is capable of remodeling other corrinoids by lower ligand replacement when provided a functional benzimidazole base. This study of corrinoid utilization and modification by *D. mccartyi* provides insight into the array of strategies that microorganisms employ in acquiring essential nutrients from the environment.

INTRODUCTION
Corrinoids are essential cofactors for enzymes that facilitate carbon skeleton rearrangements, methyl group transfers, and reductive dehalogenation (76). Members of all three domains of life use corrinoid cofactors, yet the complete biosynthesis of corrinoids, which requires approximately 30 enzymatic steps, is performed only by a subset of prokaryotes (77, 78). The dependence of certain microbes on corrinoids produced by other organisms has previously been observed in ecosystems such as the mammalian gut, marine environments, and in microbial consortia isolated from forest soil (79-81). A recent bioinformatic analysis revealed that while 76% of 540 sequenced bacterial genomes contain corrinoid-dependent enzymes, only 39% of these genomes contain the complete corrinoid biosynthesis pathway (82). This study also found that the *btuFCD* genes encoding a high affinity corrinoid transporter are present in 76% of sequenced bacterial genomes, indicating that most bacteria are capable of taking up corrinoids

from the environment (82). Based on these experimental and bioinformatic results, corrinoid cross-feeding is likely to be widespread in microbial communities.

Corrinoids are distinguished from one another in part based on the structure of the lower axial ligand, which can be a benzimidazole, purine, or phenolic compound (83) (Fig. 1). Corrinoid cofactors with different lower ligands (collectively termed cobamides) are not necessarily functionally equivalent as cofactors, as some cobamide-dependent metabolic processes have been shown to function only with a specific cobamide (84, 85). The best studied cobamide, cobalamin (also known as vitamin B_{12}), contains 5,6-dimethylbenzimidazole (DMB) as its lower ligand and is the only cobamide that is commercially available (Fig. 1A). In humans, cobalamin is clearly the preferred cobamide and may be the only corrinoid with significant biological activity. While a diverse range of corrinoids has been detected in microbial communities, very little is currently known about how microbes that import exogenous corrinoids respond to the presence of different corrinoids in the environment (86-88). The organohalide-respiring bacterium *Dehalococcoides mccartyi* strain 195 was chosen as a model organism for this study due to its obligate requirement for exogenously supplied corrinoids. *mccartyi* requires corrinoid cofactors for dechlorination and growth (89-93). Genome annotations of sequenced *D. mccartyi* isolates [strains 195, VS, BAVI, CBDB1, and GT] reveal the presence of three types of corrinoid-dependent enzymes (94-96) (http://img.jgi.doe.gov/). Genes encoding reductive dehalogenases (RDases), which catalyze successive dehalogenation reactions in organohalide respiration, are present in 10-38 copies in each of the five published genome sequences, and evidence of corrinoid cofactor involvement in RDases has been shown experimentally in strains 195 and CBDB1 (90, 93). The presence of 2-3 copies of the corrinoid-dependent ribonucleotide reductase (RNR) encoded by *nrdJ* in each strain indicates that corrinoid cofactors are also involved in DNA replication in *D. mccartyi* (Table S1). In addition, homologs of the *acsCD* genes, predicted to encode a corrinoid iron sulfur protein (CFeSP) that facilitates methyl transfer reactions in an incomplete Wood-Ljungdahl acetyl-CoA pathway, are also present (94-96) (http://img.jgi.doe.gov/). In strain 195, transcripts and proteins corresponding to the RDase gene *tceA*, two of the three *nrdJ* paralogs, and both CFeSP genes have been detected (97-99).

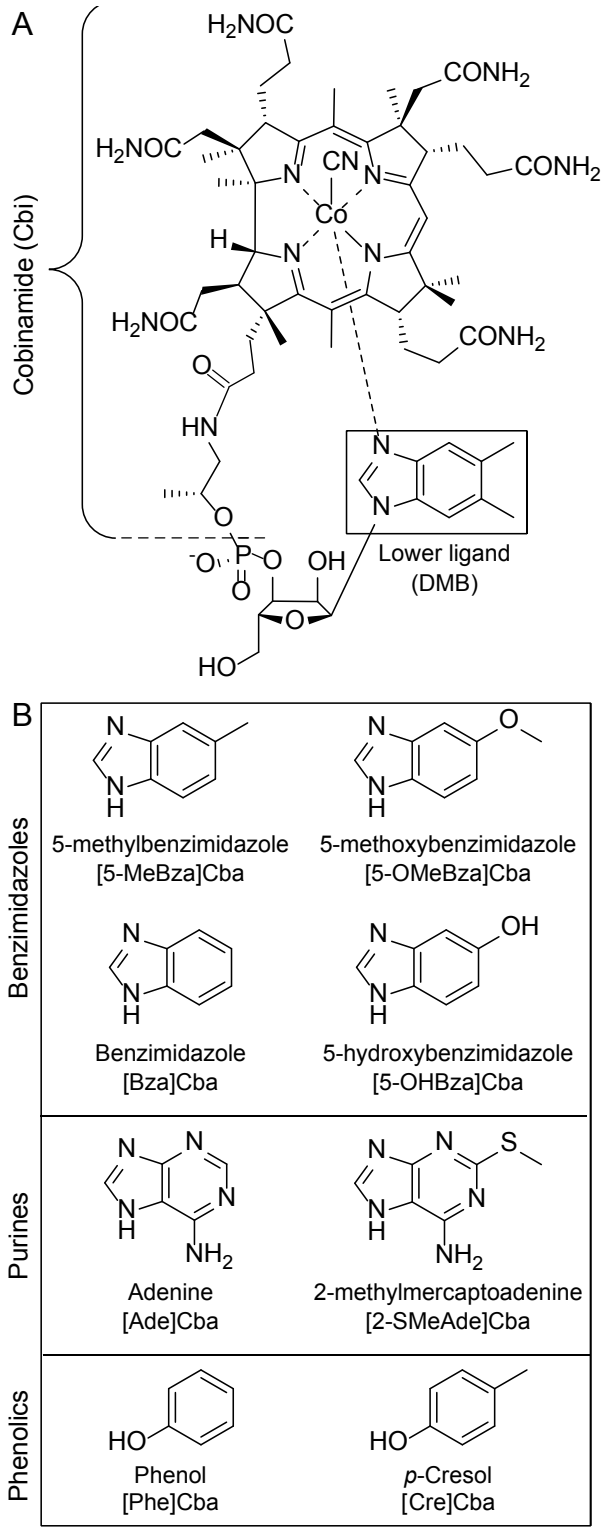

Figure 1. Structures of corrinoids and lower ligands. (A) Cyanocobalamin (vitamin B_{12}) is shown with the lower ligand, 5,6-dimethylbenzimidazole (DMB), boxed and the structure of cobinamide (Cbi) indicated by the bracket. (B) The structures of alternative lower ligands of corrinoids examined in this work are shown with the name

of the lower ligand and the abbreviation for the corresponding complete cobamide (Cba) indicated below. A cobamide is defined as a corrinoid containing the nucleotide loop and a lower ligand.

D. mccartyi plays a crucial role in the bioremediation of chlorinated solvents, as it is the only known organism capable of converting the common groundwater contaminants tetrachloroethene (PCE) and trichloroethene (TCE) to the non-toxic end product ethene (100-103). *D.* Despite their dependence on corrinoid cofactors, portions of the corrinoid biosynthesis pathway are absent from the genomes of *D. mccartyi* strains, and all isolates require corrinoid supplementation when grown in pure culture. A possible exception was found in a recent metagenomic analysis of a TCE-dechlorinating enrichment culture in which putative genes for corrin ring synthesis from the precursor uroporphyrinogen III were identified in *D. mccartyi* strain ANAS2 (104). However, neither the ability to synthesize a corrinoid *de novo* nor the ability to grow without corrinoid supplementation has been shown experimentally in this strain.

D. mccartyi has been found in many different environments including groundwater, subsurface soil, and river and marine sediments (105, 106). In these environments, *D. mccartyi* co-exists with microbes that perform a variety of corrinoid-dependent metabolic functions such as the fermentation of short-chain organic compounds, acetogenesis, and methanogenesis (76, 107-109). Microorganisms carrying out these processes have been found to produce different corrinoids with benzimidazolyl, purinyl, and phenolyl lower ligands (83, 110, 111) (Fig. 1). As *D. mccartyi* is likely to encounter multiple corrinoids in the environment, it is important to understand which corrinoids can support its growth.

The genomes of all sequenced *D. mccartyi* strains contain putative corrinoid salvaging and remodeling genes, which may help fulfill the requirement for exogenous corrinoids (94-96) (http://img.jgi.doe.gov/). Corrinoid salvaging involves the uptake and modification of an incomplete corrinoid such as cobinamide (Cbi, a corrinoid lacking a lower ligand) to form a cobamide through the attachment of an upper ligand, nucleotide loop, and lower ligand (Fig. 2) (55). The enzyme CbiZ has been shown in *Rhodobacter sphaeroides* to catalyze the removal of the lower ligand and nucleotide loop of adeninylcobamide [Ade]Cba, a corrinoid that *R. sphaeroides* can import from the environment but is incapable of using as a cofactor without modification (112, 113). The process of removing and replacing a lower ligand is termed cobamide remodeling. Corrinoid salvaging and remodeling activities have not yet been studied in *D. mccartyi* or any other organism that requires exogenous corrinoids.

Here, we demonstrate that the range of corrinoids that support dechlorination and growth of *D. mccartyi* strain 195 is restricted to just three structurally related cobamides. In addition, we find that strain 195 is capable of salvaging and remodeling a variety of nonfunctional corrinoids to form cobamides that support dechlorination and growth, as long as a benzimidazole lower ligand base is also provided. Thus, while strain 195 is incapable of producing a corrinoid or lower ligand *de novo*, it can assemble functional cobamides from cobamide precursors or from cobamides that do not function as cofactors. These activities may enable *D. mccartyi* to carry out corrinoid-dependent metabolism in microbial communities in which structurally diverse corrinoids are present.

Results

Bioinformatic analysis of corrinoid salvaging and remodeling genes in sequenced *D. mccartyi* genomes.

A genomic locus containing 11 genes, five of which are annotated as functioning in corrinoid salvaging and remodeling, is present in at least one copy in each of the five fully sequenced *D. mccartyi* genomes (Fig. 2). In each *D. mccartyi* genome, homologs of these genes are arranged in the same orientation and order. To identify additional genes that may be involved in corrinoid salvaging and remodeling, reciprocal BLAST analysis of genes at this locus was performed against the genomes of *S. enterica* serovar Typhimurium, *E. coli* K12, and *M. jannaschii*, three organisms for which experimental evidence for the functions of genes involved in corrinoid salvaging or remodeling is available (114-118). This analysis indicated that 10 of the 11 genes at this locus are likely to be involved in these processes (Fig. 2). Our model for how corrinoid salvaging and remodeling might occur in *D. mccartyi* is shown in Figure 2.

The gene *hisC*, previously annotated as encoding L-histidinol phosphate aminotransferase, was identified as being more similar to *cobD,* which encodes an enzyme that decarboxylates L-Thr-P to yield AP-P in the anaerobic corrinoid biosynthesis pathway (115) (Fig. 2). Furthermore, the gene previously annotated as *cobD* was re-annotated as *cbiB*, which encodes an enzyme that catalyzes the conversion of AP-P and Ado-Cby to Ado-Cbi-P (119) (Fig. 2). Another gene at this locus, *cbiZ*, was found to be misannotated in two of the *D. mccartyi* strains as *rdhF*. Potential homologs of *cbiZ* are also present in 2-7 copies in other regions of the *D. mccartyi* genomes.

Reciprocal best BLAST hit analysis also suggests that three genes in the locus that are annotated as *fepBDC* are more likely to encode the ABC transporter *btuFCD*, which is involved in importing corrinoids from the environment. While *fepB* was found to have the highest sequence similarity to *btuF*, which encodes the periplasmic corrinoid-binding protein, *fepD* and *fepC* are more similar to siderophore transporter components. Although ABC transporters for corrinoids, siderophores, and other substrates are difficult to distinguish from one another based on sequence alone, the presence of these genes adjacent to other corrinoid salvaging genes suggests that they function in corrinoid transport. Furthermore, a previous transcriptional study showed that expression of these genes was induced under cobalamin-limited conditions (26). Additionally, the existence of a high-affinity corrinoid transporter in *D. mccartyi* is supported by their ability to carry out growth and TCE dechlorination in the presence of as little as 0.8 nM cobalamin (97). In strain 195, previous gene expression and proteomic analyses demonstrated that eight of the 11 genes at this locus are expressed in both transcripts and proteins (97-99) (Fig. 2).

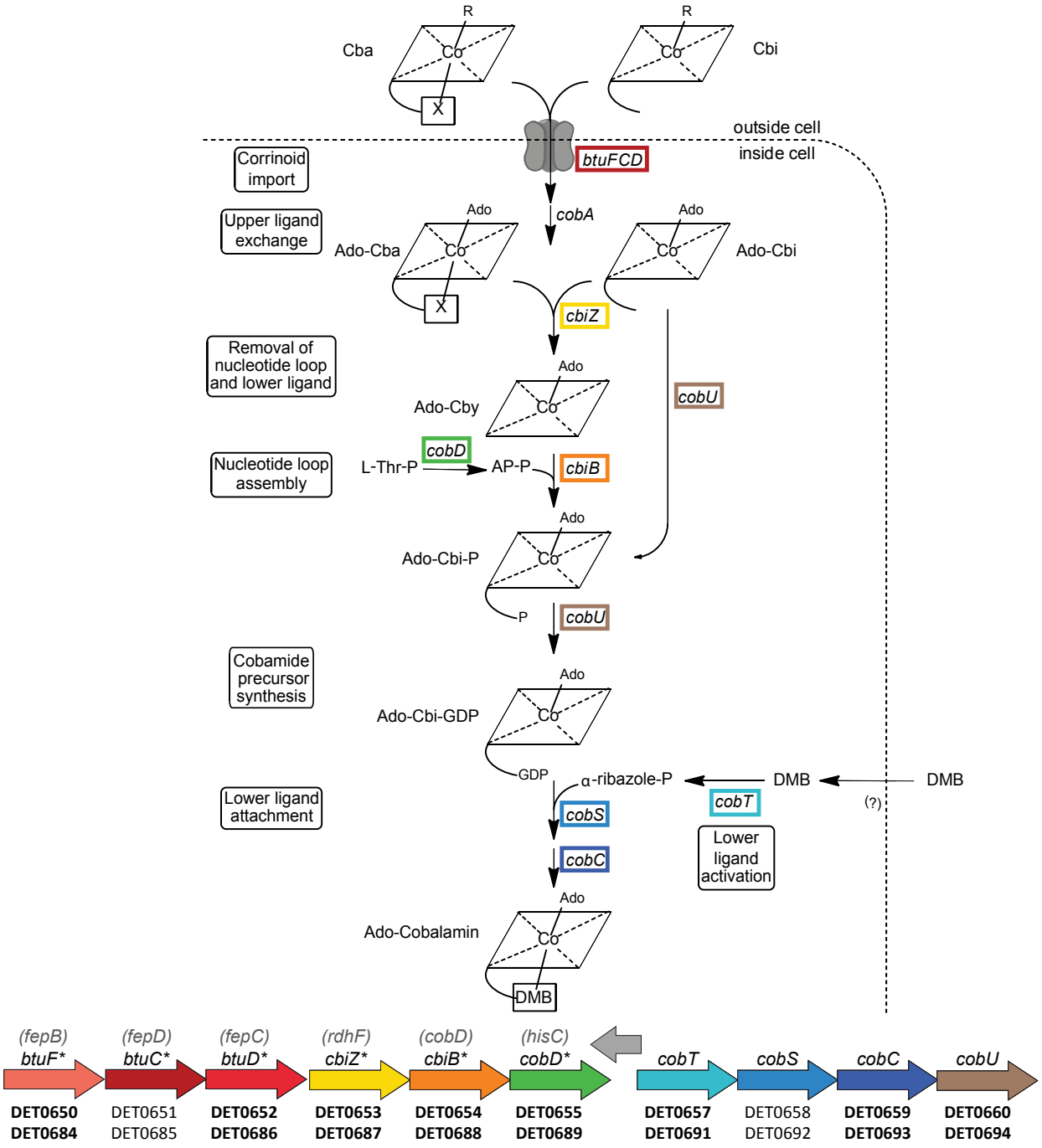

Figure 2. Model of corrinoid salvaging and remodeling pathways in *D mccartyi*. Extracellular cobamides (Cba) and Cbi are imported by a high-affinity corrinoid transporter. Within the cell, the upper ligand is exchanged with deoxyadenosine (Ado). Salvaging of cobinamide occurs through one of two redundant pathways encoded by either *cbiZ* and *cbiB* or *cobU*. Remodeling of cobamides begins with the removal of the lower ligand (X) and the nucleotide loop. Cobamides are formed from the precursor Ado-Cbi-GDP and an activated lower ligand. Putative homologs of genes involved in each step of the pathway that are encoded by *D. mccartyi* genomes are shown. Most genes in the pathway are present in a single genomic locus shown at the bottom, with the locus numbers in strain 195 shown below each gene. Locus numbers shown in bold indicate the genes that are expressed in both transcripts and proteins (97-99). Arrows represent the direction of each gene. The gray arrow indicates a conserved hypothetical open reading frame. Asterisks correspond to new annotations proposed in this work. Gene symbols in

parentheses indicate the previous annotation. Abbreviations: Ado, adenosyl; Cby, cobyric acid; P, phosphate; AP, aminopropanol; GDP, guanosine diphosphate.

Specific corrinoid requirements of strain 195.

Although *D. mccartyi* isolates are routinely supplied with cobalamin to fulfill their corrinoid requirements, it is possible that they are capable of using other corrinoids as well. Because cobamides other than cobalamin are not commercially available, we purified six additional cobamides from bacterial cultures by guided biosynthesis as described in the Materials and Methods. Parallel cultures of strain 195 were provided one of seven different corrinoids, and TCE dechlorination activity and cell growth of these cultures were compared to those of cultures supplied with cobalamin. The corrinoids tested included cobamides with phenolyl, purinyl, and benzimidazolyl lower ligands as well as Cbi. Our results showed that only two of the benzimidazolyl cobamides, cobalamin and [5-MeBza]Cba, were capable of supporting dechlorination of TCE and cell growth, while the other corrinoids tested were not (Fig. 3). Dechlorination activity of cultures grown with [5-MeBza]Cba was comparable to cultures with cobalamin (Fig. 3A). While fewer cells were produced in cultures with [5-MeBza]Cba added than in cobalamin-supplied cultures, cell growth was enhanced relative to cultures supplied with any other corrinoid (Fig. 3B). Although sequence analysis suggests that strain 195 possesses redundant pathways for Cbi salvaging (96) (Fig. 2), the addition of Cbi alone failed to support dechlorination or cell growth (Fig. 3).

