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Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities

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Summary

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 liquid chromatography tandem mass spectrometry-based corrinoid detection method to examine relationships among corrinoids, their lower ligand bases and specific microbial groups in microbial communities containing *Dehalococcoides mccartyi* that has an obligate requirement for benzimidazole-containing corrinoids for trichloroethene respiration. We found that *p*-cresolylcobamide ([p-Cre]Cba) and cobalamin were the most abundant corrinoids in the communities. It suggests that members of the family *Veillonellaceae* are associated with the production of [p-Cre]Cba. The decrease of supernatant-associated [p-Cre]Cba and the increase of biomass-associated cobalamin were

correlated with the growth of *D. mccartyi* by dechlorination. This supports the hypothesis that *D. mccartyi* is capable of fulfilling its corrinoid requirements in a community through corrinoid remodelling, in this case, by importing extracellular [p-Cre]Cba and 5,6-dimethylbenzimidazole (DMB) (the lower ligand of cobalamin), to produce cobalamin as a cofactor for dechlorination. This study also highlights the role of DMB, the lower ligand produced in all of the studied communities, in corrinoid remodelling. These findings provide novel insights on roles played by different phylogenetic groups in corrinoid production and corrinoid exchange within microbial communities. This study may also have implications for optimizing chlorinated solvent bioremediation.

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) (Banerjee and Ragsdale, 2003; Brown, 2005). Although corrinoids are synthesized solely by a subset of *Bacteria* and *Archaea*, they function as cofactors for a variety of organisms including many eukaryotes (Martens *et al*., 2002; Ryzhkova, 2003). At least 16 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) (Guimaraes *et al*., 1994; Renz, 1999; Kräutler *et al*., 2003; Allen and Stabler, 2008). Previous studies have shown that even corrinoids within one lower ligand class may not necessarily be functionally equivalent as cofactors (Yi *et al*., 2012). 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 remodelling, 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* (Escalante-Semerena, 2007; Gray and Escalante-Semerena, 2009a,b; Yan *et al*., 2012; Yi *et al*., 2012; Keller *et al*., 2013).

