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Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity

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Dehalococcoides mccartyi strains are corrinoid-auxotrophic Bacteria and axenic cultures that require vitamin B₁₂ (CN-Cbl) to conserve energy via organohalide respiration. Cultures of *D. mccartyi* strains BAV1, GT and FL2 grown with limiting amounts of 1 µg l⁻¹ CN-Cbl quickly depleted CN-Cbl, and reductive dechlorination of polychlorinated ethenes was incomplete leading to vinyl chloride (VC) accumulation. In contrast, the same cultures amended with 25 µg l⁻¹ CN-Cbl exhibited up to 2.3-fold higher dechlorination rates, 2.8–9.1-fold increased growth yields, and completely consumed growth-supporting chlorinated ethenes. To explore whether known cobamide-producing microbes supply *Dehalococcoides* with the required corrinoid cofactor, co-culture experiments were performed with the methanogen *Methanosarcina barkeri* strain Fusaro and two acetogens, *Sporomusa ovata* and *Sporomusa* sp. strain KB-1, as *Dehalococcoides* partner populations. During growth with H₂/CO₂, *M. barkeri* axenic cultures produced 4.2 ± 0.1 µg l⁻¹ extracellular cobamide (factor III), whereas the *Sporomusa* cultures produced phenolyl- and *p*-cresolyl-cobamides. Neither factor III nor the phenolic cobamides supported *Dehalococcoides* reductive dechlorination activity suggesting that *M. barkeri* and the *Sporomusa* sp. cannot fulfil *Dehalococcoides*' nutritional requirements. *Dehalococcoides* dechlorination activity and growth occurred in *M. barkeri* and *Sporomusa* sp. co-cultures amended with 10 µM 5',6'-dimethylbenzimidazole (DMB), indicating that a cobalamin is a preferred corrinoid cofactor of strains BAV1, GT and FL2 when grown with chlorinated ethenes as electron acceptors. Even though the methanogen and acetogen populations tested did not produce cobalamin, the addition of DMB enabled guided biosynthesis and generated a cobalamin that supported *Dehalococcoides*' activity and growth. Guided cobalamin biosynthesis may offer opportunities to sustain and enhance *Dehalococcoides* activity in contaminated subsurface environments.

1. Introduction

Chlorinated compounds, including tetrachloroethene (PCE) and trichloroethene (TCE), are widespread and toxic environmental pollutants [1,2]. Strains of the organohalide-respiring species *Dehalococcoides mccartyi* couple growth to the reductive dechlorination of a variety of chlorinated priority pollutants including polychlorinated biphenyls (PCBs), chlorobenzenes and chlorinated solvents. Some *D. mccartyi* strains dechlorinate PCE, TCE, dichloroethenes (DCEs) and vinyl chloride (VC) to non-toxic ethene, and thus are of great interest to restore aquifers contaminated with chlorinated ethenes [2–4].

Dehalococcoides mccartyi strains grow in completely synthetic, defined media, but cannot de novo synthesize the corrin ring and require exogenous vitamin B₁₂ (cyanocobalamin, CN-Cbl) [2]. Corrinoids, such as CN-Cbl, contain a heterocyclic tetrapyrrole ring with a central cobalt atom [5]. Some Archaea and Bacteria synthesize corrinoids with an upper β-ligand moiety and a lower α-ligand moiety, so-called cobamides, which participate in important biological processes such as methanogenesis, acetogenesis and organohalide