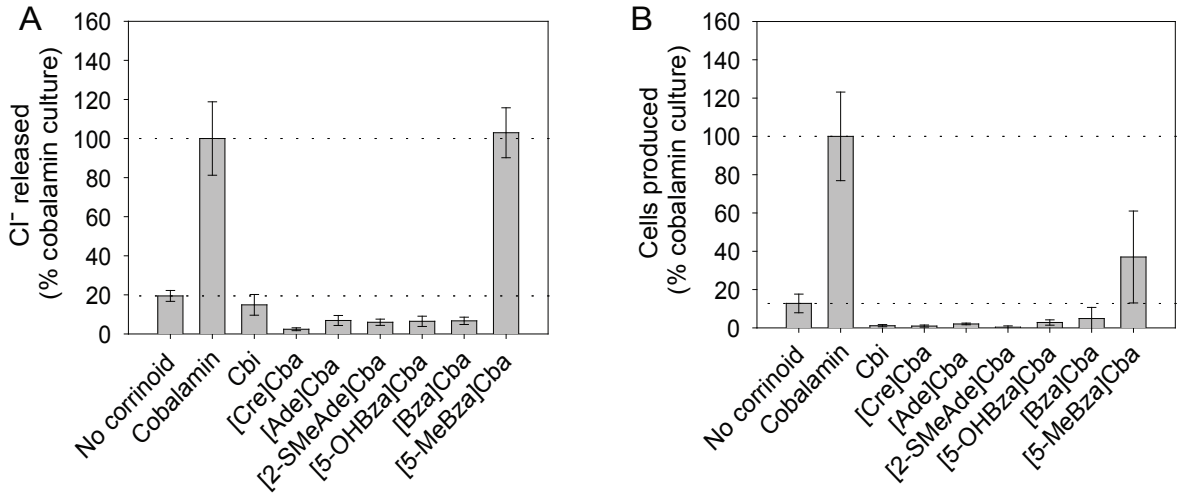

Figure 3. Dechlorination and growth of *D. mccartyi* strain 195 in the presence of different corrinoids.(A) Cl^- released from TCE dechlorination and (B) cell number are shown for cultures grown in the absence or presence of each of eight corrinoids. The data shown are the mean values from three independent experiments with error bars representing one standard deviation.

Cbi salvaging and lower ligand attachment in strain 195.

In order to investigate whether the Cbi salvaging pathways are functional in strain 195, we provided cultures with both Cbi and DMB, the lower ligand of cobalamin (Fig. 1A). We reasoned that the failure to use Cbi alone could be due to an inability to synthesize a lower ligand base such as DMB or 5-MeBza to form a functional cobamide. Our results show that cultures provided with both Cbi and DMB exhibited similar TCE dechlorination and cell growth to those of cultures with cobalamin added (Fig. 4A and B). Moreover, cobalamin was detected in corrinoid extracts from these cultures (Fig. 4C), which suggests that strain 195 is capable of salvaging Cbi, synthesizing the nucleotide loop, and activating and attaching DMB (Fig. 2).

Since we observed that the addition of Cbi plus DMB supports TCE dechlorination and growth, we next investigated whether other lower ligand bases could substitute for DMB. We provided parallel cultures of strain 195 with Cbi plus one of five lower ligand bases and measured dechlorination and growth. In cultures provided with Cbi and either Cre or 5-OHBza, dechlorination and growth did not exceed background levels (Fig. 4A and B). These results confirm those seen when cobamides containing Cre or 5-OHBza were provided to strain 195. Cre appeared to be toxic, as the cell number in these cultures decreased after incubation. A culture provided with Cbi and Bza showed a small amount of dechlorination and growth, though this effect was not observed when the complete corrinoid [Bza]Cba was provided (Fig. 3, 4). In contrast, the addition of Cbi and either 5-OMeBza or 5-MeBza resulted in levels of TCE dechlorination and growth indistinguishable from cultures grown with cobalamin or Cbi plus DMB (Fig. 4A and B). When corrinoids were extracted and analyzed from cultures provided with Cbi and 5-OMeBza, [5-OMeBza]Cba was detected (Fig. 4C).

We also investigated whether *D. mccartyi* shows preferential attachment of particular lower ligands when four benzimidazoles, DMB, 5-OMeBza 5-MeBza, and Bza, were provided in equal concentrations together with Cbi. We observed higher levels of attachment of DMB, as cobalamin represented 58% of the total extracted corrinoids, followed by nearly equal levels of [5-MeBza]Cba and [5-OMeBza]Cba at 20 and 17%, respectively (Fig. 4C). [Bza]Cba, which did not fully support dechlorination, was detected in only 4% of the total extracted corrinoids. This suggests either that these lower ligand bases are not taken up in equal amounts, or that lower ligand activation and attachment enzymes act most efficiently on benzimidazoles that support dechlorination and growth.

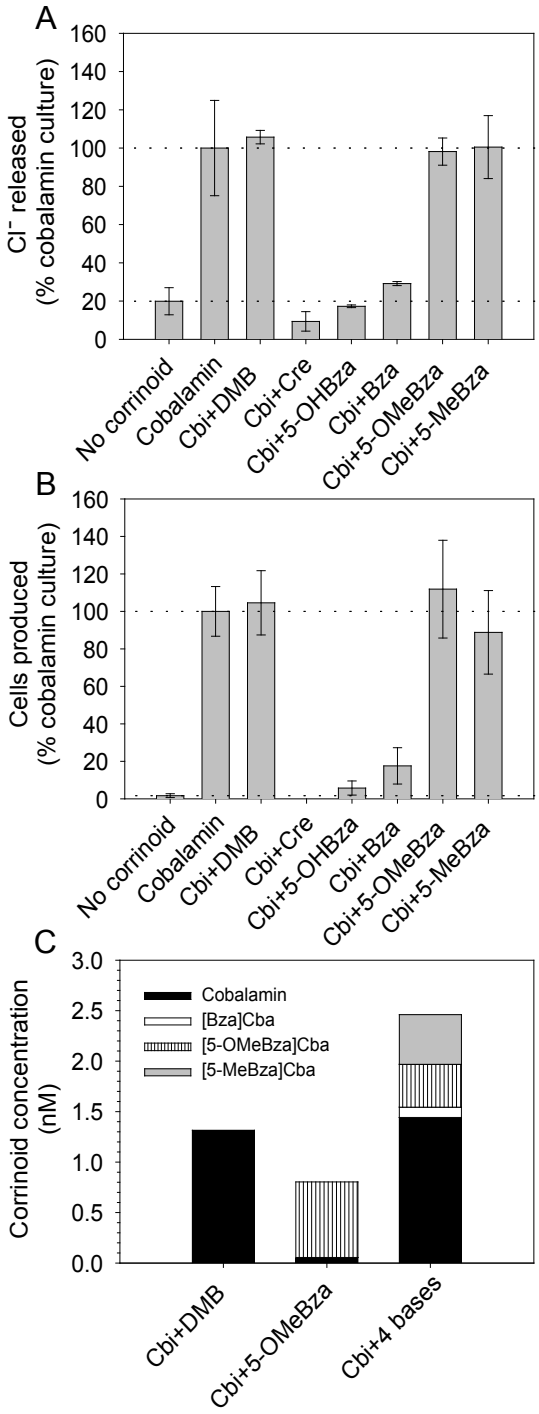

Figure 4. Dechlorination and growth of *D. mccartyi* strain 195 in the presence of Cbi plus different lower ligand bases. (A) Cl^- released from TCE dechlorination and (B) cell number are shown for cultures grown in the absence and presence of Cbi plus each of six different lower ligand bases. (C) Quantification of corrinoids extracted from cultures provided with Cbi plus DMB, 5-OMeBza, or a mixture of Bza, 5-MeBza, 5-OMeBza and DMB in equal molar concentrations (Cbi + 4 bases) is shown. The culture grown with Cbi plus 5-OMeBza contains 0.05 nM cobalamin that was present in the inoculum.

Cobamide remodeling. Our bioinformatic analysis of *D. mccartyi* genomes suggests that the complete cobamide remodeling pathway is present (Fig. 2). Although we found that some cobamides do not support TCE dechlorination or growth (Fig. 3), we reasoned that cobamide remodeling did not occur because a functional lower ligand was not provided. When DMB was provided in addition to any of the nonfunctional cobamides, dechlorination was restored to levels observed when cobalamin alone was added (Fig. 5A). Growth in these cultures was significantly improved relative to cultures with these cobamides provided alone (compare Fig. 3B and 5B).

The restoration of growth and dechlorination activity in cultures provided with a nonfunctional cobamide plus DMB suggests that the nonfunctional cobamide is converted to cobalamin, presumably through the activities of the corrinoid salvaging and remodeling pathway genes (Fig. 2). Consistent with this hypothesis, cobalamin was detected in corrinoid extracts from these cultures at levels exceeding 0.8 nM, the minimum concentration required for growth (97) (Fig. 5C). Although all nonfunctional cobamides were remodeled when DMB was provided, the efficiency of this process appeared to depend on the particular cobamide.

Replacement of the lower ligand with DMB also occurred when [5-MeBza]Cba, a cobamide capable of supporting dechlorination and growth, was provided (Fig. 5C). Similarly, when cultures were provided with cobalamin plus 5-MeBza, [5-MeBza]Cba was detected in the corrinoid extract (Fig. 5C). Together, these results demonstrate that *D. mccartyi* is capable of modifying both functional and nonfunctional corrinoids.

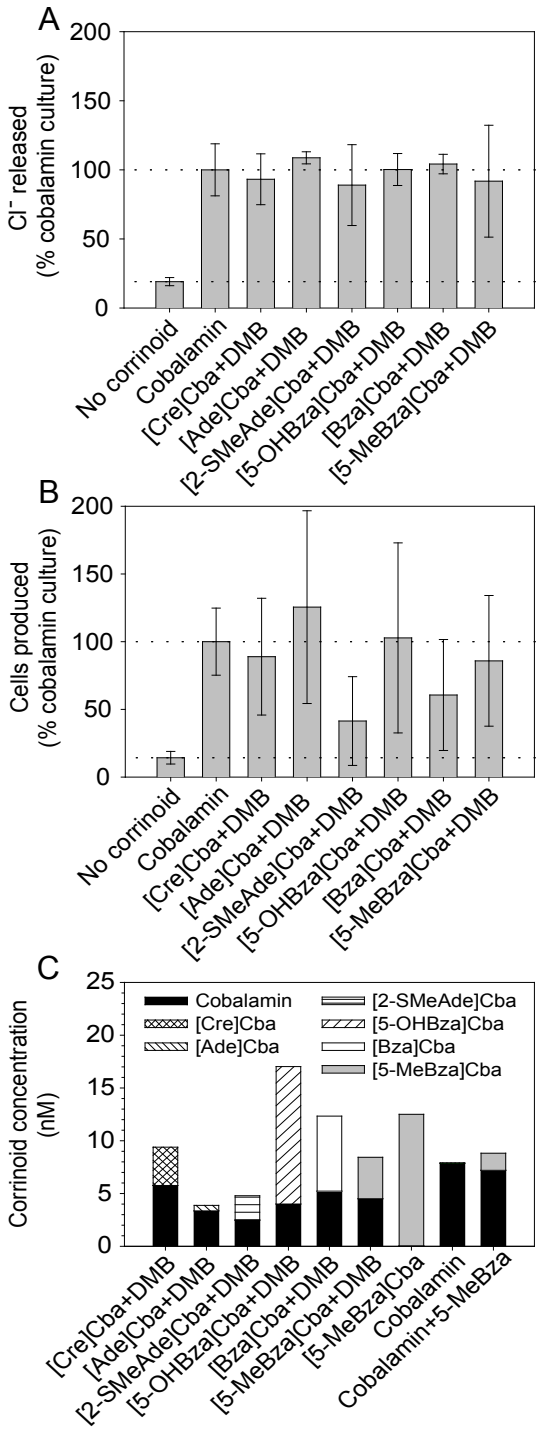

Figure 5. Modification of cobamides by *D. mccartyi* strain 195 grown in the presence of DMB. (A) Cl^- released from TCE dechlorination and (B) cell number are shown for cultures grown with each of six cobamides plus DMB. (C) Quantification of corrinoids extracted from the six cultures containing cobamide plus DMB are shown. Also shown are corrinoids present in cultures grown with [5-MeBza]Cba plus DMB and with cobalamin plus 5-MeBza.

DISCUSSION

Many microbes depend on other organisms in their environment for the biosynthesis of essential metabolites including corrinoids. Given the diversity of corrinoid structures, organisms that require exogenous corrinoids must have mechanisms to acquire the specific corrinoids that function as cofactors for their corrinoid-dependent enzymes. In mammals, the cobalamin absorption pathway effectively prevents nonfunctional corrinoids obtained from food from entering cells through a series of highly specific protein-ligand interactions (120). However, in microbes, the range of corrinoids that function as cofactors and the mechanisms by which functional corrinoids are obtained were previously less clear. In this study, we demonstrate that *D. mccartyi* strain 195 can use only three benzimidazolyl cobamides efficiently as cofactors, but can make use of a broader range of corrinoids through its corrinoid salvaging and remodeling activities. The inability to synthesize corrinoids *de novo* despite the presence of corrinoid-dependent enzymes is common among bacteria (82) and highlights the importance of corrinoid production by the microbial community.

This analysis of the corrinoid requirements of strain 195 demonstrates that the structure of the lower ligand base of a corrinoid affects its ability to function as a cofactor in dechlorination and other processes essential for cell growth. We found that strain 195 is surprisingly restrictive in its corrinoid requirements, as only a subset of the benzimidazolyl cobamides function efficiently as cofactors, in contrast to *S. enterica* or the corrinoid bioassay strain *Lactobacillus leichmannii* which can use both benzimidazolyl and purinyl cobamides (121, 122). The requirement for benzimidazolyl cobamides for cell growth may be due to the ability of these cobamides to modulate the reactivity of the upper ligand in enzymes that utilize cobamides with the lower ligand coordinated to the cobalt center (i.e., "base-on"). DNA synthesis in *D. mccartyi* is predicted to be

dependent on the class II RNR NrdJ, an enzyme that functions with the cobamide bound in the base-on form (123). In contrast, RDases and CFeSPs, the two other types of corrinoid-dependent enzymes present in *D. mccartyi*, have been shown in other organisms to function with the corrinoid cofactor bound in the "base-off" position (124-126). The requirement of *D. mccartyi* for benzimidazolyl cobamides differs from that of *Sulfurospirillum multivorans*, which uses the purinyl cobamide nor-adeninylcobamide as a cofactor for its PCE RDase, and is capable of reductive dechlorination of PCE only to dichloroethene (127). This could be due to differences in the catalytic mechanisms of the RDases or adaptations of the enzymes to the corrinoids available in their respective environments.

Our results also show that strain 195 can produce functional corrinoids by attaching DMB, 5-MeBza, or 5-OMeBza (and to a lesser extent, Bza) to Cbi. This activity is likely enabled by the combined functions of several enzymes present in all sequenced *D. mccartyi* genomes that are predicted to perform each of the steps necessary for the conversion of Cbi to a cobamide (Fig. 2). Our finding that the addition of Cbi plus 5-OHBza does not support TCE dechlorination or growth indicates that *D. mccartyi* lacks the ability to transform 5-OHBza into DMB, an activity that has been described in *Eubacterium limosum* (83). The production of different levels of each corrinoid when four benzimidazole bases were supplied together suggests that preferential attachment of specific lower ligands occurs even within a single structural class of lower ligands. Selectivity in the activation or attachment of particular lower ligands could play an important role in ensuring that functional cobamides are produced when multiple potential lower ligands are present in the environment. We are currently examining the lower ligand attachment specificity of the CobT enzyme of strain 195 *in vitro* to determine whether substrate specificity in CobT influences the attachment efficiency of different benzimidazoles.

The potential of *D. mccartyi* to make use of a variety of corrinoids is demonstrated by its ability to convert nonfunctional cobamides to cobalamin when DMB is also available. Interestingly, we found that strain 195 also remodels two functional corrinoids, cobalamin and [5-MeBza]Cba. This suggests that all corrinoids imported by strain 195 are subject to modification regardless of their ability to function as cofactors. This is in contrast to the remodeling activity observed in *R. sphaeroides* (112, 113), in which the CbiZ enzyme does not act on cobalamin. Remodeling of cobalamin has also been observed in the human gut, presumably by the action of members of the gut microbiota. A recent study shows that dietary supplementation with cobalamin results in an increase in the concentration of Cbi and five other cobamides, suggesting that cobalamin is converted to a variety of other corrinoids (87).

The ability of *D. mccartyi* to salvage and remodel corrinoids only when free DMB is provided underscores the importance of the availability of functional lower ligands. While much previous attention has been given to organisms that synthesize corrinoids, the identification of organisms that synthesize free lower ligand bases now appears to be of equal importance. Identification of the genes involved in the anaerobic biosynthesis of benzimidazoles would broaden our understanding of the organisms that contribute to lower ligand production in microbial communities.

The corrinoid salvaging and remodeling abilities of *D. mccartyi* demonstrate how a microbe can rely on corrinoid-dependent metabolism without the need to produce a large cofactor *de novo*. Bioinformatic analyses have shown that many other microbes lack the complete corrinoid biosynthetic pathway though they possess corrinoid-dependent enzymes. Strategies used by *D. mccartyi* to acquire functional corrinoids may be common in microbial communities. Furthermore, this analysis of corrinoid acquisition by *D. mccartyi* brings us closer to

understanding the means by which microbes obtain nutrients from other organisms in environmental communities.

MATERIALS AND METHODS

Identification of corrinoid salvaging and remodeling genes in *D. mccartyi* strains.