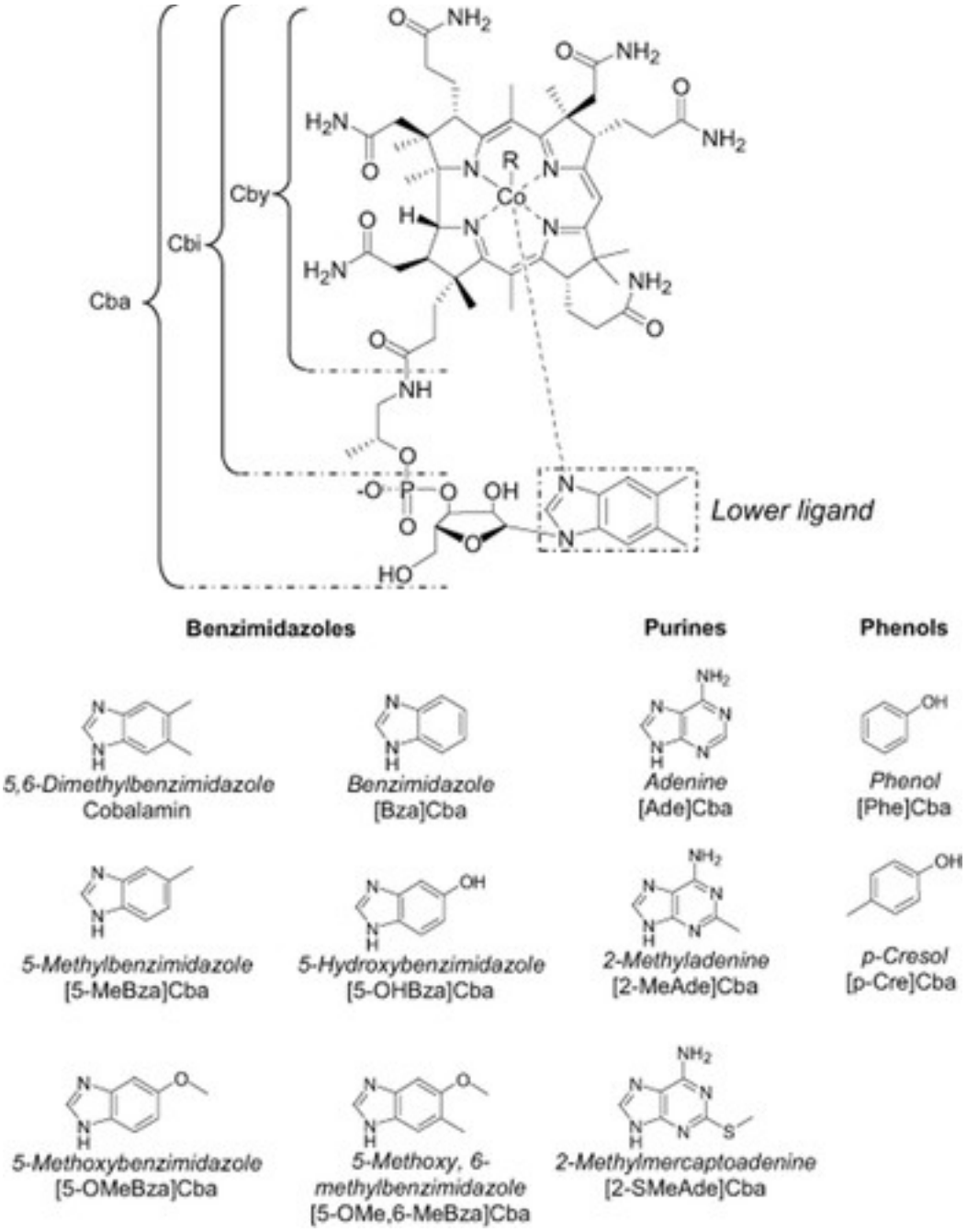

Figure 1

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Structures of corrinoids and lower ligand bases together with abbreviated designation. The names of the lower ligands are italicized, below which are the abbreviations of the corresponding cobamides. Cby, cobyric acid; Cbi, cobinamide; Cba, cobamide.

Dehalococcoides mccartyi strains are the only known bacteria capable of complete dechlorination of the common groundwater contaminants tetrachloroethene and trichloroethene (TCE) to the innocuous end-product ethene (Maymó-Gatell *et al*., 1997; He *et al*., 2003;

Cupples *et al*., 2004). Corrinoids are essential cofactors for RDases, the enzymes that catalyze organohalide respiration in many different microorganisms, including *D. mccartyi* strains (Maymó-Gatell *et al*., 1997; Siebert *et al*., 2002; Kräutler *et al*., 2003; Maillard *et al*., 2003; Müller *et al*., 2004; Seshadri *et al*., 2005; Reinhold *et al*., 2012; Schipp *et al*., 2013). The corrinoid cofactors bind to the RDases, serving as the enzyme-active site, where dehalogenation occurs either through halide elimination forming an organocobalt adduct or by using Co(I) as the electron donor (Banerjee and Ragsdale, 2003). Genomic analyses of the sequenced *D. mccartyi* strains indicate that they are unable to produce corrinoids *de novo*, and therefore exogenous cobalamin is regularly added to *D. mccartyi* isolates and *D. mccartyi*-containing communities to enhance dechlorination performance (Lesage *et al*., 1996; Richardson *et al*., 2002; He *et al*., 2003; Cupples *et al*., 2004; Duhamel *et al*., 2004; Schipp *et al*., 2013). We recently showed that [5-MeBza]Cba and [5-OMeBza]Cba also support growth and dechlorination in *D. mccartyi* strain 195 (strain 195) (Yi *et al*., 2012). However, other corrinoids do not function as cofactors for strain 195, indicating a strict requirement for specific benzimidazole cobamides.

D. mccartyi coexists with corrinoid-producing organisms such as acetogens, methanogens and sulfate-reducing bacteria in dechlorinating communities (Kräutler *et al*., 1988; Stupperich and Krautler, 1988; Stupperich *et al*., 1988; Guimaraes *et al*., 1994; Renz, 1999) and is likely to encounter a variety of different corrinoids in its environment. Although none of the sequenced *D. mccartyi* strains possesses the complete corrinoid biosynthesis pathway, genes encoding corrinoid salvaging and remodelling pathways have been identified in each strain (Yi *et al*., 2012; Schipp *et al*., 2013). The remodelling of seven non-functional corrinoids (i.e. [Ade]Cba, [2-SMeAde]Cba, [5-OHBza]Cba, [Bza]Cba, [5-MeBza]Cba, [p-Cre]Cba and Cbi) has been observed in a number of *D. mccartyi* strains in isolation or defined consortia. When provided with the lower ligand of cobalamin, 5,6-dimethylbenzimidazole (DMB), strain 195 was able to grow and dechlorinate TCE by remodelling nonfunctional corrinoids to cobalamin (Yi *et al*., 2012). *D. mccartyi* strains incubated with corrinoid-producing microorganisms *Geobacter sulfurreducens*, *Methanosarcina barkeri* or *Sporomusa* sp. also exhibited dechlorination activity when DMB was added to the cobalamin-free medium (Yan *et al*., 2012; 2013). Similar dechlorination activities have been observed in enrichments grown without exogenous cobalamin (Men *et al*., 2013), leading to the hypothesis that other bacteria provide corrinoids to *D. mccartyi* that can be used directly or after remodelling. However, comprehensive profiles of corrinoids produced by other bacteria and corrinoid remodelling in dechlorinating microbial communities have not been reported. Additionally, little is known about the generation and availability of DMB in anaerobic communities, although the availability of DMB is crucial for

corrinoid remodelling by *D. mccartyi* according to previous studies with pure cultures and defined consortia (Yan *et al*., 2012; 2013; Yi *et al*., 2012).

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 (Chanarin and Muir, 1982; Guggisberg *et al*., 2012; Yan *et al*., 2013). 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 13 corrinoids and 3 benzimidazoles in two sets of dechlorinating enrichments derived from geographically different inocula (Supporting Information Table S1). One set of enrichments was subcultures of ANAS, a long-term TCE dechlorinating enrichment initially derived from contaminated soil in California (CA; Freeborn *et al*., 2005), grown with and without exogenous 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) (Men *et al*., 2013).