The amino acid sequences corresponding to experimentally characterized genes involved in corrinoid salvaging and remodeling pathways in three microorganisms, *Salmonella enterica* serovar Typhimurium, *Escherichia coli* K12, and *Methanocaldococcus jannaschii,* were compared to sequences in each of the *D. mccartyi* genomes using reciprocal BLAST analysis (Table S2). In the first step, each characterized protein sequence was queried against each *D. mccartyi* genome to identify the highest scoring orthologs using BLASTP (128). In the second step, reciprocal BLASTP searches were conducted using *D. mccartyi* ortholog sequences to query against the genome containing the characterized protein of interest. An Expect (E) value of $<10^{-17}$, query coverage of at least 48%, and a minimum of 24% sequence identity were used as stringency thresholds for determining a valid best hit.

Extraction and purification of cobamides.

[Bza]Cba, [5-MeBza]Cba, [2-SMeAde]Cba, and [Cre]Cba (see Fig. 1 for abbreviation descriptions) were extracted from bacterial cultures and purified as described previously (87). [Ade]Cba was extracted from 6 L of *S. enterica* serovar Typhimurium strain LT2 grown aerobically for 48 h at 37 °C in NCE medium containing 10 mM glycerol, 80 mM 1,2-propanediol and 1 μM Cbi, as described previously (112). [5-OHBza]Cba was extracted from 4 L of *Methanosarcina barkeri* strain Fusaro grown anaerobically for 14 days at 34 °C in defined mineral salt medium containing a N_2/CO_2 headspace, 0.5% (v/v) methanol and a modified Wolin vitamin solution without cobalamin (89). Cells were harvested by centrifugation (8,000×*g*, 4 °C, 20 min). Cell pellets were resuspended in 20 ml of methanol with 20 mg KCN per gram (wet weight) of cells and incubated at 60 °C for 1.5 h with vortex mixing every 20 min. Cellular debris was removed by centrifugation at 40,000×*g* for 1 h, and the supernatant was dried in a rotary evaporator. Samples were resuspended in 10-20 ml deionized water, desalted with a C18 Sep-Pak cartridge (Waters Associates, Milford, MA), and eluted in 2 ml methanol. The eluates were dried overnight in a vacuum desiccator, resuspended in deionized water, and stored at -80 °C.

HPLC purification was performed with an Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector. Samples were injected onto an Agilent Eclipse plus C18 column (5 μm, 9.4 x 250 mm) at 45 °C, with a flow rate of 1.8 ml min^{-1}. Samples were separated using a solvent system consisting of A, 0.1% formic acid in water and B, 0.1% formic acid in methanol with a linear gradient of 10 to 40% solvent B over 17 min. Fractions were collected using an Agilent 1200 series fraction collector and dried under vacuum. The concentrations of cobamides were measured spectrophotometrically at 367.5 nm in deionized water. A molar extinction coefficient of 30,800 mol^{-1} cm^{-1} was used for quantification (87). The identity of each corrinoid was confirmed by mass spectrometry.

D. mccartyi strain 195 culture conditions.

D. mccartyi strain 195 (100) was grown anaerobically in a defined mineral salt medium containing 7 µl TCE and 2 mM sodium acetate as the electron acceptor and carbon source, respectively (129). Hydrogen was provided as the electron donor in a headspace of H_2/CO_2 (80:20, vol:vol). The medium was amended with a modified Wolin vitamin solution excluding cobalamin. When indicated, 36.7 nM of cobalamin, an alternate purified corrinoid, Cbi, and/or the lower ligand bases Cre, 5-OHBza, Bza, 5-OMeBza, 5-MeBza, or DMB were added to the culture (89). The cultures amended with different corrinoids were incubated for 7-14 days, until the TCE was depleted from the culture provided with cobalamin.

Extraction and analysis of corrinoids from _D. mccartyi_ 195.

Three to six 100-ml cultures of strain 195 were harvested by centrifugation at 15,000×g, 4 °C for 15 min after 18 to 21 days of growth, when 95-98% of two sequential doses of 7 µl TCE were depleted. The cell pellets were then resuspended in methanol and stored at -20 °C until extraction. The culture supernatants were applied to a C18 Sep-Pak cartridge (Waters Associates, Milford, MA), washed with 50 ml water and eluted with 3 ml methanol. Corrinoid extractions from cell pellets and supernatants were performed as described above. Corrinoid concentrations reported represent the amount present in the cell pellet and supernatant combined.

Analytical methods.

Chloroethenes and ethene in the culture headspace were measured by gas chromatography (Agilent 7890A, Agilent technologies, Palo Alto, CA) with a 30-m J&W capillary column and a flame ionization detector as described previously (89, 129). Cl^- released was calculated from metabolic dechlorination from TCE to vinyl chloride (VC) as described (130). Cell number in the cultures was determined by quantitative real-time PCR with primers specific to the *D. mccartyi* strain 195 *tceA* gene, using a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA) as previously described (131). Dechlorination and cell yield are reported as the percentage of the average level observed in cultures containing cobalamin.

Corrinoid extracts from strain 195 were analyzed with an Agilent Technologies 6410 liquid chromatograph-triple quadrupole mass spectrometer (88). Briefly, samples were injected onto a ZORBAX Eclipse Plus C18 column (1.8 µm, 50 x 3.0 mm) (Agilent Technologies, Santa Clara, CA) using an Agilent 1200 series autosampler. The flow rate was 0.5 ml min^{-1} and elution solvents consisted of 0.1% formic acid in water (A) and methanol (B). Samples were eluted with a solvent program that was started at 18% B, increased to 21% B in a linear gradient over 3 min, and remained at 21% B for 2 min. The tandem triple quadrupole mass spectrometry was set for multiple reaction monitoring for quantification of each corrinoid except [Cre]Cba in positive electrospray ionization mode with the fragmentor voltage set to 135 V and the collision energy set to 45 V. The limit of quantification for this method was 5 nM. [Cre]Cba was quantified using selective ion mode (SIM) with a limit of quantification of 0.2 µM.

Chapter 3

Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities

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Abstract
Cobalamin and other corrinoids are essential cofactors for many organisms. The majority of microbes with corrinoid-dependent enzymes do not produce corrinoids *de novo*, and instead must acquire corrinoids produced by other organisms in their environment. However, the profile of corrinoids produced in corrinoid-dependent microbial communities, as well as the exchange and modification of corrinoids among community members have not been well studied. In this study, we applied a newly developed LC/MS/MS–based corrinoid detection method to examine relationships between corrinoids, their lower ligand bases, and specific microbial groups in microbial communities containing *Dehalococcoides mccartyi* (Dhc), which has an obligate requirement for benzimidazolyl corrinoids for trichloroethene-respiration. We found that *p*-cresolylcobamide ([Cre]Cba) and cobalamin were the most abundant corrinoids in these communities. In addition, we detected free 5, 6-dimethylbenzimidazole (DMB, the lower ligand of cobalamin) in the supernatants of these communities. Growth of Dhc was correlated with a decrease in supernatant-associated [Cre]Cba and increase of biomass-associated cobalamin. These findings support the hypothesis that Dhc is capable of fulfilling its corrinoid requirements in a community through corrinoid remodeling, in this case, by importing extracellular [Cre]Cba and DMB to produce cobalamin as a cofactor for dechlorination. These findings also provide novel insights into roles played by different phylogenetic groups in corrinoid production and corrinoid cross-feeding within microbial communities and may also have implications for optimizing chlorinated solvent bioremediation.

List of Acronyms
Dhc: *Dehalococcoides mccartyi*
Dhc195: *Dehalococcoides mccartyi* strain 195
PCE: tetrachloroethene
TCE: trichloroethene
Cby: cobyric acid
Cbi: cobinamide
Cba: cobamide
DMB: 5,6-dimethylbenzimidazole
OTU: operational taxonomic unit
PDS: summation of OTUs related to *Pelosinus, Dendrosporobacter*, and *Sporotalea*

Introduction

Corrinoids, which include cobalamin (vitamin B_{12}) and other structurally related compounds (Fig. 1), are a family of cofactors that function in three classes of enzymes: isomerases, methyltransferases, and reductive dehalogenases (RDases) (132, 133). Although corrinoids are synthesized solely by a subset of bacteria and archaea, they function as cofactors for a variety of organisms including many eukaryotes (134, 135). At least sixteen corrinoids with structural variability in the lower axial ligand have been identified, and can be classified into three groups defined by the structure of the lower ligand: benzimidazole, purine, or phenolic cobamides (Fig. 1) (136-138). Previous studies have shown that even corrinoids within one lower ligand class may not necessarily be functionally equivalent as cofactors (139). Therefore, organisms that rely on corrinoids produced by other members of their community must have mechanisms to obtain corrinoids with the appropriate lower ligand. Corrinoid remodeling, in which an organism removes the lower ligand of an imported corrinoid and replaces it with its functional lower ligand, has been demonstrated in a number of microorganisms including *Dehalococcoides mccartyi* (all strains belonging to *D. mccartyi* are denoted "Dhc" in this study) (112, 113, 139-141).

Dhc strains are the only known bacteria capable of complete dechlorination of the common groundwater contaminants tetrachloroethene (PCE), and trichloroethene (TCE) to the innocuous end product ethene (103, 142, 143). Corrinoids are essential cofactors for reductive dehalogenases (RDases), the enzymes that catalyze organohalide respiration in many different microorganisms, including Dhc strains (96, 143-146). However, genomic analyses of the sequenced Dhc strains indicate that they are unable to produce corrinoids *de novo*, and therefore exogenous cobalamin is regularly added to Dhc isolates and Dhc-containing communities to enhance dechlorination performance (103, 107, 142, 147, 148). We recently showed that [5-MeBza]Cba and [5-OMeBza]Cba also support growth and dechlorination in Dhc strain 195 (Dhc195) (139). However, other corrinoids do not function as cofactors for Dhc195, indicating a strict requirement for specific benzimidazolyl cobamides.

Dhc coexists with corrinoid-producing organisms such as acetogens, methanogens, and sulfate-reducing bacteria in dechlorinating communities (137, 138, 149, 150), and is likely to encounter a variety of different corrinoids in its environment. Though none of the sequenced Dhc strains possesses the complete corrinoid biosynthesis pathway, genes encoding corrinoid salvaging and remodeling pathways have been identified in each strain (139). The remodeling of seven nonfunctional corrinoids ([Ade]Cba, [2-SMeAde]Cba, [5-OHBza]Cba, [Bza]Cba, [5-MeBza]Cba, [Cre]Cba and Cbi) in the presence of exogenously supplied DMB, has been observed in a number of Dhc strains in pure culture or defined consortia (139, 141). Dechlorination occurs at similar levels in enrichment cultures containing Dhc with or without cobalamin added (151), suggesting that in it's natural environment, other bacteria provide Dhc with corrinoids that can either be used directly or remodeled by Dhc to a functional form. However, comprehensive profiles of corrinoids in dechlorinating microbial communities have not been reported and it is unknown whether corrinoid remodeling occurs in these communities. Additionally, little is known about the generation and availability of DMB in anaerobic communities, although the availability of DMB is crucial for corrinoid remodeling by Dhc according to previous studies on pure cultures and defined consortia (139, 141, 152).

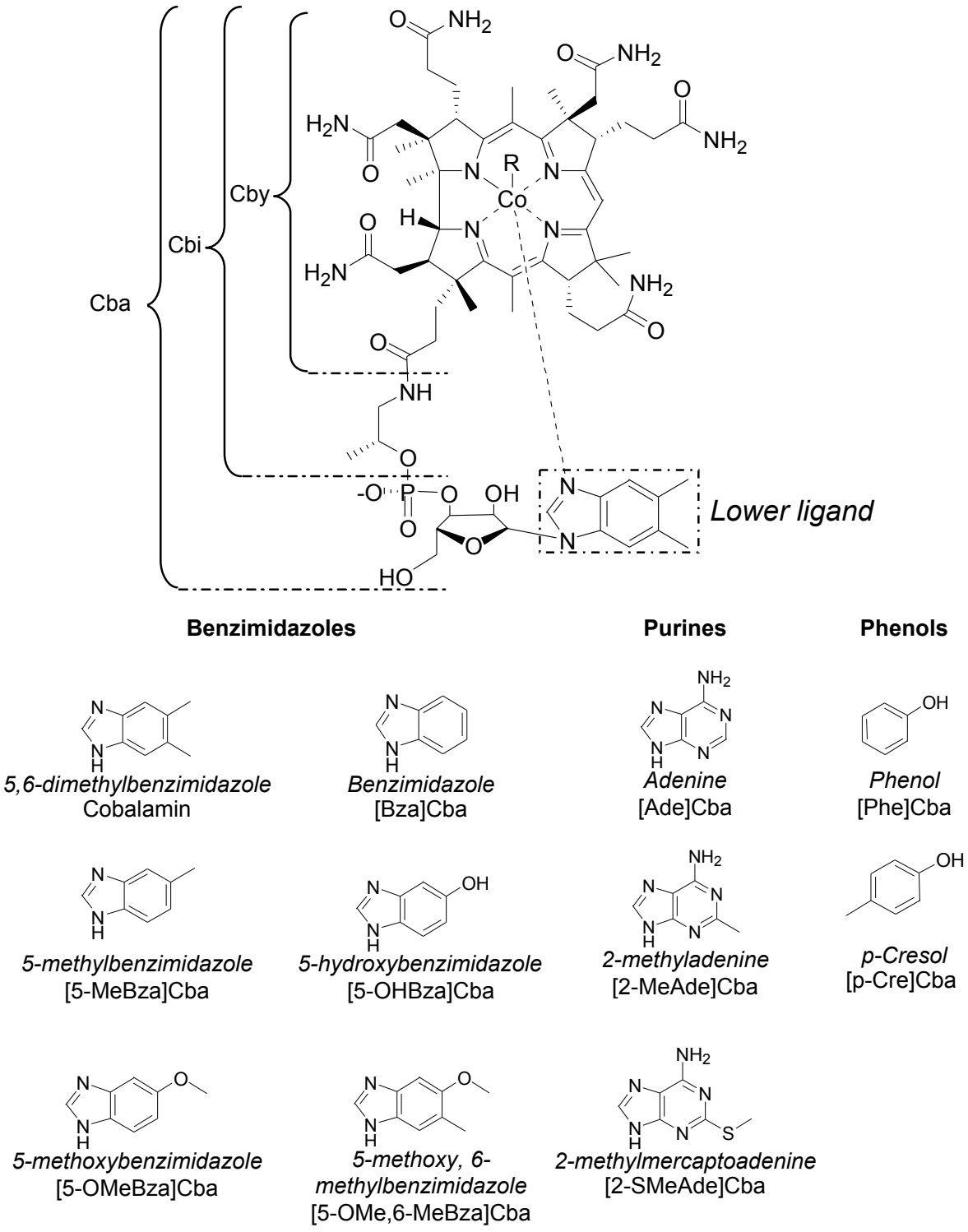


Fig. 1. Structures of corrinoids and lower ligand bases together with abbreviated designation. The names of the lower ligands are italicized and the abbreviation of each cobamide is given below. Cby: cobyric acid; Cbi: cobinamide; Cba: cobamide.

Limitations of analytical techniques for differentiating and quantifying corrinoids impede our understanding of corrinoid content and functions associated with specific corrinoids in microbial communities. Previous studies have largely relied on bioassays to analyze corrinoid content, although these do not allow identification of specific corrinoids or the detection of corrinoid forms that cannot be utilized by chosen bioassay strains (153, 154). Indeed, the range of corrinoids that bioassays can detect is unknown. Moreover, no methods have been reported for detecting free benzimidazole lower ligands present in complex mixtures. In this study, we established a liquid chromatography tandem mass spectrometry (LC/MS/MS) method that is able to not only differentiate various corrinoids and benzimidazole lower ligands, but also to quantify them at low levels.

We successfully applied the LC/MS/MS method to examine thirteen corrinoids and three benzimidazoles in two sets of dechlorinating enrichments derived from geographically different inocula (Table 1). One set of enrichments was inoculated with ANAS, a long-term TCE dechlorinating enrichment initially derived from contaminated soil in California (155), grown with and without added cobalamin (SANASB12 and SANAS). The other set of enrichments was inoculated with microbial cells collected from contaminated groundwater from a field site in New Jersey (NJ enrichments), and grown under four different conditions exploring two parameters: low and high TCE amendment (resulting in uninhibited and inhibited methanogenic activity, respectively) and with and without cobalamin amendment (LoTCEB12 and HiTCEB12, LoTCE and HiTCE) (151).

In this study, we examine the contributions of specific microbial groups to corrinoid production and modification by analyzing corrinoid profiles in the two sets of enrichments as well as in cultures exposed to growth perturbations. This represents the first comprehensive analysis of corrinoid and lower ligand profiles in dechlorinating microbial communities, and allows us to better understand the roles played by different microbial groups in corrinoid production, modification by corrinoid scavengers, and the ecological interactions associated with corrinoid producers and corrinoid auxotrophs.

Results

Development and validation of an analytical method for the detection of corrinoids and free benzimidazoles.

In order to measure the composition of corrinoids in microbial communities, we developed a new LC/MS/MS-based analytical method for quantification of thirteen different corrinoids. Combined with the 1000× concentration by solid phase extraction prior to LC/MS/MS, the overall limit of detection was 200 pM for phenolic corrinoids, and 1-2 pM for all other corrinoids. The overall limit of quantification was 1 nM for phenolic corrinoids, and 2-5 pM for all other corrinoids. This method was validated by a direct comparison with a previously described LC/MS analytical method (136). We found concentrations of detectable corrinoids to be comparable using the two methods, with the exception of Cbi, for which the LC/MS/MS method yielded a lower limit of detection. The LC/MS/MS method was also able to measure three benzimidazoles: DMB, 5-MeBza, and 5-OMeBza with an overall limit of detection of 1 pM and an overall limit of quantification of 2 pM. Subsequent experiments in this study relied solely on the newly established LC/MS/MS method.

Corrinoid and benzimidazole profiles in TCE-dechlorinating enrichments.

We applied the LC/MS/MS method to the two sets of enrichments that reductively dechlorinate TCE. The enrichment conditions, a description of subculture feeding regimes, as well as Dhc growth are listed in Table 1. The ANAS subcultures contained bacterial species related most closely to Dhc, *Clostridium* sp., *Eubacterium* sp., *Bacteroides* sp., *Citrobacter* sp., *Spirochaeta* sp., and δ-proteobacteria (155). In the NJ enrichments, besides Dhc, 7 other Bacterial operational taxonomic units (OTUs) have been identified, which, according to the closest genus, were designated in Genbank as *Pelosinus*_GW, *Dendrosporobacter*_GW, *Sporotalea*_GW, *Desulfovibrio*_GW, *Clostridium*_GW, *Spirochaetes*_GW, and *Bacteroides*_GW (151), "GW" (short for groundwater) is used in order to differentiate the OTU name from the genus name.