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 on corrinoid production of the community, corrinoid 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 13 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 (Supporting Information Table S2). This method was validated by a direct comparison with a previously described liquid chromatography mass spectrometry (LC/MS) analytical method (Allen and Stabler, 2008). 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 (Supporting Information Table S3). 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 (Supporting Information Table S2). 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 *D. mccartyi* growth are listed in Supporting Information Table S1. The ANAS subcultures contained bacterial species related most closely to *D. mccartyi*, *Clostridium* sp., *Eubacterium* sp., *Bacteroides* sp., *Citrobacter* sp., *Spirochaeta* sp. and δ-proteobacteria (Freeborn *et al*., 2005). In the NJ enrichments, besides *D. mccartyi*, seven 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 (Men *et al*., 2013); 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 [p-Cre]Cba, although smaller amounts of [5-MeBza]Cba, [Ade]Cba, [2-SMeAde]Cba and Cbi were also present (Fig. 2A and Supporting Information Table S4). 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 strain 195 (He *et*

al., 2007). 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 with 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 and Supporting Information Table S5).

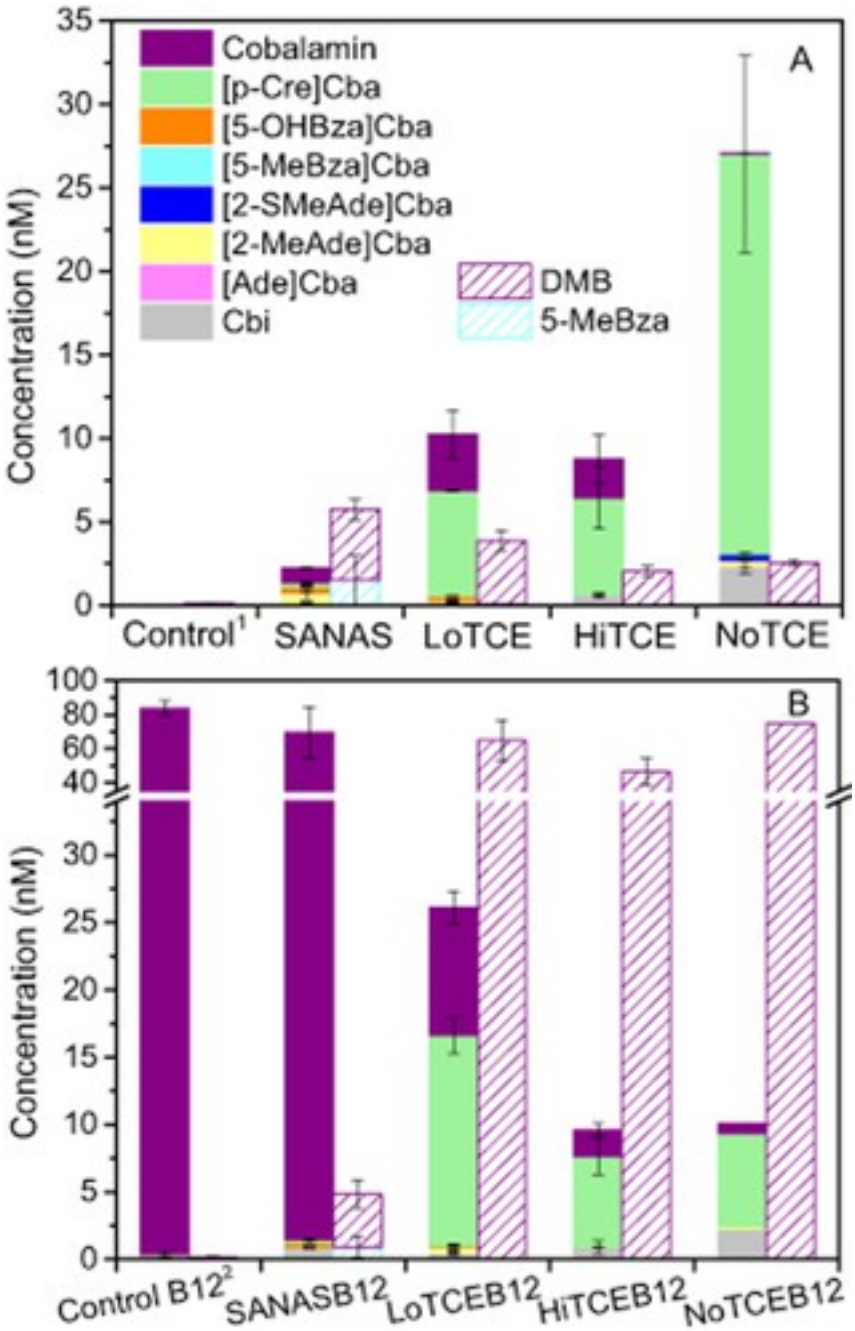

Figure 2
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Corrinoid and benzimidazole lower ligand concentrations in B_{12}-unamended (A) and B_{12}-amended (B) enrichments at the end of 13–18 days' subculturing cycle, error bars represents standard deviation, $n = 3$. [1]Abiotic control without 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 corrinoid profiles of the NJ enrichments differed from SANAS and SANASB12. In the NJ enrichments without exogenous cobalamin (LoTCE and HiTCE), [p-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 *D. mccartyi*. Free DMB was detected at levels similar to those detected in SANAS (Fig. 2A). Interestingly, greater [p-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 (Supporting Information Table S4). 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).

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. 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 (Supporting Information Table S4). One possible reason for the disappearance of cobalamin in the two NJ enrichments is the remodelling of cobalamin, however, the generation of other corrinoids by corrinoid remodelling was not sufficient to account for the decrease of cobalamin in LoTCEB12 and HiTCEB12. [p-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 and Supporting Information Table S5), 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 days feeding cycle. Concentrations of [p-Cre]Cba and cobalamin, the most abundant corrinoids in these cultures, as well as DMB are shown in Fig. 3. In all cultures, [p-Cre]Cba accumulated to near maximum levels during the first 2–3 days of incubation. Subsequently, [p-Cre]Cba levels declined substantially in cultures without cobalamin added (LoTCE and HiTCE) (Fig. 3A and C), while those in the B_{12}-amended cultures (LoTCEB12 and HiTCEB12) exhibited little change (Fig. 3B and D). Cobalamin in LoTCE and HiTCE cultures increased from 0 to 1–2 nM during the first 2 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 and 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 and D).

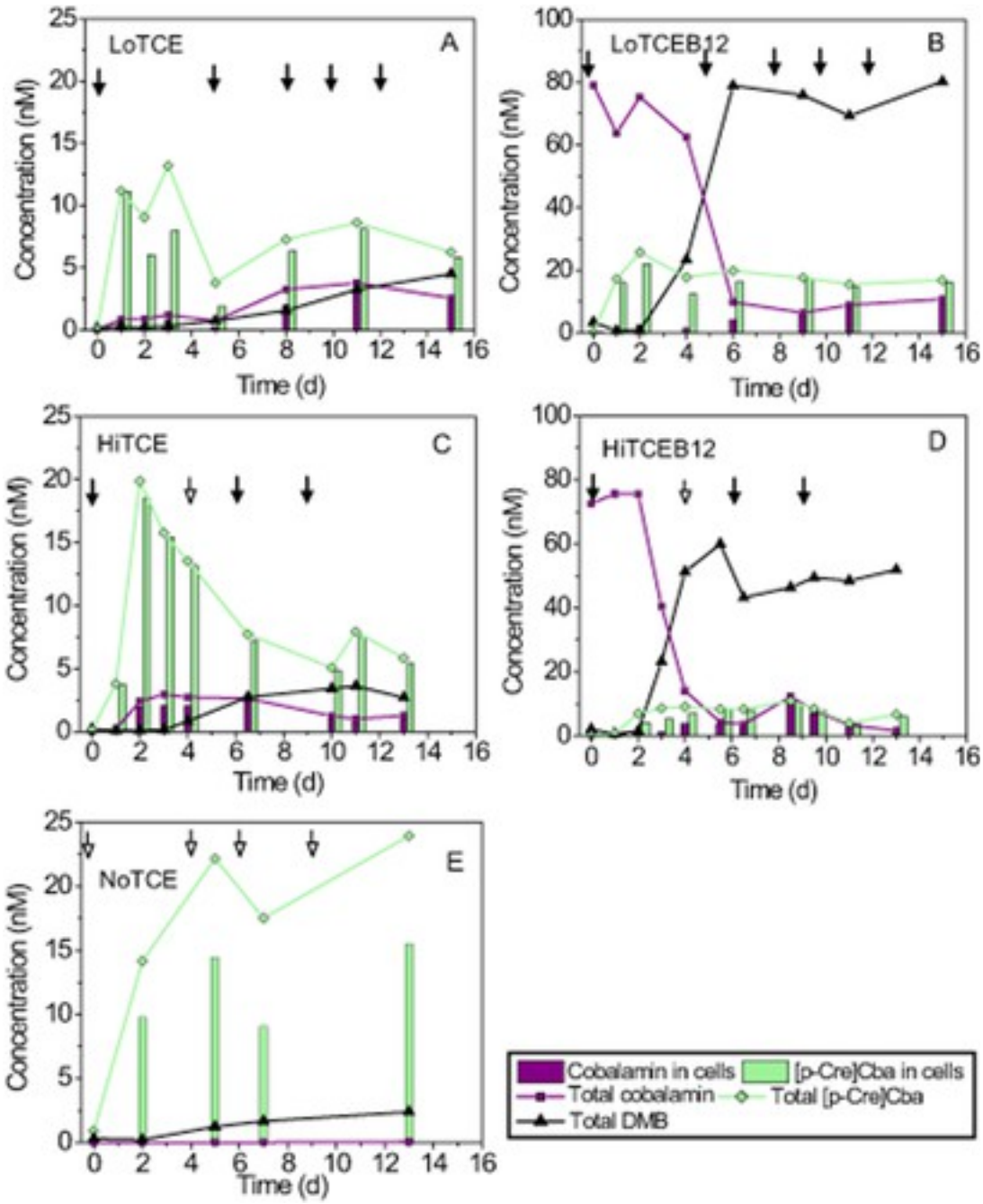

Figure 3

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Temporal changes of [p-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 Supporting Information Table S1. Note: Y-axis scales in A, C and E are different from those in B and D).