Corrinoids were quantified in each of the enrichments after the added TCE was degraded (13-14 days for NJ enrichments and 18 days for SANAS and SANASB12), or in the case of the NoTCE enrichment, after 13 days. The major corrinoids detected in SANAS were cobalamin, [2-MeAde]Cba, [5-OHBza]Cba, and [Cre]Cba, though smaller amounts of [5-MeBza]Cba, [Ade]Cba, [2-SMeAde]Cba, and Cbi were also present (Fig. 2A). Of the corrinoids in SANAS, only cobalamin was present at concentrations above 0.74 nM (c.a. 1 μg/L), the reported minimum requirement for growth of Dhc195 (89). In SANASB12, 80% of the added cobalamin was detected. With the exception of [2-SMeAde]Cba, corrinoids detected in SANAS were also present in SANASB12 (Fig. 2B). The level of [2-MeAde]Cba decreased, and Cbi increased in SANASB12 compared to SANAS. Similar levels of DMB and 5-MeBza lower ligand bases were detected in SANAS and SANASB12, consistent with the detection of both cobalamin and [5-MeBza]Cba (Fig. 2).

The corrinoid profiles of the NJ enrichments differed from SANAS and SANASB12. In the NJ enrichments without exogenous cobalamin (LoTCE and HiTCE), [Cre]Cba was the most abundant corrinoid, followed by cobalamin (Fig. 2A). Cobalamin was detected at levels above 0.74 nM in LoTCE and HiTCE (3.2 nM and 2.2 nM, respectively), Similar to SANAS (Fig. 2A), indicating that the amount of cobalamin present was sufficient for the growth of Dhc. Free DMB was detected at levels similar to those detected in SANAS (Fig. 2A). Interestingly, greater [Cre]Cba concentrations were detected in LoTCEB12 and HiTCEB12 (enrichments with exogenous cobalamin) than in LoTCE and HiTCE. [2-MeAde]Cba, [Ade]Cba, and Cbi were also detected in the four NJ enrichments, but at levels lower than 0.74 nM (Table S3). Neither [5-MeBza]Cba nor its associated lower ligand 5-MeBza was detected in the NJ enrichments (Fig. 2). Notably, [5-OHBza]Cba was only present in methanogenic NJ enrichments, LoTCE and LoTCEB12 (Fig. 2).

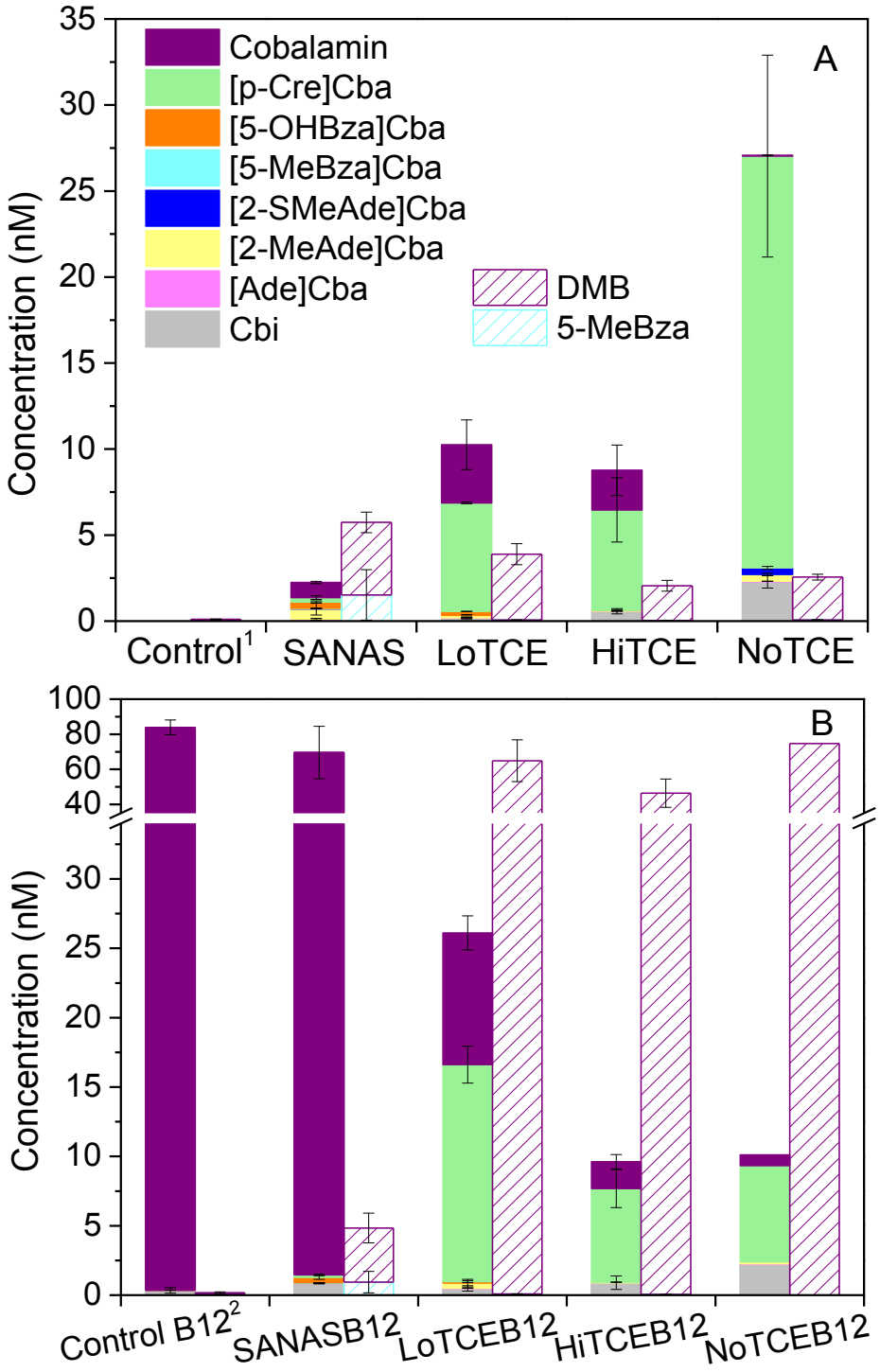


Fig. 2. Corrinoid and benzimidazole lower ligand concentrations in B_{12}-unamended (A) and B_{12}-amended (B) enrichments at the end of 13-18 days' subculturing cycle, error bars represent standard deviation, n=3 ([1]Abiotic control without exogenous vitamin B_{12}; [2]Abiotic control with 74 nM (c.a.100 µg/L) vitamin B_{12}; Note: (A) and (B) share the same legend, but different y axis scales. Most of the cobalamin in SANASB12 was detected in the supernatant, while most corrinoids were detected in the cell pellets of the other enrichments. Lower ligands were mostly detected in the supernatant).

The fate of exogenously added cobalamin was substantially different in SANASB12 versus the NJ enrichments (LoTCEB12 and HiTCEB12). In the SANASB12, 68 nM out of 83 nM added cobalamin was detected (Fig. 2B), and the majority (80%) was in the culture supernatant; whereas 90% of the other corrinoids were detected in the SANASB12 cell pellet (Table S3). In contrast, in LoTCEB12 and HiTCEB12, only 9.45 nM and 1.95 nM out of 83 nM added cobalamin was detected, respectively, most of which was in the cell pellet (Table S3). One possible explanation for the disappearance of cobalamin in the two NJ enrichments is the remodeling of cobalamin, however, the generation of other corrinoids by corrinoid remodeling was not sufficient to account for the decrease of cobalamin in LoTCEB12 and HiTCEB12. [Cre]Cba increased by 9.3 nM and 1.3 nM in LoTCEB12 and HiTCEB12, compared with LoTCE and HiTCE, respectively, but no increase was observed for the other corrinoids targeted in this study. The decrease in cobalamin was accompanied by the apparent liberation of DMB, which is reflected by the profiles of free DMB. In contrast to SANASB12, where only 3.7 nM DMB was detected in the culture supernatant, 45-64 nM DMB was found in the supernatant of the two B_{12}-amended NJ enrichments (Fig. 2B), suggesting that DMB was cleaved from the amended cobalamin by bacteria present in the NJ enrichment that are different from those in SANASB12.

In order to examine the dynamics of corrinoid production and modification, temporal changes of the corrinoids and lower ligand bases in the four NJ enrichments were monitored over a 13-15 day feeding cycle. Concentrations of [Cre]Cba and cobalamin, the most abundant corrinoids in these cultures, as well as DMB are shown in Fig. 3. In all cultures, [Cre]Cba accumulated to near maximum levels during the first 2-3 days of incubation. Subsequently, [Cre]Cba levels declined substantially in cultures without cobalamin added (LoTCE and HiTCE) (Fig. 3A & C), while those in the B_{12}-amended cultures (LoTCEB12 and HiTCEB12) exhibited little change (Fig. 3B & D). Cobalamin in LoTCE and HiTCE cultures increased from 0 to 1-2 nM during the first two days, and reached the highest levels at day 11 and day 3, respectively. Free DMB slowly accumulated in the supernatant, and reached maximum concentrations by day 15 for LoTCE and by day 11 for HiTCE (Fig. 3A & C). In LoTCEB12 and HiTCEB12, added cobalamin was primarily detected in the supernatant during the first 2-4 days of incubation, but subsequently decreased dramatically accompanied by an increase in free DMB in the supernatant (Fig. 3B & D).

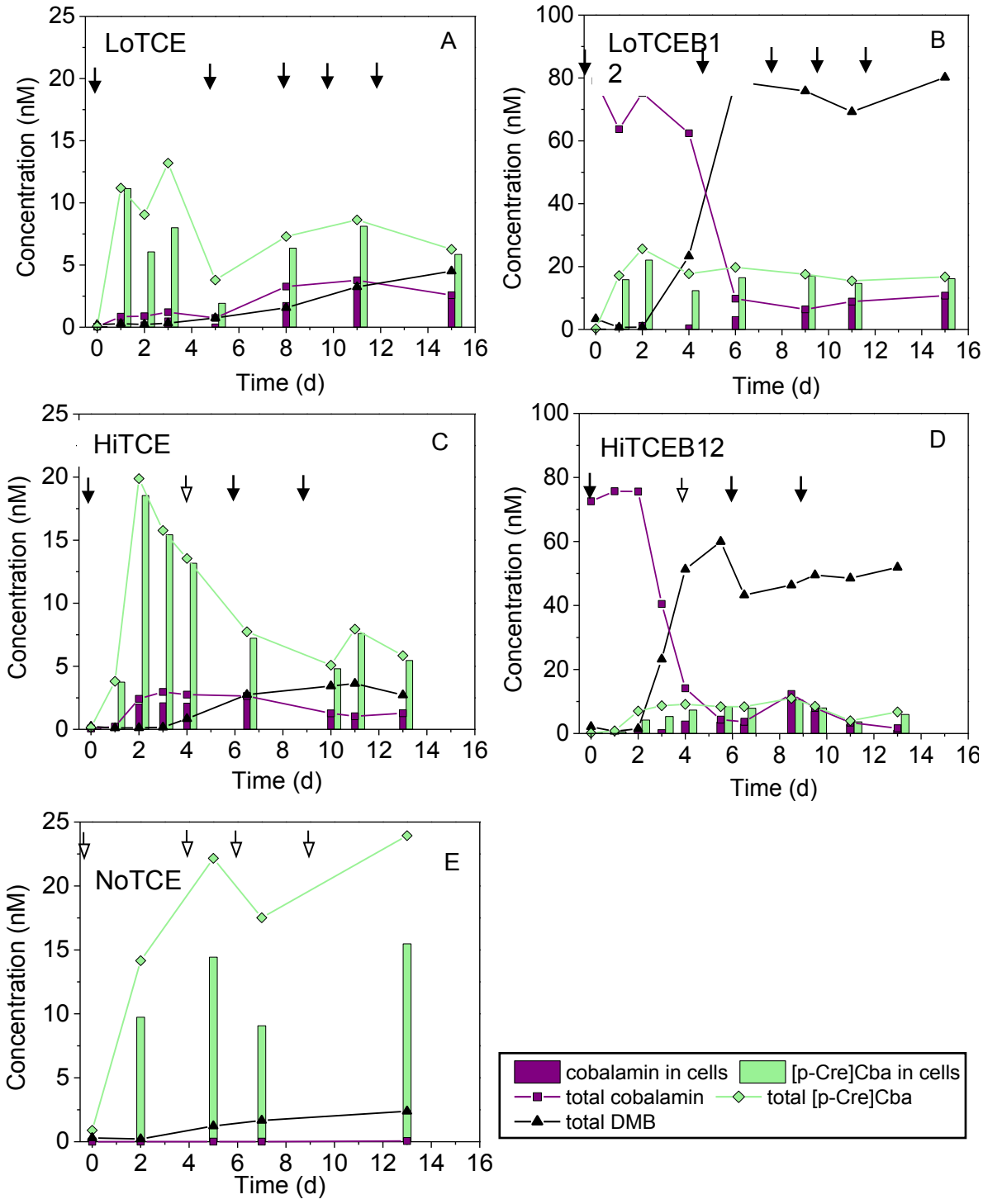


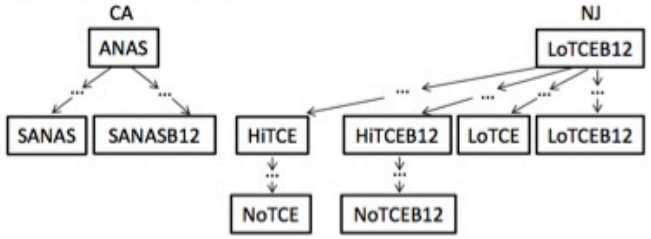
Fig. 3. Temporal changes of [Cre]Cba, Cobalamin, and DMB in groundwater enrichments (A: LoTCE, B: LoTCEB12, C: HiTCE, D: HiTCEB12, E: NoTCE. ↓ indicates amendments of lactate and TCE, ⇩ indicates amendment of lactate only, added amounts are according to Table 1. Note: Y-axis scales in A, C, E are different from those in B & D).

Table 1. Growth conditions of different enrichment cultures

Enrichments[a]	Lactate (mmol)		TCE (μmol)		Vitamin B_{12} (nM)	Dechlorination products	Methane produced	Dhc 16S gene copy number ($\times 10^8$ copies/mL)
	Feeding regimen[b]	Total	Feeding regimen[b]	Total	One-time addition			
SANAS	48	48	22	22	0	Ethene	+	1.3 ± 0.05
SANASB12	48	48	22	22	74	Ethene	+	1.7 ± 0.3
LoTCE	38, 4, 4, 4, 2	52	22, 55, 55, 55, 33	220	0	VC & ethene	+	1.1 ± 0.07
LoTCEB12	38, 4, 4, 4, 2	52	22, 55, 55, 55, 33	220	74	VC & ethene	+	1.0 ± 0.05
HiTCE	38, 4, 5, 5	52	77, 77, 66	220	0	VC & ethene	-	1.1 ± 0.3
HiTCEB12	38, 4, 5, 5	52	77, 77, 66	220	74	VC & ethene	-	1.7 ± 0.2
NoTCE	38, 4, 5, 5	52	0	0	0	None	-	L.D.[c]
NoTCEB12	38, 4, 5, 5	52	0	0	74	None	-	L.D.[c]

[a] The hierarchy of different enrichments is indicated in the following diagram.
[b] Each number represents an individual amendment.
[c] Low detection, < 10^4 copies/mL.

Hierarchy of enrichment cultures:



Note: "→" indicates one subculturing event; "…" indicates more than 3 subculturing events.

The effect of dechlorination metabolism and growth of Dhc on the corrinoid profiles of NJ enrichments.

Since free DMB (a prerequisite for corrinoid remodeling by Dhc195) was detected in the supernatant of the cultures without exogenous cobalamin, we hypothesized that Dhc strains in the NJ enrichments generate cobalamin by remodeling other corrinoids with DMB. To test this hypothesis, we examined whether active dechlorination and Dhc cell growth affected the corrinoid profile of the community. To limit the growth of Dhc, we constructed enrichments NoTCE and NoTCEB12 by subculturing HiTCE and HiTCEB12 cultures without TCE (Table 1).

We analyzed the corrinoid profiles of NoTCE and NoTCEB12 after 6 subculturing events when the growth of Dhc in these two cultures was significantly inhibited ($<10^4$ cells/mL compared to 10^9 total Bacteria). Interestingly, only trace amounts of cobalamin were detected in NoTCE throughout the entire incubation (< 0.1 nM versus 2.3 nM in HiTCE) (Fig. 2A and Fig. 3E), while [Cre]Cba was produced at concentrations as high as 24 nM. These results, together with the observed decrease in [Cre]Cba in the HiTCE culture after day 2 (Fig. 3C) suggest that Dhc may be responsible for remodeling [Cre]Cba to form cobalamin in these cultures. Moreover, about 20-50% of total [Cre]Cba in NoTCE was detected in the supernatant (Fig. 3E), while in HiTCE, almost all of the [Cre]Cba was detected in the cell pellets (Fig. 3C). Despite the trace production of cobalamin, the concentration of free DMB in NoTCE was similar to that found in HiTCE (Fig. 2A), confirming anaerobic production of DMB in these communities, a result corroborated by alternative DMB detection methods (*Sinorhizobium meliloti bluB* bioassays, data not shown). Similar to other B_{12}-amended NJ enrichments, a low concentration of cobalamin was detected in NoTCEB12, while a high concentration of DMB was generated (Fig. 2B). This result, in the absence of actively growing Dhc, indicates that microorganisms other than Dhc were active in modification of the amended cobalamin.