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

Because free DMB (a prerequisite for corrinoid remodelling by strain 195) was detected in the supernatant of the cultures without exogenous cobalamin, we hypothesized that *D. mccartyi* strains in the NJ enrichments generate cobalamin by remodeling other corrinoids with DMB. To test this hypothesis, we examined whether active dechlorination and *D. mccartyi* cell growth affected the corrinoid profile of the community. To limit the growth of *D. mccartyi*, we constructed enrichments NoTCE and NoTCEB12 by subculturing HiTCE and HiTCEB12 cultures without TCE (Supporting Information Table S1>).

We analyzed the corrinoid profiles of NoTCE and NoTCEB12 after six subculturing events when the growth of *D. mccartyi* in these two cultures was significantly inhibited (< 10^4cells/ml compared with 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) (Figs 2A and 3E), while [p-Cre]Cba was produced at concentrations as high as 24 nM. These results, together with the observed decrease in [p-Cre]Cba in the HiTCE culture after day 2 (Fig. 3C) suggest that *D. mccartyi* may be responsible for remodelling [p-Cre]Cba to form cobalamin in these cultures. Moreover, about 20–50% of total [p-Cre]Cba in NoTCE was detected in the supernatant (Fig. 3E), while in HiTCE, almost all of the [p-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, Supporting Information Fig. S1). 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 *D. mccartyi*, indicates that microorganisms other than *D. mccartyi* were active in modification of the added cobalamin.

Corrinoid profiles were also determined when *D. mccartyi* 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 (Supporting Information Table S6). HiTCE$_{-/-}$ was HiTCE subcultured without TCE for two feeding cycles (Supporting Information Table S6). As expected, *D. mccartyi* 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 *D. mccartyi* 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. *D. mccartyi* and the other seven OTUs previously identified in the NJ enrichments (Men *et al*., 2013) have been quantified by quantitative polymerase chain reaction (qPCR; Fig. 4B). Interestingly, numbers of the three dominant OTUs, *Pelosinus*_GW, *Dendrosporobacter*_GW and *Sporotalea*_GW (collectively designated 'PDS') exhibited a fivefold increase in the two perturbed cultures compared to HiTCE (Fig. 4B), accompanied by an increase in [p-Cre]Cba concentrations (Fig. 4A).