Corrinoid profiles were also determined when Dhc activity was perturbed over the course of two feeding cycles. HiTCE$_{-/+}$ was constructed by subculturing HiTCE into TCE-free medium, incubating for one feeding cycle (13 days) and then re-amending 77 µmol of TCE on day 14 for another feeding cycle (Table 2). HiTCE$_{-/-}$ was HiTCE subcultured without TCE for two feeding cycles (Table 2). As expected, Dhc numbers were about 10 times lower in HiTCE$_{-/-}$ than in HiTCE (Fig. 4B), and in HiTCE$_{-/+}$, they were about double the amount in HiTCE$_{-/-}$, indicating a rebound in Dhc caused by the TCE re-amended during the second feeding cycle. The concentration of cobalamin was 86% lower in HiTCE$_{-/-}$ than in HiTCE (Fig. 4A), while in HiTCE$_{-/+}$, it rebounded to two times the concentration in HiTCE. No substantial difference in other corrinoids or in free DMB was observed between the two perturbed cultures. Dhc and the other seven OTUs previously identified in the NJ enrichments (151) have been quantified by qPCR (Fig. 4B). Interestingly, numbers of the three dominant OTUs, *Pelosinus*_GW, *Dendrosporobacter*_GW and *Sporotalea*_GW (collectively designated "PDS") exhibited a 5-fold increase in the two perturbed cultures compared to HiTCE (Fig. 4B), accompanied by an increase in [Cre]Cba concentrations (Fig. 4A).

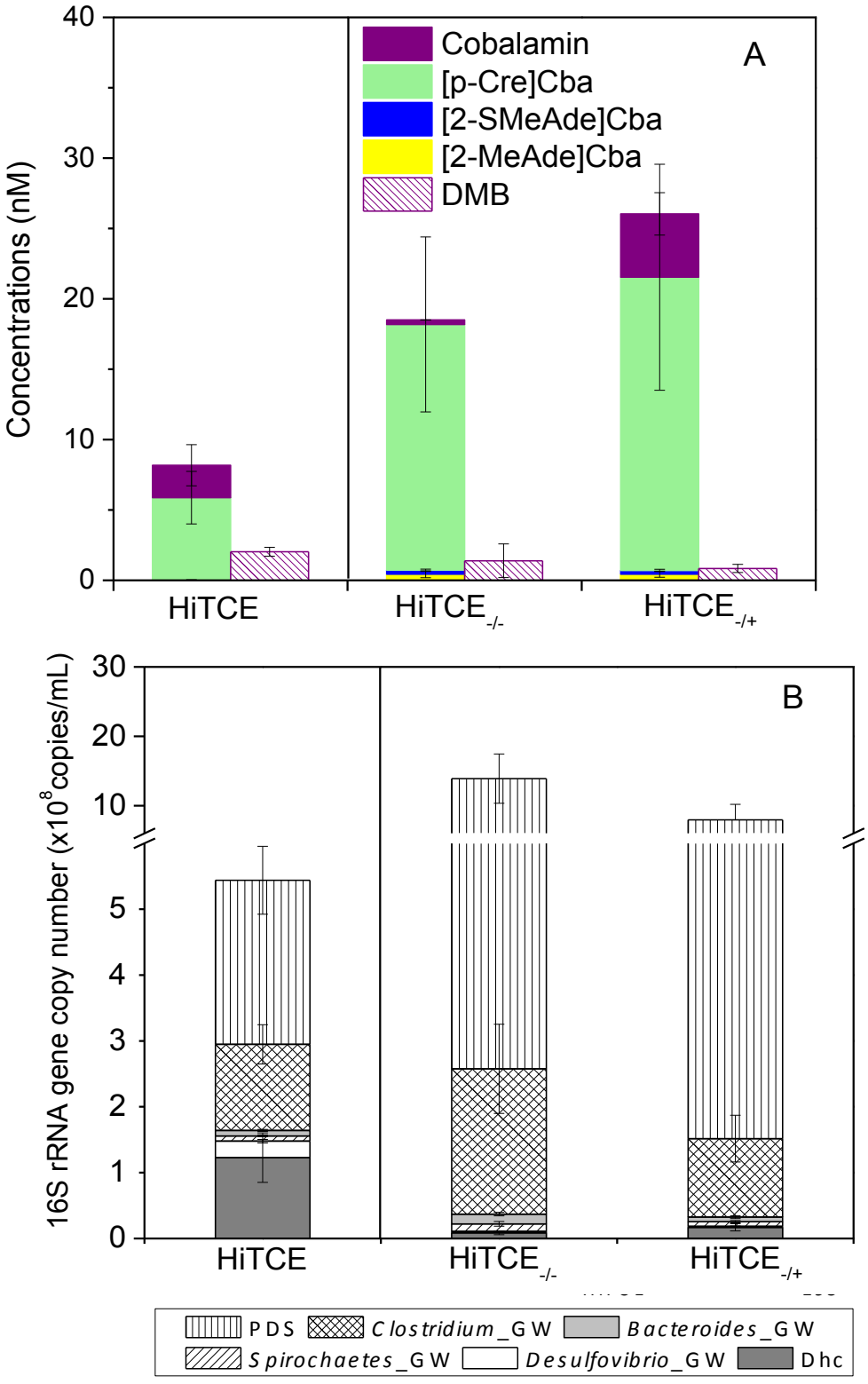


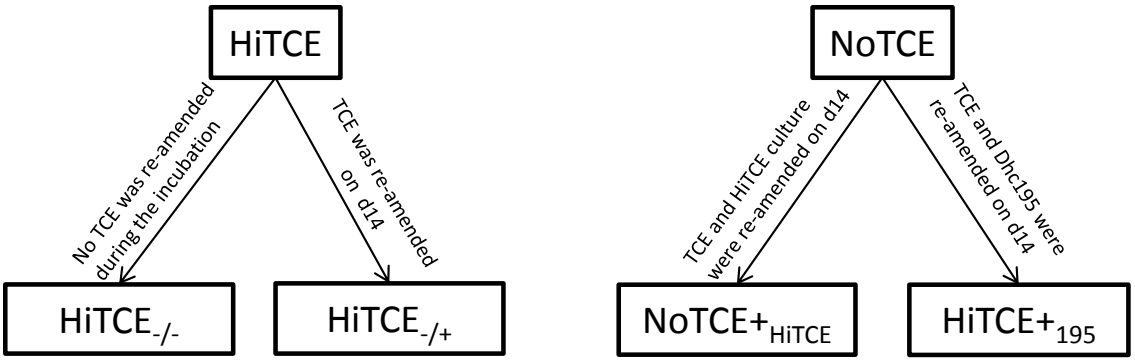
Fig. 4. Comparison of corrinoid and lower ligand production (A) and 16S rRNA gene copy numbers of the OTUs (B) between HiTCE$_{-/-}$ and HiTCE$_{-/+}$, HiTCE is shown as reference. Error bars represents standard deviation, n=3. (PDS represents the summation of *Pelosinus*_GW, *Dendrosporobacter*_GW and *Sporotalea*_GW).

When we attempted to evaluate whether the production of cobalamin would be recovered when TCE was re-amended to the NoTCE culture, no dechlorination occurred even after a prolonged incubation (60 days), likely due to the extremely low Dhc numbers resulting from multiple sub-culturing events. Therefore, NoTCE was subsequently bioaugmented with HiTCE, the original inoculation culture for NoTCE, to construct NoTCE+$_{HiTCE}$ (Table 2). NoTCE+$_{HiTCE}$ dechlorinated TCE to VC and ethene after 14 days, together with a 32-fold increase in the cobalamin concentration and a 10^3-time increase in the Dhc cell number. To determine whether these effects could be specifically attributed to Dhc, NoTCE was also bioaugmented with Dhc195 isolate (1%, v/v) to construct NoTCE+$_{195}$ (Table 2), in which substantial increases were observed in the cobalamin concentration (92-fold) and the Dhc cell number (10^3-time), similar to NoTCE+$_{HiTCE}$ (Fig. 5). Interestingly, increases in *Desulfovibrio* were also observed in both bioaugmented cultures (20-fold in NoTCE+$_{HiTCE}$ and 3-fold in NoTCE$_{+195}$) compared with NoTCE (Fig. 5B).

Table 2. Growth conditions of perturbed cultures

Perturbation over one feeding cycle	Lactate (mmol)		TCE added on 14th day of incubatoin (μmol)	Dhc strain inoculated with TCE addition	Vitamin B_{12} (μg/L)	Dechlorination products	Methane produced
	Feeding regimen	Total					
HiTCE$_{-/-}$	38, 4, 5, 5	52	0	-	0	VC & ethene	-
HiTCE$_{-/+}$	38, 4, 5, 5	52	7	-	0	VC & ethene	-
NoTCE+$_{HiTCE}$	38, 4, 5, 5	52	4	HiTCE	0	VC & ethene	-
NoTCE+$_{195}$	38, 4, 5, 5	52	7	Strain 195	0	VC & ethene	-

Construction of perturbed cultures：



Note: "→" indicates one subculturing event. All four perturbed cultures were cultivated for 28 days before the corrinoid and cell growth measurements.

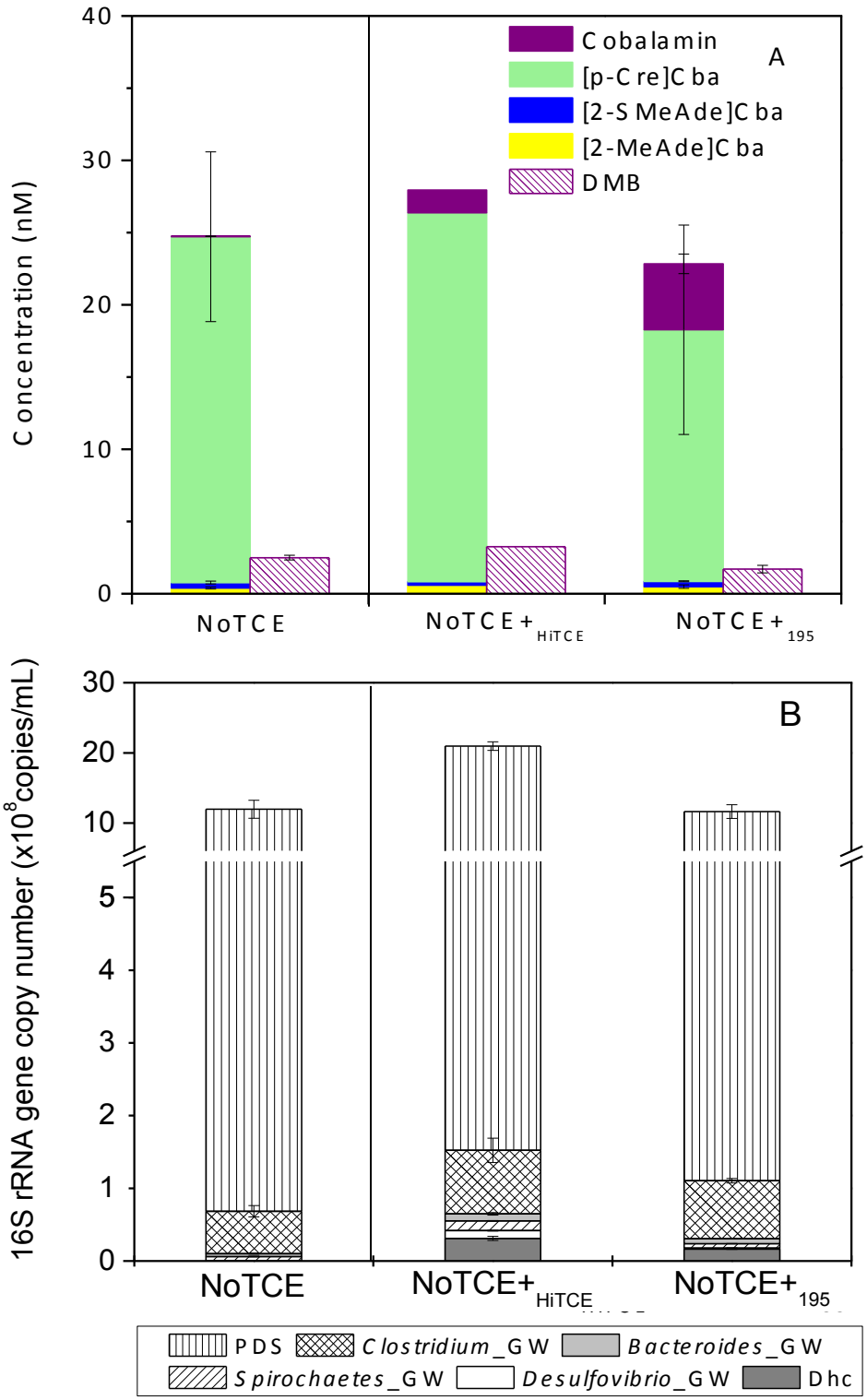


Fig. 5. Comparison of corrinoid and lower ligand production (A) and 16S rRNA gene copy numbers of the OTUs (B) between NoTCE+$_{HiTCE}$, and NoTCE+$_{195}$, NoTCE is shown as reference. Error bars represent standard deviation, n=3. (PDS represents the summation of *Pelosinus*_GW, *Dendrosporobacter*_GW and *Sporotalea*_GW).

Discussion

The exchange of metabolites among members of microbial communities enables the catabolism of complex substrates and supports the growth of auxotrophic microbes. Better understanding of the roles played by nutrient providers and scavengers in communities will help to elucidate ecological relationships among community members. Corrinoids are produced by less than half of the microbes that have corrinoid-dependent enzymes, and as such, the exchange of corrinoid cofactors is crucial for functionally integrated microbial communities (134). Since different corrinoids do not function equivalently as cofactors, some microbes require specific corrinoids for survival. Microorganisms in a community that require specific corrinoids have three options: 1) *de novo* biosynthesis; 2) import of the specific corrinoid generated by other community members; or 3) salvaging and remodeling corrinoids with an appropriate free lower ligand. Previous studies have examined the prevalence of genes involved in corrinoid biosynthesis, import, and modification among sequenced microbes, but there are few known biomarkers indicative of the specific corrinoid an organism produces in communities. The traditional corrinoid bioassays are not capable of distinguishing between different corrinoids (154). By using a crude extraction procedure combined with LC/MS/MS, the new corrinoid detection method developed in this study enables the identification of the specific corrinoids present in a microbial community, which may have important implications for community function. The analysis of corrinoid profiles in microbial communities, such as the corrinoid-dependent dechlorinating enrichments studied here, will contribute to our understanding of the roles played by specific microbial groups in corrinoid production and modification in the environment.

The dechlorinating enrichments evaluated in this study were able to sustain robust continuous growth of Dhc in the absence of cobalamin amendment. However, corrinoid profiles and community structures differed in the enrichments inoculated from different locations (CA or NJ). In CA derived SANAS, cobalamin was the dominant corrinoid detected at concentrations above the minimal requirement of Dhc, while in the NJ enrichments, [Cre]Cba was the most abundant corrinoid. The dominance of different corrinoids is likely due to the difference in the microbial compositions of these two sets of enrichments. The dominance of cobalamin in SANAS is likely attributed to the *de novo* anaerobic cobalamin biosynthesis, which has been shown in *Acetobacterium woodii*, *Eubacterium limosum*, and *Clostridium barkeri* (149, 156), and members of these genera have been identified in ANAS, the original inoculum of SANAS (107, 155). In addition, recent metagenomic analysis of ANAS found that one Dhc strain (ANAS2) in this community possesses a nearly complete corrinoid biosynthesis pathway (157), however the function has not been confirmed in this strain. In contrast, the abundance of [Cre]Cba in NJ enrichments is likely related to the dominance of PDS strains in these enrichments, which were not detected in ANAS subcultures. PDS belong to the family *Veillonellaceae* that includes *Sporomusa ovata*, which until recently was the only organism known to produce [Cre]Cba (149, 158-161). Genomic analysis reveals that all of the genes involved in corrinoid biosynthesis, as well as genes homologous to the *p*-cresol lower ligand activation genes *arsAB* in *Sporomusa ovata* (160) are present in sequenced *Pelosinus* strains (https://www.ncbi.nlm.nih.gov/). This suggests that *Pelosinus* spp. are likely able to biosynthesize [Cre]Cba.

Previous studies have shown that cobalamin, [5-MeBza]Cba, and [5-OMeBza]Cba are the only corrinoids able to support Dhc growth (139). [5-MeBza]Cba, another functional corrinoid for Dhc (139), has been detected in SANAS but not in NJ enrichments. This corrinoid has been previously identified in a variety of sulfate-reducing bacteria, including *Desulfobulbus propionicus* (150). Members of the genus *Desulfobulbus* were detected in ANAS subcultures (155), but not in NJ enrichments (151), suggesting that these microorganisms may be involved in the production of [5-MeBza]Cba in SANAS cultures.

The effect of community structure on corrinoid profiles was also reflected by the detection of [5-OHBza]Cba only in methanogenic communities. This is consistent with previous studies, which have shown that [5-OHBza]Cba is produced by a number of methanogens (162-166). Methanogens have been shown to be able to produce various corrinoids, and the role of methanogens in communities containing Dhc has long been of interest. However, given that the amounts of [5-OHBza]Cba in all enrichments were very low, and that NJ enrichments with inhibited methanogenic activity and no detectable [5-OHBza]Cba also successfully supported Dhc growth and dechlorination, the contribution of methanogens to the corrinoid supply of Dhc is likely relatively small in the communities examined in this study.

According to our previous study, Dhc is the only dechlorinating bacterial species found in the NJ enrichments (151). The correlation between cobalamin production and Dhc growth in B_{12}-unamended NJ enrichments suggests corrinoid salvaging and remodeling carried out by Dhc. Genes encoding enzymes involved in the corrinoid remodeling pathway, including the amidohydrolase CbiZ and the adenosylcobinamide-phosphate guanylyltransferase CobU (140), are present in all sequenced Dhc strains (96, 139, 167, 168), as well as the Dhc strains in the NJ enrichments (151). A previous study with Dhc195 revealed that this strain is capable of remodeling added nonfunctional corrinoids including [Cre]Cba into cobalamin in the presence of DMB (139). Results of this study suggest that the Dhc strains in the NJ enrichments employ remodeling to obtain cobalamin: when the growth of Dhc was inhibited, little cobalamin was produced, and the level of [Cre]Cba increased; when the growth of Dhc was restored in perturbed cultures, cobalamin concentrations rebounded. This correlation between cobalamin concentration and Dhc growth supports the hypothesis that Dhc acquires corrinoids and free DMB from the other microbes and remodels them into cobalamin. The dominance of [Cre]Cba in the NJ enrichments indicates that [Cre]Cba, possibly produced by PDS strains, serves as a major substrate for corrinoid remodeling by Dhc. The low level of [Cre]Cba in the supernatant of HiTCE and the increase of [Cre]Cba in the supernatant of NoTCE (Fig. 3C&E) strongly favor the hypothesis that [Cre]Cba is released into the culture supernatant by its producers where it is then salvaged by Dhc for corrinoid remodeling.

A notable finding of this study is the detection of free DMB in community supernatants of all cultures examined. Studies of Dhc isolates (139) and constructed co-cultures (141, 152) indicate the importance of DMB in corrinoid remodeling. This study is the first to detect and quantify DMB in microbial communities and provides evidence for endogenous DMB production (at nM levels) in anaerobic microbial communities enriched from contaminated soil and groundwater. Although the only known physiological role of DMB is as the lower ligand of cobalamin (156, 169), the generation of DMB in the absence of cobalamin in the NoTCE enrichment suggests that DMB is produced independently of cobalamin biosynthesis. Under aerobic conditions, DMB is biosynthesized from flavin mononucleotide (FMN) catalyzed by the enzyme BluB (169-171). However, information on anaerobic DMB production is still very limited. Previous labeling studies showed that the anaerobe *Eubacterium limosum* synthesizes

DMB from substrates such as glutamine, glycine and formate, which are also used in purine-nucleotide biosynthesis (156). However, the enzymes involved in anaerobic DMB biosynthesis have not yet been identified. Due to the importance of DMB in the corrinoid remodeling processes of Dhc and other cobalamin-salvaging anaerobes, further investigations are needed to understand DMB synthesis in anaerobic communities.

In summary, this study sheds light on the correlation between corrinoid production and community structure, the corrinoid salvaging and modification in Dhc-containing communities, as well as ecological relationships between Dhc and other community members. Greater insights into corrinoid production, modification, and utilization in microbial communities will help us better understand how nutrient exchange shapes these communities, and what ecological roles are played by individual community members.

Materials and methods

Cultures and growth conditions. SANASB12 and SANAS are the subcultures of ANAS, a well-maintained TCE-dechlorinating enrichment characterized in previous studies (107, 155, 172). They were constructed by inoculating 5 mL ANAS culture (5%, v/v) into 95 mL basal medium with N_2/CO_2 headspace (90:10, v/v) and 74 nM (c.a. 100 μg/L) vitamin B_{12} (SANASB12) and without the addition of B_{12} (SANAS). The composition of the basal medium was the same as described elsewhere (89), except that a modified Wolin vitamin stock excluding B_{12} was used (173). It contained (per liter) 1 g of NaCl, 0.5 g of $MgCl_2 \cdot 6H_2O$, 0.2 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.3 g of KCl, 0.015 g of $CaCl_2$ $2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 ml of a trace element solution (174), 1 ml of a Na_2SeO_3-Na_2WO_4 solution (175), and 10 mg of resazurin. Cultures were amended with 48 mM lactate as both carbon source and electron donor, and 2 μL TCE (ca. 0.2 mM, final concentration) was supplied as the terminal electron acceptor. Both SANASB12 and SANAS completely dechlorinated TCE to ethene. Experiments were carried out after five 5% (v/v) subculturing events for SANASB12 and three subculturing events for SANAS.

Another four dechlorinating enrichments (LoTCEB12, LoTCE, HiTCEB12 and HiTCE) used in this study were originally inoculated with contaminated groundwater from New Jersey and maintained under conditions listed in Table 1. The high initial TCE concentration (Table 1) in HiTCEB12 and HiTCE cultures resulted in the inhibition of methanogenesis due to the toxicity of TCE to methanogens. These enrichments are capable of dechlorinating TCE to VC and ethene as described elsewhere (151). In order to investigate the effects of the presence of Dhc on corrinoid profiles, two enrichments were subsequently constructed from HiTCE and HiTCEB12 using the same growth condition, but with no TCE added (denoted "NoTCE", "NoTCEB12", respectively) (Table 1). Experiments were carried out after 40 subculturing events for LoTCE, LoTCEB12, HiTCE and HiTCEB12, and 6 subculturing events for NoTCE and NoTCEB12.

Corrinoid biosynthesis and purification. [Ade]Cba and [2-MeAde]Cba were extracted from *Salmonella enterica* serovar Typhimurium strain LT2, and [5-OHBza]Cba was extracted from *Methanosarcina barkeri* strain Fusaro using cyanidation and solid phase extraction as previously described (112, 139). Briefly, cells were collected, resuspended in 20 mL methanol with 20 mg KCN per gram of cells, and incubated at 60 °C for 1.5 hr with periodic mixing. Samples were then dried and resuspended in 20 mL deionized (DI) water, desalted using a C_{18} Sep-Pak

cartridge (Waters Associates, Milford, MA) and eluted in 2 mL methanol. The eluates were dried and redissolved in DI water. [Bza]Cba, [5-MeBza]Cba, [5-OMeBza]Cba, [5-OMe, 6-MeBza]Cba, [2-SMeAdeCba], [Phe]Cba and [Cre]Cba were extracted from bacterial cultures and purified as described previously (136). A molar extinction coefficient of 30,800 M^{-1} cm^{-1} at 367.5 nm was used for quantification (136). The identity of each corrinoid was confirmed by mass spectrometry.

Monocyanocobyric acid standard was prepared as described (176), with the following changes: after evaporation to dryness on a rotary evaporator, the reaction mixture residue was dissolved in 0.1 mM KCN in DI water. 5 mL aliquots were desalted with a C_{18} Sep-Pak cartridge, and eluted in 3 mL methanol. The eluates were dried, resuspended in deionized water, and stored at -80 °C. Monocyanocobyric acid was purified using the solvent gradient as described (176).

Extraction of corrinoid and lower ligand bases. Cell pellets were collected from 200-300 mL cultures by centrifugation at 15,000 × g for 10 min at 4 °C and stored at -80 °C. Supernatants were passed through a 0.2 μm filter and loaded onto a Sep-Pak C_{18} cartridge. The cartridge was then washed with 50 mL DI water, and eluted with 3 mL 100% methanol. The eluate was stored at -80 °C. Methanol extraction, cyanidation and desalting were carried out as described above. The dried extracts were dissolved in 200-300 μL milliQ water. All samples were stored at -20 °C prior to LC/MS/MS analysis.

Analytical methods. Chlorinated ethenes and ethene were measured by an Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector (Agilent, Santa Clara, CA), as described elsewhere (107, 151).

Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was performed using an Agilent 6410 liquid chromatograph-triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Samples were loaded onto an Agilent Eclipse Plus C_{18} column, 1.8 μm, 3.0 × 50 mm (Agilent Technologies, Santa Clara, CA) with temperature maintained at 40 °C. LC was performed at 0.5 mL/min with initial mobile phase conditions of 82% miliQ water with 0.1% formic acid (A) and 18% methanol with 0.1% formic acid (B) held for 3 min, increased to 21% B immediately and held for 2 min, increased to 100% B over 0.1min and held for 1 min, decreased back to 18% B over 0.1 min and held for 3.8 min. The injection volume was 10 μL. The fragmentor voltage was set at 135 V for MS2 scan, and the collision energy was 45 V for product ion scan. Multiple reaction monitoring (MRM) was used to capture the signature transition of each corrinoid and lower ligand for quantitative analysis. Corrinoids lacking a lower ligand base (Cby and Cbi) and the phenolic corrinoids ([Phe]Cba and [Cre]Cba) were identified by their unique product ions. All other corrinoids are qualified and quantified by tracking the transition of the doubly charged molecular ion to two dominant product ions corresponding to the singly charged lower ligand base, and an ion of unknown structure with an m/z of 912 (136). Benzimidazoles were quantified by monitoring the transition from the singly charged molecular ion to unique product ions (Table S1).

Two parallel sets of samples were prepared for the LC/MS/MS method validation. In each set cell pellets and supernatant were separated by centrifugation at 15,000 × g for 15 min at 4 °C. Two fresh media controls with different cyanocobalamin concentrations were prepared in parallel. One set of samples was measured using extraction and detection methods described by

Allen and Stabler (136), while the other set was extracted and analyzed by the method described in this study.

Biological triplicates were performed for GC measurements. For end-point corrinoid and lower ligand detection, measurements were carried out on one subculture, and measurements on three subsequential subcultures were reported as biological triplicates. For temporal analyses, measurements were carried out on one subculture.

Bioassays for DMB detection. Calcofluor analysis of DMB using a *Sinorhizobium meliloti bluB* mutant strain was performed as previously described (169, 171). A modified quantitative bioassay was further performed as described by Croft and Taga (personal communication). Briefly, *S. meliloti bluB* was grown in M9 minimal media supplemented with 1 mg/mL L-methionine, and was inoculated with serially diluted DMB standards or enrichment samples to a total of 200 µL in a 96-well plate. Calcofluor was added for the final 5 hr, and the fluorescent phenotype was measured by excitation at 360 nm and emission at 460 nm.

DNA isolation and quantification by quantitative PCR (qPCR). Genomic DNA was extracted from 1.5 mL culture using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. qPCR was applied using SYBR Green reagent (Applied Biosystems, Foster City, CA) and primer sets targeting 16S rRNA gene sequences of the OTUs of interest as described elsewhere (151).

Chapter 4

Identification of host-associated early corrinoid precursor auxotrophs

Abstract

Tetrapyrroles such as heme, chlorophyll, and corrinoids are essential cofactors. The biosynthetic pathways of this diverse family of molecules all begin with the production of 5-aminolevulinic acid (ALA), and proceed through three additional common precursors before diverging. We sought to identify tetrapyrrole precursor auxotrophs from among bacteria that possess an otherwise complete corrinoid biosynthesis pathway, and to test whether these organisms were capable of scavenging tetrapyrrole precursors from the environment. We identified 39 such auxotrophs based on bioinformatic analysis. Three putative ALA auxotrophs were tested experimentally, and all were capable of scavenging ALA from the growth medium for corrinoid production. Interestingly, all 39 putative tetrapyrrole precursor auxotrophs are animal host-associated, raising the question of whether host-produced tetrapyrrole precursors might be scavenged by corrinoid-producing members of the microbiota.

Introduction

Corrinoids are cofactors for diverse metabolic processes across all three domains of life, but are produced solely by a subset of prokaryotes(9). The corrinoid biosynthesis pathway is one of the longest biosynthetic pathways known, and involves approximately 30 enzymatic steps(9, 42, 77, 177). Genomic analyses estimate that while 76% of bacteria encode corrinoid-dependent enzymes, fewer than half of these encode the complete corrinoid biosynthesis pathway(10, 11, 64). As such, the majority of corrinoid-dependent organisms rely on exogenously produced corrinoids. In addition to scavenging complete corrinoids, some archaea and bacteria are capable of taking up the late corrinoid precursor cobinamide and carrying out the remaining steps necessary to convert it into a complete corrinoid, a process known as cobinamide salvaging(11, 55, 68, 69). This process requires only a small subset of the genes required for *de novo* corrinoid biosynthesis, and some bacteria, such as *Escherichia coli* encode only this portion of the pathway(11, 55). In this study, we provide evidence that early tetrapyrrole precursors can also be scavenged, and fed into the corrinoid biosynthesis pathway. From heme to chlorophyll to corrinoids, tetrapyrroles play diverse and essential roles in metabolism, and have thus earned the nickname the “pigments of life”(40). The early steps in tetrapyrrole biosynthesis are highly conserved, as are the intermediate products (Figure 1). The first universal precursor, 5-aminolevulinic acid (ALA), is produced via one of two unrelated biosynthetic routes. The Shemin or C_4 pathway, utilized by animals, fungi, and α-proteobacteria, involves the condensation of glycine and succinyl-CoA by a single enzyme, 5-aminolevulinic acid synthase (ALAS)(178-181). In contrast, plants, archaea and most bacteria produce ALA in two steps via the C_5-pathway, starting from glutamyl-tRNA(181-187). Glutamyl-tRNA reductase (GluTR, encoded by *hemA*) converts glutamyl-tRNA into glutamate-1-semialdehyde (GSA) which is then converted into ALA by glutamate-1-semialdehyde-2,1-aminomutase (GSA-T, encoded by *hemL*)(186, 188). From ALA, the pathway proceeds through the formation of porphobilinogen (PBG) via porphobilinogen synthase (PBGS, encoded by *hemB*), to hydroxymethylbilane via hydroxymethylbilane synthase (HMBS, encoded by *hemC*), and finally, to the last common tetrapyrrole precursor, uroporphyrinogen III (Uro’III) via uroporphyrinogen III synthase, encoded by *hemD* (188-192). The production of Uro’III marks a branch point in tetrapyrrole biosynthesis –corrinoids, siroheme, and coenzyme F_{430} share one final common precursor -

precorrin-2, while heme and chlorophyll biosynthesis pathways proceed through three additional common precursors before diverging (Figure 1)(40, 182, 193, 194). Tetrapyrrole precursors have been identified in biological samples such as human urine (ALA and PBG) and feces (Uro'III) and swine manure (ALA), as well as in culture supernatants of a number of different microbes(195-202). Organisms that produce heme or chlorophyll must avoid the accumulation of protoporphyrin IX, a precursor downstream of Uro'III, which can lead to oxidative damage to the cell(203). One method for doing so is the excretion of excess tetrapyrrole precursors(200, 201).

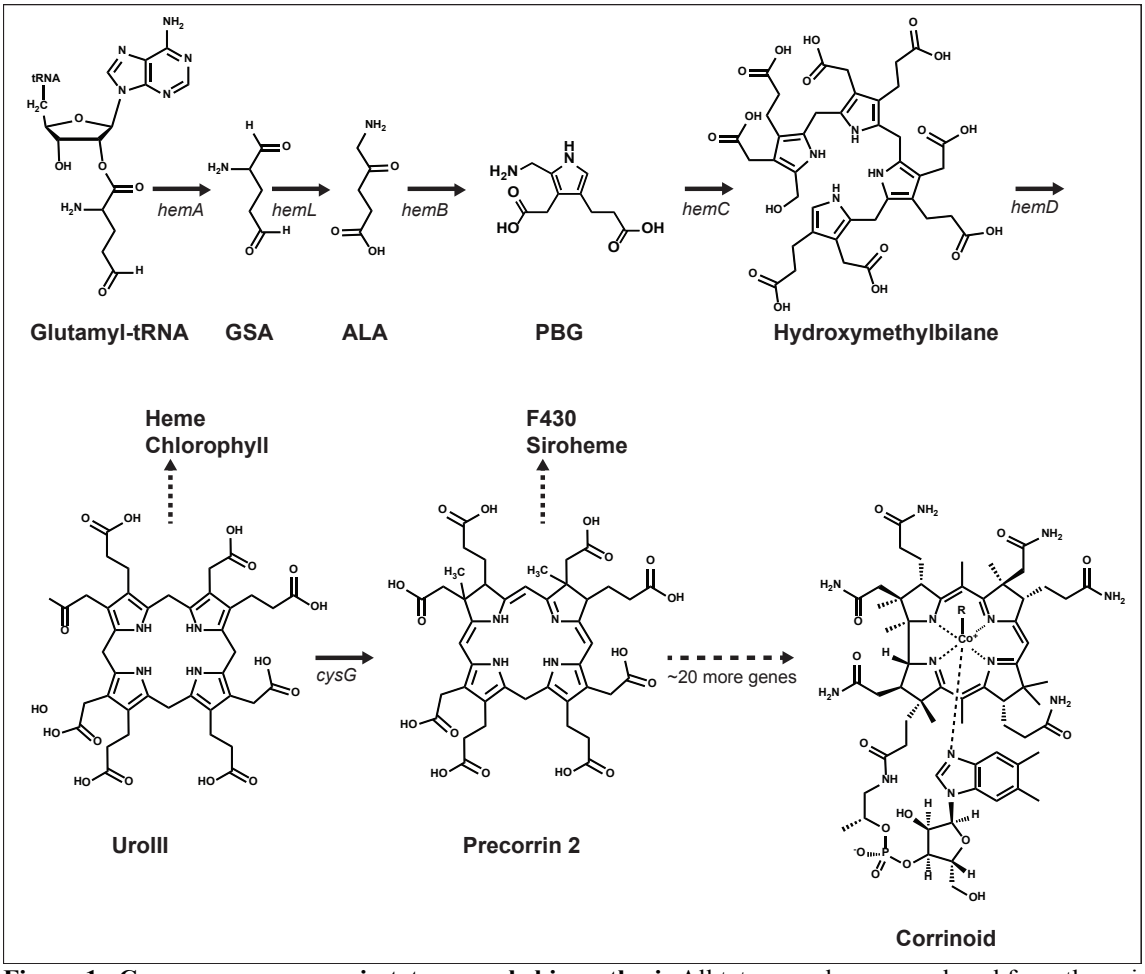


Figure 1. Common precursors in tetrapyrrole biosynthesis All tetrapyrroles are produced from the universal precursor, ALA. Two pathways exist for ALA production; the C_5 pathway from glutamyl tRNA, which is present in plants, Archaea, and most bacteria is shown here. From ALA, the biosynthesis of PBG, hydroxymethylbilane, and Uro'III (the last common tetrapyrrole intermediate) is evolutionarily conserved across all domains. The production of Uro'III marks a branch point in tetrapyrrole biosynthesis.

Given the ubiquity of tetrapyrrole biosynthesis pathways among organisms, the presence of common biosynthetic intermediates, and the metabolic burden of maintaining complete tetrapyrrole biosynthetic pathways, we hypothesized that some microbes may scavenge common intermediates from the environment in order to produce the tetrapyrrole(s) they require. To test this hypothesis, we examined corrinoid biosynthesis pathways encoded in bacterial and archaeal genomes available in the Joint Genome Institute Integrated Microbial Genomes database

(JGI/IMG)(204), identified candidates lacking genes necessary for the production of common tetrapyrrole intermediates but encoding an otherwise complete corrinoid biosynthesis pathway, and tested the ability of three of these microbes to grow and produce corrinoids in the presence and absence of the first universal tetrapyrrole precursor, ALA. We found that several bacteria are auxotrophs for ALA or other tetrapyrrole precursors, suggesting that these compounds may be shared among organisms in the environment.