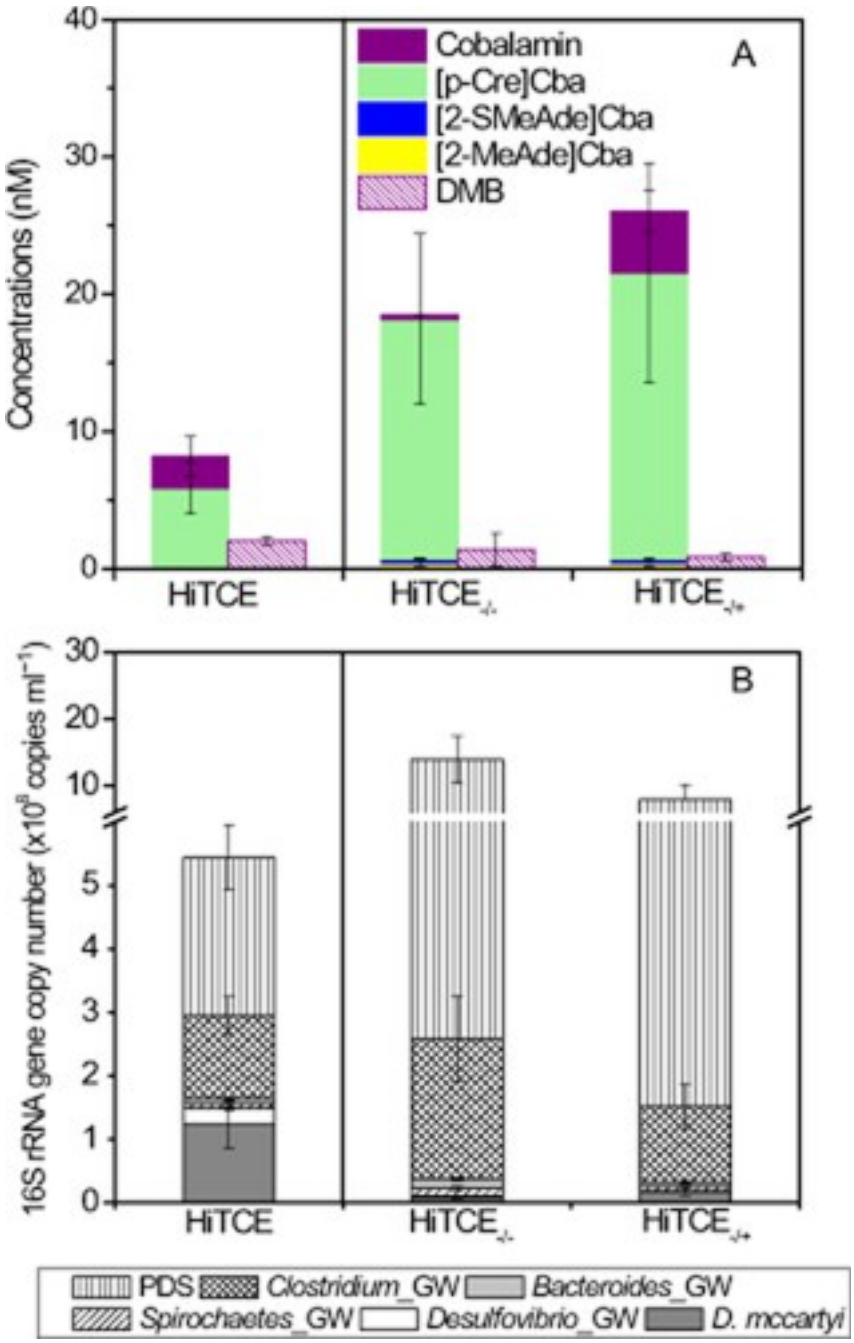

Figure 4

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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 *D. mccartyi* numbers resulting from multiple subculturing events. Therefore, NoTCE was subsequently bioaugmented with HiTCE, the original inoculation culture for NoTCE, to construct NoTCE+$_{HiTCE}$ (Supporting Information Table S6). NoTCE+$_{HiTCE}$ dechlorinated TCE to vinyl chloride (VC) and ethene after 14 days, together with a 32-fold increase in the cobalamin concentration and a 10^3-time increase in the *D. mccartyi* cell number. To determine whether these effects could be specifically attributed to *D. mccartyi*, NoTCE was also bioaugmented with strain 195 isolate (1%, v/v) to construct NoTCE+$_{195}$ (Supporting Information Table S6), in which substantial increases were observed in the cobalamin concentration (92-fold) and the *D. mccartyi* 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 threefold in NoTCE$_{+195}$) compared with NoTCE (Fig. 5B).

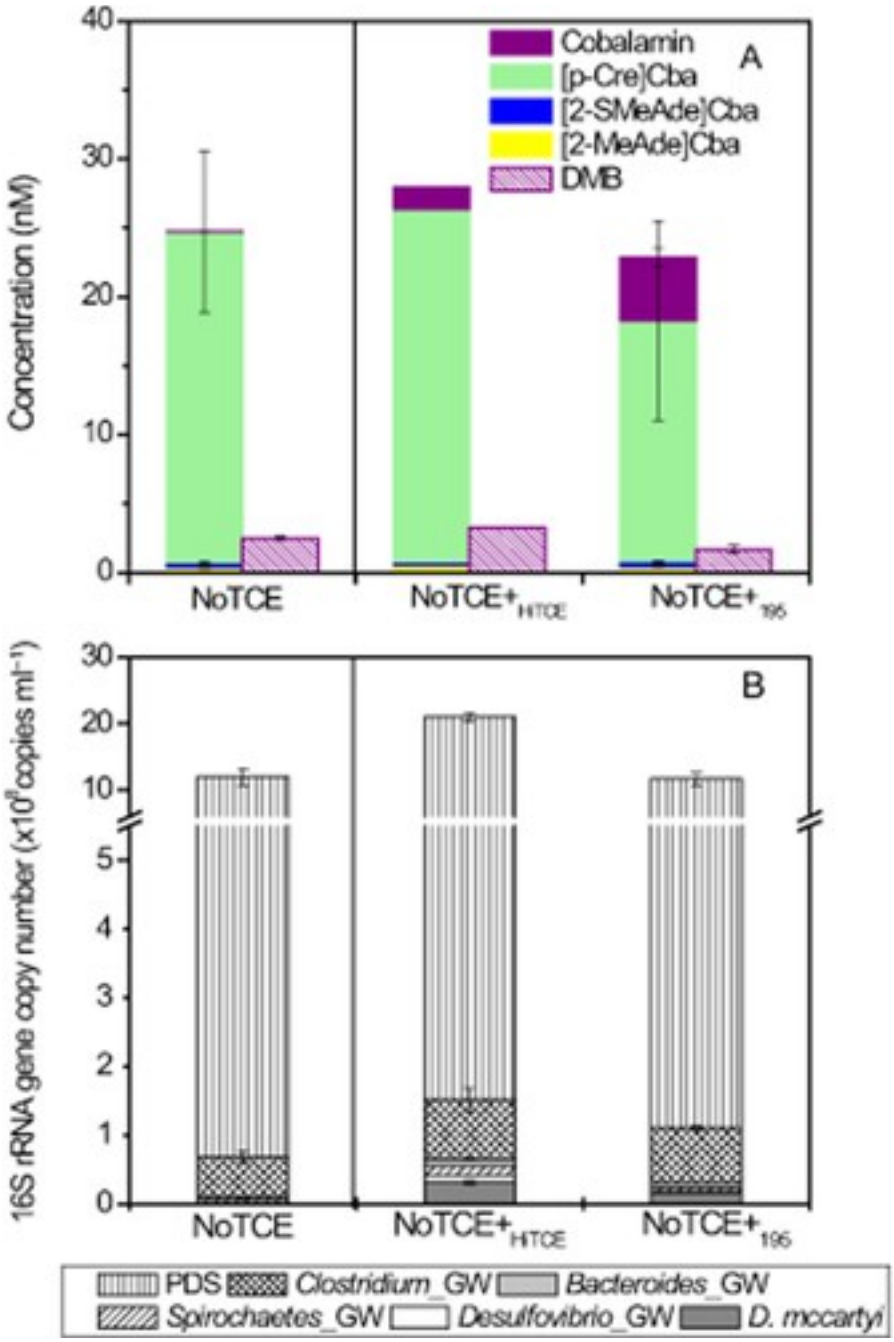

Figure 5

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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 (Martens *et al*., 2002). Because 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: (i) *de novo* biosynthesis; (ii) import of the specific corrinoid generated by other community members or (iii) salvaging and remodelling 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 (Guggisberg *et al*., 2012). Moreover, the total corrinoids detected by bioassays are limited to the corrinoid utilization capabilities of the applied bacterium, potentially resulting in underestimation. For example, Yan and colleagues (2013) reported that [p-Cre]Cba and [Phe]Cba were not detectable using the *Lactobacillus delbrueckii* bioassay. This might be due to the absence of the coordination between the Co ion and the lower ligand (base-off conformation) in the two cobamides, making them non-functional in *L. delbrueckii*. By applying 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 the 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 *D. mccartyi* in the absence of exogenous 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 *D. mccartyi*, while in the NJ enrichments, [p-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 (Stupperich and Krautler, 1988; Stupperich *et al*., 1988; Munder *et al*., 1992), and members of these genera have been identified in ANAS, the original inoculum of SANAS (Richardson *et al*., 2002; Freeborn *et al*., 2005). In addition, recent metagenomic analysis of ANAS found that one *D. mccartyi* strain (ANAS2) in this community possesses a nearly complete corrinoid biosynthesis pathway (Brisson *et al*., 2012), however the function has not been confirmed in this strain. In contrast, the abundance of [p-Cre]Cba in NJ enrichments is likely related to the dominance of *Pelosinus*-like bacteria in *Veillonellaceae* family (Men *et al*., 2013), which were not detected in ANAS subcultures. According to our recent study, *Pelosinus fermentans* strain R7 is another bacterium to generate [p-Cre]Cba, following previously reported *Sporomusa ovata* and *Veillonella parvula* (Stupperich and Krautler, 1988; Stupperich *et al*., 1988; 1989; Chan and Escalante-Semerena, 2011; Crofts *et al*., 2013; Men *et al*., 2014).

The effect of community structure on corrinoid profiles was also reflected by the detection of [5-OHBza]Cba in only the methanogenic enrichments. This is consistent with previous studies which have shown that [5-OHBza]Cba is produced by a number of methanogens (Pol *et al*., 1982; Whitman and Wolfe, 1984; Kräutler *et al*., 1987; Stupperich *et al*., 1987; Stupperich and Krautler, 1988). Methanogens have been shown to be able to produce various corrinoids, and the role of methanogens in communities containing *D. mccartyi* 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 *D. mccartyi* growth and dechlorination, the contribution of methanogens to the corrinoid supply of *D. mccartyi* is relatively small in the communities examined in this study.

D. mccartyi is the only dechlorinating bacterial species found in the NJ enrichments (Men *et al*., 2013). The correlation between cobalamin production and *D. mccartyi* growth in B_{12}-unamended NJ enrichments suggests corrinoid salvaging and remodelling carried out by *D. mccartyi*. Genes encoding enzymes involved in the corrinoid remodelling pathway, including the amidohydrolase CbiZ and the adenosylcobinamide-phosphate guanylyltransferase CobU (Escalante-Semerena, 2007), are present in all sequenced *D. mccartyi* strains (Kube *et al*., 2005; Seshadri *et al*., 2005; McMurdie *et al*., 2009; Yi *et al*., 2012), as well as the *D. mccartyi*strains in the NJ enrichments (Men *et al*., 2013). A previous study with strain 195 revealed that this strain is capable of remodelling added non-functional corrinoids including [p-Cre]Cba into cobalamin in the presence of DMB (Yi *et al*., 2012). Results of this study suggest that the *D. mccartyi* strains in the NJ enrichments also employ the remodelling mechanism to obtain

cobalamin: when the growth of *D. mccartyi* was inhibited, little cobalamin was produced, and the level of [p-Cre]Cba increased; when the growth of *D. mccartyi* was restored in perturbed cultures, cobalamin concentrations rebounded. This correlation between cobalamin concentration and *D. mccartyi* growth supports the hypothesis that *D. mccartyi* acquires corrinoids and free DMB from the other microbes and remodels them into cobalamin. The dominance of [p-Cre]Cba in the NJ enrichments indicates that [p-Cre]Cba, likely produced by *Pelosinus*-like strains, serves as a major substrate for corrinoid remodelling by *D. mccartyi*. The low level of [p-Cre]Cba in the supernatant of HiTCE and the increase of [p-Cre]Cba in the supernatant of NoTCE (Fig. 3C and E) strongly favour the hypothesis that [p-Cre]Cba was exchanged between its producers and *D. mccartyi* by being released into culture supernatant, and was salvaged by *D. mccartyi* for corrinoid remodelling. This hypothesis has recently been tested with experiments in which *Pelosinus fermentans*strain R7 successfully supported the growth of strain 195 in B_{12}-unamended defined consortia by generating [p-Cre]Cba in the presence of DMB. These results highlight the potential role of *Pelosinus*-like species on corrinoid exchange in the NJ enrichments (Men *et al*., 2014).