Results and Discussion

Identification of putative early corrinoid precursor auxotrophs

We hypothesized that if tetrapyrrole precursors are commonly scavenged from the environment for use as intermediates in corrinoid biosynthesis, the pressure to maintain the genes encoding the enzymes involved in their production might be relaxed and these genes may be lost from some genomes. To identify potential tetrapyrrole precursor scavengers, we performed a comparative genomic search for bacterial or archaeal genomes that were missing genes involved in the initial steps of tetrapyrrole production, but retained the rest of the corrinoid biosynthesis pathway. Using the "function profile" tool on JGI's Integrated Microbial Genomes (IMG) webserver (204) to identify genes involved in corrinoid biosynthesis, we analyzed 5,988 archaeal and bacterial genomes and identified 39 bacteria missing genes involved in ALA, PBG, or precorrin-2 production (Fig. 1, Tables 1-3). One interesting feature of the PBG and precorrin-2 auxotrophs is that these organisms are missing not only the gene involved in PBG or precorrin-2 production (*hemB* and *cysG*, respectively) but also all of the other genes involved in the production of upstream precursors (Fig. 1).

No potential tetrapyrrole precursor scavengers were identified among the Archaea, while a diverse group of bacteria including members of six different phyla are represented (Tables 1-3). Surprisingly, all of the potential tetrapyrrole precursor scavengers identified in this study are host-associated, while only 32% of the sequenced bacterial genomes within the IMG database are specifically identified as such(204). The majority of the potential scavengers are associated with the human gastrointestinal tract or oral cavity, though organisms from the bovine rumen and the termite hindgut were also identified (Tables 1-3). While most of these bacteria are considered to be commensals, 12 animal pathogens, including *Clostridium difficile* and *Clostridium botulinum* were also identified (Tables 1-3). One interpretation of this finding is that tetrapyrrole precursors are provided by the host, either through production by host cells, or for gut-associated microbes, as part of the host's diet. Precedence for the cross-feeding of tetrapyrrole precursors from an animal host to its resident microbes has been established for an insect-bacterial mutualism based on bioinformatic analysis. Cross-feeding of Uro'III, a precursor produced by the host for heme biosynthesis, may explain how the cicada endosymbiont *Hodgkinia cicadacola* is able to produce methionine using a corrinoid-dependent methionine synthase despite its incomplete corrinoid biosynthesis pathway, which contains only the genes downstream of Uro'III production(205). Other members of the host microbiota are another potential source of tetrapyrrole precursors. Tetrapyrrole precursors have been detected in supernatants from a variety of different microbes(198-202). A recent study by Kanto et al. found that ALA was present in swine manure, and that levels of ALA increased in the manure over a 28 day period of incubation, which suggests that microbes in this material contribute to its tetrapyrrole precursor content (31). A study comparing two species of *Fibrobacter*, prominent members of the bovine rumen microbiota, found that *hemA* was present in *Fibrobacter succinogenes*, but absent in

Fibrobacter intestinalis(206). *F. intestinalis* was found to require ALA, porphobilinogen, or cobalamin supplementation for growth in pure culture, but not in co-culture with *F. succinogenes*, demonstrating that cross-feeding of one or more of these molecules occurred between these two species when co-cultured(206).

Table 1. Putative ALA auxotrophs

Organism	Phylum	Class	Order	Family	Environment[1]	Pathogen[2]	Ref.
Clostridium botulinum (12 strains)	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	contaminated food products human gut	y	(207-216)
Clostridium sporogenes (3 strains)	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	human gut	n	(217-219)
Clostridium scindens ATCC 35704	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(220)
Lachnospiraceae bacterium 5_1_57FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Johnsonella ignava ATCC 51276	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human oral cavity	n	(222)
Clostridium difficile (8 strains)	Firmicutes	Clostridia	Clostridiales	Peptostrepto-coccaceae	human gut	y	(223-225)
Peptostreptococcus stomatis 17678	Firmicutes	Clostridia	Clostridiales	Peptostrepto -coccaceae	human oral cavity	y	(226)
Jonquetella anthropi E3_33 E1	Synergistetes	Synergistia	Synergistales	Synergistaceae	human oral cavity	y	(227)
Treponema primitia ZAS_2	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	termite gut	n	(228)

Table 2. Putative PBG auxotrophs

Organism	Phylum	Class	Order	Family	Environment[1]	Pathogen[2]	Ref.
Eubacterium infirmum F0142	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	human oral cavity	y	(229)
Ruminococcus gnavus ATCC 29149	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(230)
Ruminococcus torques ATCC 27756	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(231)
Eubacterium rectale (3 strains)	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(232)
Clostridium citroniae WAL-17108	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	y	(233)
Clostridium clostridioforme 2_1_48FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	y	(234)
Clostridium cf. saccharolyticum K10	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(235)
Clostridium sp. D5	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(236)
Clostridium sp M62/1	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(236)
Clostridium symbiosum WAL-14163	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(237)
Dorea formicigenerans (2 strains)	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 1_1_57FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 1_4_56FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 2_1_46FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 2_1_58FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 3_1_46FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)

Lachnospiraceae bacterium 6_1_37FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(236)
Lachnospiraceae bacterium 8_1_57FAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Lachnospiraceae bacterium 9_1_43BFAA	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	human gut	n	(221)
Ruminococcaceae bacterium D16	Firmicutes	Clostridia	Clostridiales	Rumino-coccaceae	bovine rumen	n	(238)
Aerococcus urinae ACS-120-V-Col10a	Firmicutes	Bacilli	Lacto-bacillales	Aerococcaceae	human urogenital tract	y	(239)
Fusobacterium mortiferum ATCC 9817	Fusobacteria	Fuso-bacteria	Fuso-bacteriales	Fusobacteriaceae	human oral cavity	y	(240)
Bartonella tamiae (2 strains)	Proteo-bacteria	a-proteo bacteria	Rhizobiales	Bartonellaceae	human blood	y	(241)

Table 3. Putative precorrin-2 auxotrophs

Organism	**Phylum**	**Class**	**Order**	**Family**	**Environment[1]**	**Pathogen[2]**	**Ref.**
Shuttleworthia satelles DSM 14600	Firmicutes	Clostridia	Clostridiales	Lachno-spiraceae	human oral cavity	n	(221)
Ruminococcus flavefaciens FD-1	Firmicutes	Clostridia	Clostridiales	Rumino-coccaceae	bovine rumen	n	(242)
Megasphaera micronuciformis F0359	Firmicutes	Negativicutes	Seleno-monadales	Veillonellaceae	human oral cavity	n	(243)
Megasphaera genomosp. UPII 135-E	Firmicutes	Negativicutes	Seleno-monadales	Veillonellaceae	human urogenital tract	n	(236)
Collinsella tanakaei TIY 12063	Actino-bacteria	Actino-bacteria	Corio-bacteriales	Corio-bacteriaceae	human gut	n	(244)
Treponema pedis T A4	Spirochaetes	Spirochaetes	Spiro-chaetales	Spirochaetaceae	bovine and porcine skin	y*	(245)
Treponema denticola (6 strains)	Spirochaetes	Spirochaetes	Spiro-chaetales	Spirochaetaceae	human oral cavity	y	(236, 246, 247)

1. Environment refers to the source from which the bacterium was isolated or is commonly detected
2. Human pathogen, unless otherwise noted
y: yes
n: no
*pathogen of pigs and cows

Phylogenetic distribution of corrinoid precursor auxotrophs

The largest number of candidate corrinoid precursor auxotrophs are members of the family Lachnospiraceae (Tables 1-3). The majority of the Lachnospiraceae candidates are putative PBG auxotrophs, lacking the first three genes involved in tetrapyrrole biosynthesis, though ALA auxotrophs (lacking only the first two genes), and a precorrin-2 auxotroph (lacking the first 6 genes) were also identified. An analysis of the 16S rDNA sequences of Lachnospiraceae members from each of these categories as well as those that possess the complete corrinoid biosynthesis pathway suggests that the loss of genes involved in early tetrapyrrole precursor biosynthesis occurred multiple times within this family (Fig. 2). Loss of *hemA* and *hemL*, the genes required for ALA biosynthesis, appears to have occurred once within the Clostridiaceae (fig.3). Within the species *Clostridium botulinum*, which is polyphyletic, all sequenced group 1 *C. botulinum* strains, as well as the most closely related non-toxigenic clostridium, *C. sporogenes* are ALA auxotrophs, while other *C. botulinum* strains, which are most closely related to different clostridia, are not (Fig. 3)(248). Within the Bartonellaceae, *Bartonella tamiae,* a PBG auxotroph is the only sequenced member that possesses any corrinoid biosynthesis genes, which may suggest that this organism acquired these genes through horizontal gene transfer (Fig. 4).

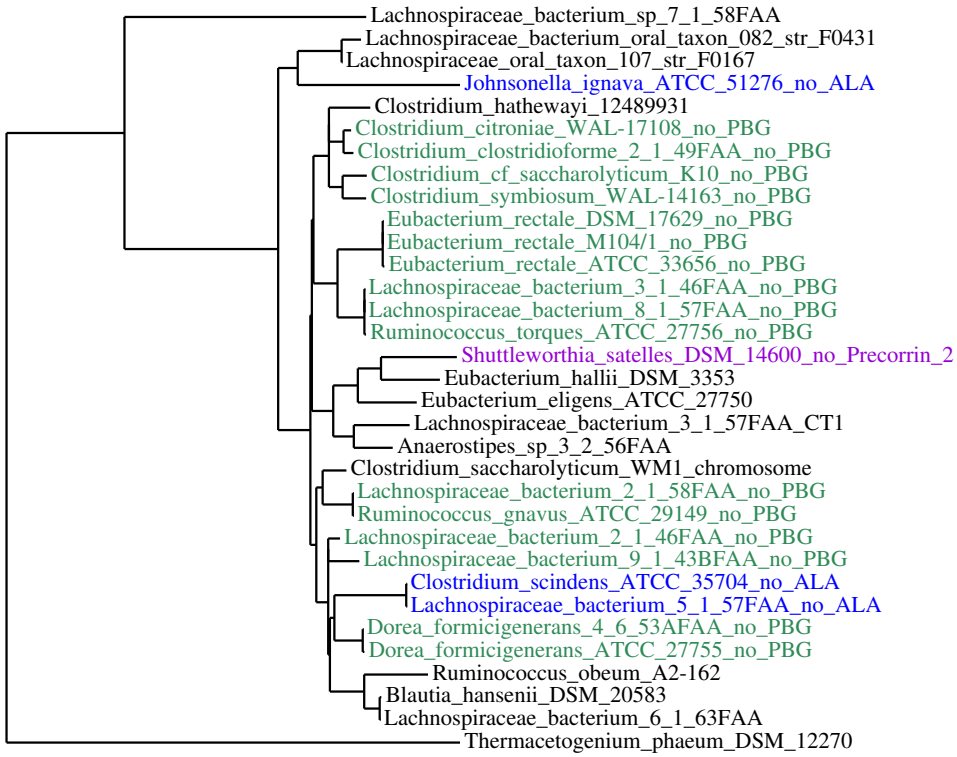


Figure 2. Distribution of tetrapyrrole precursor auxotrophs among the Lachnospiraceae Maximum likelihood phylogenetic tree of 16S rDNA sequences of members of the Lachnospiraceae family that possess the complete corrinoid biosynthesis pathway (black), or lack homologs of *hemA* and *hemL* (blue, ALA auxotrophs), *hemALB* (green, PBG auxotrophs), or *hemALBCD* (pink, precorrin-2 auxotrophs).

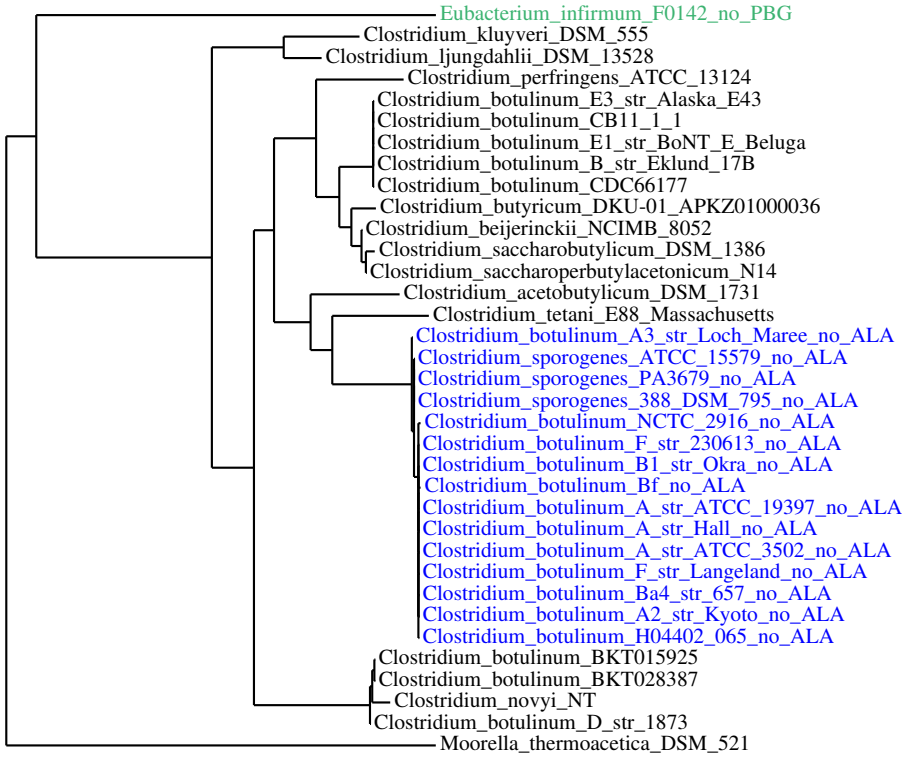


Figure 3. Distribution of tetrapyrrole precursor auxotrophs among the Clostridiaceae
Maximum likelihood phylogenetic tree of 16S rDNA sequences of members of the Clostridiaceae family that possess the complete corrinoid biosynthesis pathway (black), or lack homologs of *hemA* and *hemL* (blue, ALA auxotrophs), or *hemALB* (green, PBG auxotroph).

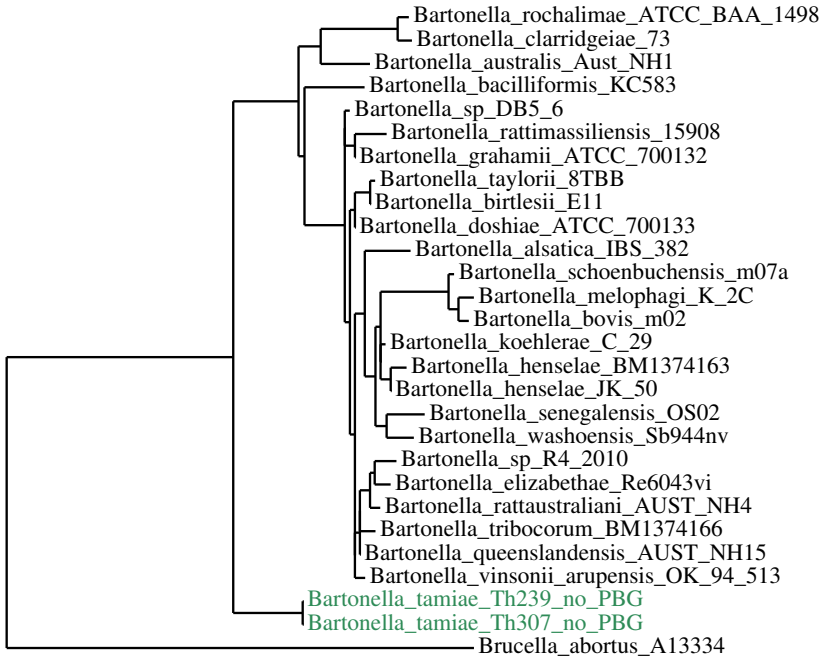


Figure 4. Distribution of tetrapyrrole precursor auxotrophs among the Bartonellaceae

Maximum likelihood phylogenetic tree of 16S rDNA sequences of members of the Bartonellaceae family that lack any corrinoid biosynthesis genes (black), or lack homologs of *hemALB* (green, PBG auxotrophs).

Experimental validation of bioinformatic results

The absence of tetrapyrrole precursor biosynthesis genes could indicate either the ability to scavenge precursors or an evolutionary step in the loss of a functional corrinoid biosynthesis pathway. Indeed, previous genomic analyses show that loss of the corrinoid biosynthesis pathway has occurred repeatedly in multiple lineages(249). We therefore tested the ability of three candidates, *Clostridium scindens* ATCC 35704, *Clostridium sporogenes* ATCC 15579, and *Treponema primitia* ZAS-2 from the Lachnospiraceae, Clostridiaceae and Spirochaetaceae families, respectively, to scavenge ALA from the medium by monitoring corrinoid production in the presence or absence of ALA.

High performance liquid chromatography (HPLC) analysis of corrinoid extracts showed that addition of ALA to the medium led to corrinoid production in all three organisms (Figure 5). In each case, multiple peaks with UV-Vis spectra characteristic of corrinoids were present. The spectra of both peaks in *C. sporogenes* was characteristic of a corrinoid in the "base-off" configuration (that is, without a lower ligand coordinated to the cobalt) typically observed in corrinoid intermediates. This result was unsurprising, since *C. sporogenes*, like *C. botulinum* and *Listeria innocua* lacks cobT, the enzyme responsible for lower ligand activation, a step that must occur before the lower ligand can be attached to the corrinoid(250). *L. innocua* presumably circumvents this issue by instead importing the downstream products of *cobT*, activated (ribosylated) lower ligands, from the environment via the cblT transporter. Homologs of cblT have also been identified in *C. sporogenes*(250).