Besides RDases, isomerases involved in fermentation pathways are also corrinoid dependent (Banerjee and Ragsdale, 2003). Recently, *Pelosinus* sp. strain HCF1 has been reported to ferment lactate to propionate and acetate through methylmalonyl-CoA pathway (Beller *et al*., 2013), in which methylmalonyl-CoA mutase is a corrinoid-dependent isomerase (Banerjee and Ragsdale, 2003). Given that a total of 5.3 mmol lactate was provided to the NJ enrichments per feeding cycle, most of which was fermented to propionate and acetate (Men *et al*., 2013), it is possible that the [p-Cre]Cba detected in the NJ enrichments is generated for this fermentation process.

A notable finding of this study is the detection of free DMB in community supernatants of all cultures examined. Studies of *D. mccartyi* isolates (Yi *et al*., 2012) and constructed co-cultures (Yan *et al*., 2012; 2013) indicate the importance of DMB in corrinoid remodelling. 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 (Munder *et al*., 1992; Taga *et al*., 2007), 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 catalyzed by the enzyme BluB (Campbell *et al*., 2006; Gray and Escalante-Semerena, 2007; Taga *et al*., 2007). However, information on anaerobic DMB

production is still very limited. Previous labelling 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 (Munder *et al*., 1992). However, the enzymes involved in anaerobic DMB biosynthesis have not yet been identified. Due to the importance of DMB in the corrinoid remodelling processes of *D. mccartyi* 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 *D. mccartyi*-containing communities, as well as ecological relationships between *D. mccartyi* 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.

Experimental procedures

Cultures and growth conditions

SANASB12 and SANAS are the subcultures of ANAS, a well-maintained TCE-dechlorinating enrichment characterized in previous studies (Richardson *et al*., 2002; Freeborn *et al*., 2005; Lee *et al*., 2006). They were constructed by inoculating 5 ml of ANAS culture (5%, v/v) into 95 ml of 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 (He *et al*., 2007), except that a modified Wolin vitamin stock excluding B_{12} was used (Wolin *et al*., 1963). 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 (Pfennig, 1974), 1 ml of a Na_2SeO_3-Na_2WO_4solution (Brysch *et al*., 1987), and 10 mg of resazurin. Cultures were amended with 48 mmol 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 NJ and maintained under conditions listed in Supporting Information Table S1. The high initial TCE concentration

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 (Men *et al*., 2013). In order to investigate the effects of the presence of *D. mccartyi* 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) (Supporting Information Table S1). Experiments were carried out after 40 subculturing events for LoTCE, LoTCEB12, HiTCE and HiTCEB12, and six 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 *M. barkeri* strain Fusaro using cyanidation and solid phase extraction as previously described (Gray and Escalante-Semerena, 2009a; Yi *et al*., 2012). Briefly, cells were collected, resuspended in 20 ml of methanol with 20 mg of KCN per gram of cells and incubated at 60°C for 1.5 h with periodic mixing. Samples were then dried and resuspended in 20 ml of deionized (DI) water, desalted using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) and eluted in 2 ml of 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 [p-Cre]Cba were extracted from bacterial cultures and purified as described previously (Allen and Stabler, 2008). A molar extinction coefficient of 30 800 M^{-1} cm^{-1} at 367.5 nm was used for quantification (Allen and Stabler, 2008). The identity of each corrinoid was confirmed by mass spectrometry.

Monocyanocobyric acid standard was prepared as described (Butler *et al*., 2006), 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. Five millilitres of aliquots were desalted with a C_{18} Sep-Pak cartridge, and eluted in 3 ml of methanol. The eluates were dried, resuspended in DI water and stored at −80°C. Monocyanocobyric acid was purified using the solvent gradient as described (Butler *et al*., 2006).

Extraction of corrinoid and lower ligand bases

Cell pellets were collected from 200 to 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 of DI water, and eluted with 3 ml of 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 (Richardson *et al*., 2002; Men *et al*., 2013).

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^{-1} 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 0.1 min 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 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 [p-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 (Allen and Stabler, 2008). Benzimidazoles were quantified by monitoring the transition from the singly charged molecular ion to unique product ions (Supporting Information Table S2).

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 (2008), 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 (Campbell *et al*., 2006; Taga *et al*., 2007). A modified quantitative bioassay was further performed as described by Croft and Taga (pers. comm.). Briefly, *S. meliloti bluB* was grown in M9 minimal media supplemented with 1 mg ml^{-1} of 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 h, and the fluorescent phenotype was measured by excitation at 360 nm and emission at 460 nm.

DNA isolation and quantification by qPCR

Genomic DNA was extracted from 1.5 ml of 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 (Men *et al*., 2013).

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