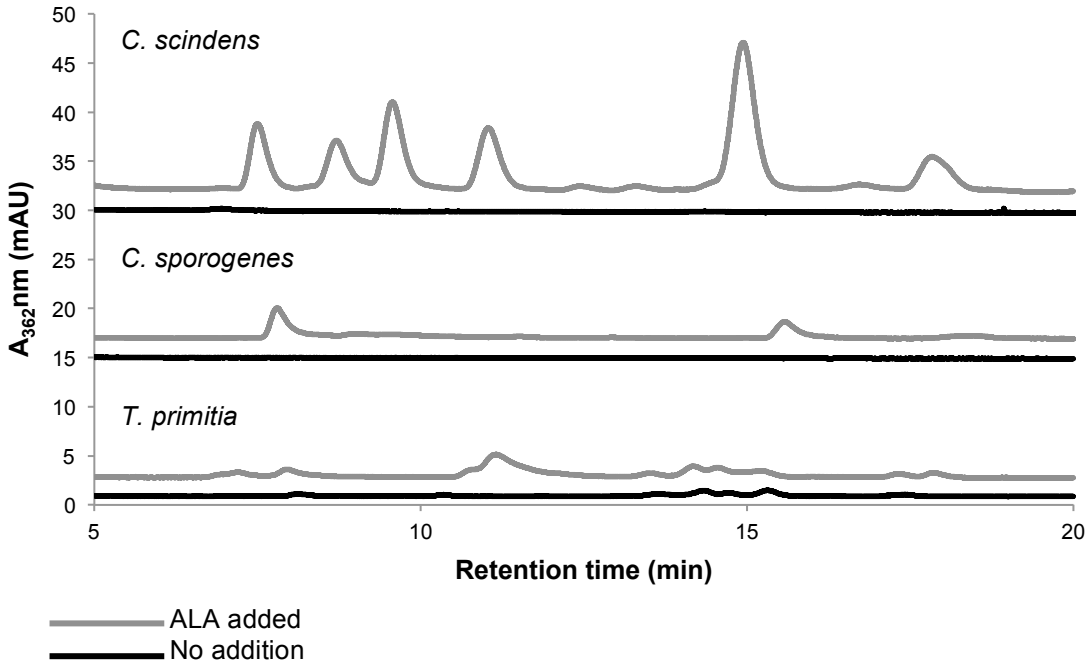


Figure 5. Corrinoid production in the presence and absence of ALA

HPLC analysis of corrinoid extracts from *C. scindens, C. sporogenes,* and *T. primitia* grown with (gray trace) or without 1mM ALA added (black trace).

In *T. primitia*, the addition of ALA led to increased corrinoid production, showing that it is capable of ALA scavenging. Low levels of corrinoids were also observed in the absence of ALA addition, which suggests that another source of ALA or other tetrapyrrole precursor is available to *T. primitia*, either through biosynthesis or in the growth medium (Fig. 5). *T. primitia* requires a complex medium containing *Saccharomyces cerevisiae* autolysate, a possible source of tetrapyrrole precursors, suggesting that the latter may be the case(251). *Saccharomyces cerevisiae* produces ALA, PBG, and Uro'III for heme biosynthesis, and ALA has been shown to accumulate in yeast cells under anaerobic conditions (252). An alternative explanation for this result is that *T. primitia* possesses a method of ALA production other than those encoded by the Shemin or C_5 pathways. *T. primitia*'s complex nutritional requirements make it difficult to resolve this issue.

As a member of the termite gut microbiota, *T. primitia* relies on corrinoid-dependent CO_2-reductive acetogenesis for growth(251). The ability of *T. primitia* to scavenge ALA was confirmed by monitoring growth in medium supplemented with cobalamin or ALA, or without supplementation. *T. primitia*'s growth rate and $O.D._{650}$ at stationary phase achieved similar levels when cobalamin or ALA was added, but were lower in medium without supplementation (Fig.). In *E.coli*, ALA transport is mediated by the dipeptide transport system encoded by the *dpp* operon(253). A BLAST search of the *T. primitia* genome revealed the presence of *dpp* homologs, however, the current lack of tools for genetic manipulation of *T. primitia* makes it difficult to assess whether *T. primitia* uses this system for ALA transport. An analysis of the tetrapyrrole precursor content of the termite hindgut could help to determine whether *T. primitia* has access to ALA in its natural environment.

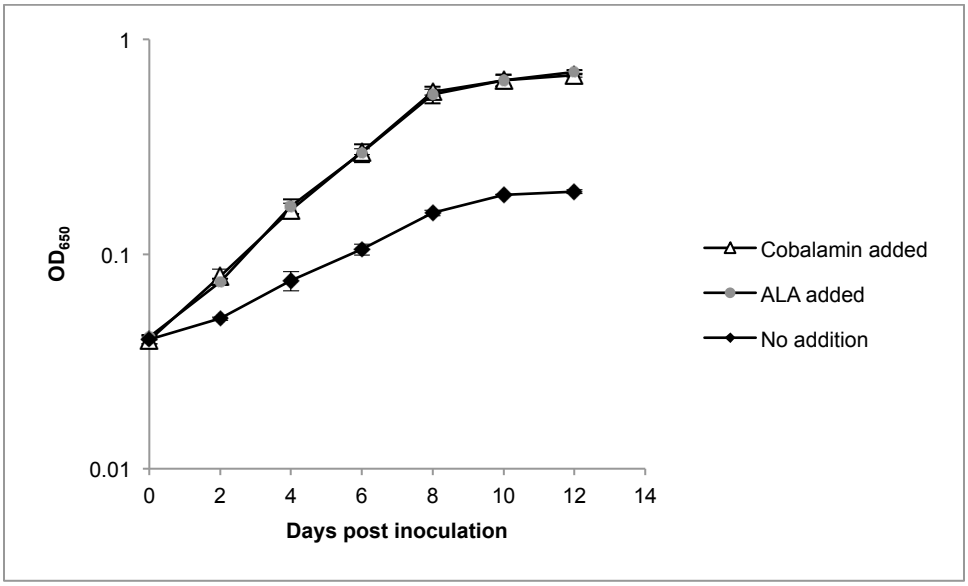


Figure 6. Growth of *T. primitia* with and without cobalamin or ALA Culture density (OD_{650}) of *T. primitia* cultures grown with 37nM cobalamin (white triangles), or 1mM ALA added (gray circles), or with no addition (black diamonds). All cultures were grown in triplicate, and the data shown are the mean values, with error bars representing the standard deviation.

While the results of these experiments demonstrate that *C. scindens*, *C. sporogenes*, and *T. primitia* are all capable of scavenging ALA, the question of whether this activity is important

for their survival in the environment remains. Both *C. sporogenes* and *T. primitia* encode homologs of the Btu corrinoid transporter, and as such, may fulfill their corrinoid requirements by importing corrinoids produced by other microbes instead of scavenging ALA for corrinoid biosynthesis. However, corrinoid transporter genes were not detected in *C. scindens,* which suggests that ALA scavenging may be the primary means of fulfilling corrinoid requirements in this organism, which possesses a corrinoid-dependent methionine synthase encoded by *metH* and lacks the corrinoid-independent *metE*. A member of the human gut microbiota, *C. scindens* has recently been shown to play an important role in host resistance to colonization by *C. difficile*, another organism identified in this study as an ALA auxotroph(254).

Conclusions and Future Directions

In this study, I identified 39 putative corrinoid precursor auxotrophs, all lacking genes involved in tetrapyrrole precursor biosynthesis. I showed that three of these auxotrophs, C. scindens, C. sporogenes, and T. primita are capable of scavenging ALA from the growth medium for use in corrinoid production. These findings suggest that the bacteria identified in this study can fulfill their corrinoid requirements by scavenging tetrapyrrole precursors from the environment, and carrying out the remaining steps in corrinoid biosynthesis.

The range of tetrapyrrole precursors that the bacteria identified in this study can scavenge remains to be investigated, as does the level of precursors in the environments that they occupy, such as the rumen. The ability of microbes to take up exogenous ALA is well known, and is the basis of some forms of antimicrobial photodynamic therapy(255, 256). In addition, studies have revealed the ability of various microbes to take up exogenous PBG(206, 257, 258). Excess ALA and PBG produced by human cells is excreted in the urine, and thus would be available to microbes colonizing the urinary tract, such as *Aerococcus urinae*, a pathogen which causes urinary tract infections(195, 239). Excess Uro'III is excreted in human feces, however, it is unknown whether this molecule can be scavenged(259, 260). To our knowledge, the amount of precorrin-2 (a precursor of corrinoids, siroheme, and factor F_{430}) in human feces has not yet been investigated.

As previously mentioned, accumulation of tetrapyrrole precursors can be toxic; thus, microbes that scavenge these molecules from the environment could contribute to community stability by keeping tetrapyrrole precursors at safe levels(201).

The finding that all of the tetrapyrrole precursor auxotrophs identified in this study are host-associated raises the question of whether tetrapyrrole precursors produced by a host organism can be scavenged by corrinoid-producing members of the microbiota. The injection of labeled succinyl CoA and glycine, the substrates of ALA synthase in animals, into a host followed by monitoring fecal corrinoid content could reveal whether this occurs.

Materials and Methods

Phylogenetic analyses:

A comparative genomic search was carried out using JGI's Integrated Microbial Genomes (IMG) webserver [1]. The initial search included all archaeal and bacterial genomes as of August 2013. An additional search was performed including only bacterial genomes of finished status as of March 2014, bringing the total number of genomes searched to 5,988. "Function Profile" was

performed using enzymes, or "functions", involved in the biosynthesis of cobalamin and its precursors. Functions were selected from the "porphyrin and chlorophyll metabolism" KEGG pathway via EC numbers. Functions from both the anaerobic and aerobic biosynthesis pathways were included in the search. The two pathways were differentiated in downstream analyses. The function profile search was carried out with 500 genomes at a time, as limited on IMG's webserver. The results of the function profile search of all 5,988 genomes were compiled and manipulated in Microsoft Excel. Organisms with genomes of unfinished status were identified as tetrapyrrole precursor auxotrophs only if the genome assembly contained fewer than 150 scaffolds. In order to rule out false positives due to inadequate sequence coverage, inspection of the scaffold containing tetrapyrrole precursor biosynthesis genes was carried out to ensure that these genes were not located at the end of a scaffold.

For the construction of phylogenetic trees, 16S rDNA sequences were obtained from the IMG webserver or from the Ribosomal Database Project(204, 261). Tree construction was carried out using the "one-click" mode on the phylogeny.fr webserver(262).

Growth conditions:
Treponema primitia str. ZAS-2 was grown at room temperature in anaerobic 4YACo medium with an 80% H_2, 20% CO_2 headspace as previously described (251),with the following changes: For cobalamin added cultures, the final concentration of cyanocobalamin was reduced from 4.42 μM to 37 nM. No addition and ALA added cultures were obtained from cobalamin added cultures, by serially passaging cells (three transfers) into cobalamin-free medium or in cobalamin-free medium containing 1 mM ALA before being used as inocula for growth experiments. Growth was monitored spectrophotometrically (OD_{650}). All growth experiments were performed in triplicate.

Clostridium scindens ATCC 35704 was grown at 37C under 80% N_2, 20% CO_2 in an anaerobic defined mineral salts medium of the following composition (g/L): NaCl, 1; $MgCl_2 \cdot 6H_2O$, 0.5; KH_2PO_4, 0.2; NH_4Cl, 0.3; KCl, 0.3; $CaCl_2 \cdot 2\ H_2O$, 0.015. In addition, 2.29 g of N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, free acid), 2 ml of a trace element solution, 1ml of a Na_2SeO_3-Na_2WO_4 solution, 10 mg of resazurin, and 40mg each of the amino acids arginine, cysteine, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were added per liter. After the medium was boiled and cooled under N_2, the gas was switched to an 80% N_2, 20% CO_2 mix, and the reductants $Na_2S \cdot 9\ H_2O$ and L-cysteine were added to final concentrations of 0.2mM each. Next, 2.52 g $NaHCO_3$ (30mM final concentration) was added to the medium, and the pH was adjusted to 7. The medium was dispensed under 80% N_2, 20% CO_2 in 10 ml aliquots in 25 ml Balch tubes, or for large volumes, 1L in 2L pyrex bottles, and tubes and bottles were sealed with butyl stoppers and aluminum crimp seals, and autoclaved for 30 min. After autoclaved medium cooled to room temperature, glucose was added to a final concentration of 25mM, and 100ul (tubes) or 1mL (bottles) Wolin vitamin solution (prepared without cobalamin) was added. 1mM ALA was added after autoclaving to ALA-added cultures.
The trace element stock solution contained (g/L) Nitriloacetic acid, 1.11; $MnSO_4 \cdot H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $ZnCl_2$, 0.1, $NiCl_2 \cdot 6H_2O$, 0.05, $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2 \cdot 12\ H_2O$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01.

The Na_2SeO_3-Na_2WO_4 solution contained (g/L) Na_2SeO_3 5H2O, 0.006; Na_2WO_4 2H2O, 0.008; NaOH, 0.5.
Clostridium sporogenes ATCC 15579 was grown in the same medium described for *Clostridium scindens*, with the following changes: cysteine, serine, and threonine were omitted, and 1ml of a vitamin solution containing (mg/L) nicotinic acid, 500; thiamine HCl , 50; biotin, 5; and p-aminobenzoic acid, 5 was added per liter of medium.

All cultures were inoculated from exponential phase cultures to an OD_{600} (C. sporogenes and C. scindens) or OD_{650} (*T. primitia*) of 0.04.

Corrinoid extractions: Cells were harvested by centrifugation (8,000×*g*, 4 °C, 20min). Cell pellets were resuspended in 20 ml of methanol with 20 mg KCN per gram of cells and incubated at 60 °C for 1.5 h with vortex mixing every 20 minutes. Cellular debris was removed by centrifugation at 40,000×*g* for 1 h, and the supernatant was dried in a rotary evaporator. Samples were resuspended in 10-20 ml deionized water, desalted with a C18 Sep-Pak cartridge (Waters Associates, Milford, MA), and eluted in 2 ml methanol. The eluates were dried overnight in a vacuum desiccator, resuspended in deionized water, and stored at -80 °C.

HPLC analysis was performed with an Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector set at 362 and 525 nm. 50 to 100ul samples were injected onto an Agilent Eclipse XDB C18 column (5 µm, 4.6 x 150 mm) at 35 °C, with 0.5ml min^{-1} flow rate. Samples were separated using acidified water and methanol (0.1% formic acid) with a linear gradient of 18% to 30% methanol over 20 min.

Chapter 5

Summary

Prior to my work in *D. mccartyi*, an environmentally important bacterium used in the bioremediation of chlorinated solvents which has an obligate requirement for exogenously produced corrinoids, very little was known about how microbes that import exogenous corrinoids respond to the presence of different corrinoids in the environment. Previous work on the range of corrinoids capable of serving as cofactors in any one organism was limited to the analysis of three corrinoids, at most. Fundamental questions about whether corrinoids from different structural classes could be imported into bacterial cells had not been examined, and the ability to remodel corrinoids had never been demonstrated in an organism that lacked the ability to produce corrinoids *de novo*. Moreover, the only previous work on corrinoid remodeling in a bacterium suggested that only nonfunctional corrinoids could be remodeled.

One factor that has limited the scope of corrinoid research in the past is that although as many as 16 structurally diverse corrinoids have been detected, only two corrinoids, cobalamin and cobinamide, are commercially available. In order to conduct a comprehensive study of the array of corrinoids that *D. mccartyi* can use as cofactors, I purified six non-commercially available corrinoids from bacterial cultures. Using these corrinoids, I demonstrated that *D. mccartyi* was capable of using only three benzimidazolyl corrinoids. However, I showed that four additional corrinoids could be remodeled into functional cofactors in *D. mccartyi* if a benzimidazole was added to the growth medium. In addition, I showed that in *D. mccartyi*, functional and nonfunctional corrinoids alike could be remodeled, as long as a benzimidazole was supplied. These results demonstrate that corrinoid remodeling allows *D. mccartyi* to utilize a broad range of corrinoids present in the environment.

D. mccartyi's requirement for benzimidazoles in corrinoid remodeling raised the question of whether these molecules were present in the microbial communities in which *D. mccartyi* exists. The only known biological role of benzimidazoles is as the lower ligand of a corrinoid. While previous studies had explored the corrinoid content of various biological and environmental samples, the free (that is, not attached to a corrinoid) benzimidazole content of a microbial community had never been studied. Therefore, together with my collaborators Shan Yi, Yujie Men, and Lisa Alvarez-Cohen, I developed quantitative LC/MS/MS-based detection methods for free benzimidazoles and complete corrinoids, and applied these methods to the analysis of TCE-dechlorinating microbial communities containing *D. mccartyi* enriched from solvent-contaminated soil and groundwater. I found that DMB is present in these communities as well as evidence to suggest that corrinoid remodeling was actively occurring within these communities. Moverover, I uncovered a microbial tug of war over the two most abundant corrinoids in the community: [Cre]Cba, which *D. mccartyi* is unable to use without remodeling, and cobalamin, which functions as a cofactor for *D. mccartyi* as is. Reductive dehalogenation of chlorinated solvents is a corrinoid dependent process, and bioremediation of contaminated groundwater is often augmented with cobalamin. However, I showed that addition of cobalamin resulted in a two-fold increase in the amount of [Cre]Cba detected in the community, and a 16-fold increase in the amount of free DMB in the supernatant, which suggested that the added cobalamin is converted into [Cre]Cba. I also found evidence that suggests that *D. mccartyi* remodels [Cre]Cba present in the supernatant into cobalamin. When *D. mccartyi* is eliminated from the community, the amount of [Cre]Cba in the supernatant increased 5-fold, while cobalamin levels drop. Re-inoculation of the community with *D. mccartyi* reversed these

changes. Thus, while *D. mccartyi* is incapable of *de novo* corrinoid production, it likely produces cobalamin in its community by remodeling other corrinoids. The results of these analyses shed light not only on the corrinoid and free benzimidazole content of *D. mccartyi*'s natural environment, but also on the dynamics of corrinoid cross-feeding and modification within the community, as well as the contributions of specific phylogenetic groups to the corrinoid composition.

While my work in *D. mccartyi* examined the cross-feeding of complete corrinoids, late corrinoid intermediates such as cobinamide, and lower ligands such as DMB, I was also interested in exploring whether early corrinoid precursors are cross-fed among microbes. Together with my collaborator Andrew Han, I carried out a comparative analysis of prokaryotic genomes present in the Integrated Microbial Genomes database, and identified 39 bacteria that were missing genes involved in the first steps of corrinoid biosynthesis, though they possessed all other genes necessary for corrinoid production. All tetrapyrroles, including corrinoids, are produced from the same precursors. Since the genes that are absent in these bacteria are those required for the production of the universal tetrapyrrole precursors, it is possible that any organism carrying out tetrapyrrole production could serve as a source for precursors for these organisms. Interestingly, all 39 bacteria I identified as potential tetrapyrrole precursor scavengers are animal host-associated, raising the question of whether tetrapyrrole precursors produced by a host animal can be scavenged for corrinoid production.

Across all of these experiments, I have investigated the strategies that microbes use to fulfill their corrinoid requirements, and how interactions between members of microbial communities, and potentially, between an animal host and its microbiota allow organisms to rely on corrinoid-dependent forms of metabolism without the need to produce these complex cofactors *de novo*.

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