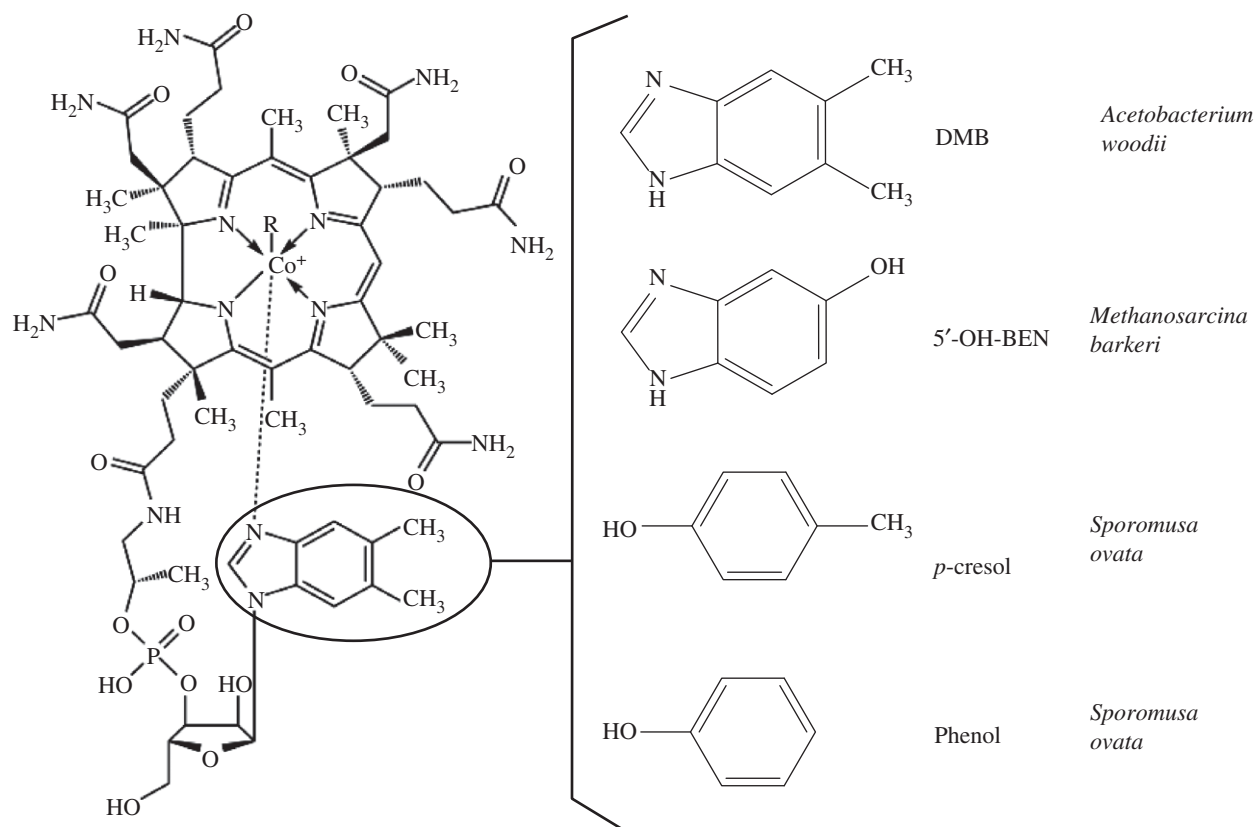


Figure 1. Structures of cobalamin and the lower α -ligands of several characterized cobamides. In vitamin B₁₂ (cyanocobalamin, CN-Cbl), a cyano group is the upper β -ligand and DMB is the lower α -ligand (circled). The structures were redrawn from [16,17]. DMB, 5',6'-dimethylbenzimidazole; 5'-OH-BEN, 5'-hydroxybenzimidazole.

respiration [5–7]. Cobamides with 5',6'-dimethylbenzimidazole (DMB) as the lower α -ligand are cobalamins such as adenosylcobalamin, hydroxycobalamin and cyanocobalamin (CN-Cbl). A cobalamin or a cobamide serve as cofactors for the PCE and TCE reductive dehalogenases (RDases) of several organohalide-respiring genera such as *Dehalobacter*, *Desulfotobacterium* and *Sulfurospirillum*, and exemplify the crucial roles these corrinoids play in the energy metabolism of organohalide-respiring Bacteria [7–10].

Genome sequence analysis demonstrated that *D. mccartyi* strains are corrinoid auxotrophs and lack the ability for de novo corrinoid biosynthesis [11,12]. A previous survey demonstrated that only about half (209 out of 410) CN-Cbl-requiring Bacteria possessed genes encoding corrinoid biosynthesis pathways [11]. Genes encoding the BtuBFC type CN-Cbl/cobamide transport system were found in most (more than 90%) of the corrinoid-auxotrophic Bacteria [11], indicating external CN-Cbl/cobamide uptake rather than de novo biosynthesis is a common strategy of microorganisms to acquire this essential cofactor. Thus, it is not surprising that *Dehalococcoides* also possess BtuBFC gene homologues as well as cobinamide (i.e. a cobamide lacking the lower α -ligand) salvage genes (*cobU* and *cblZ*) to save the energy cost associated with adenosylcobalamin (Ado-Cbl) de novo biosynthesis [11].

Enhanced *Dehalococcoides* growth and faster dechlorination rates were observed in co- or tri-cultures of *D. mccartyi* strain 195 and corrinoid-producers such as *Acetobacterium woodii*, *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* Hildenborough, suggesting that strain 195 benefited from the corrinoids produced in the co-cultures [13,14]. Recently, interspecies cobalamin transfer from *Geobacter lovleyi* to *D. mccartyi* strains BAV1 and FL2 was demonstrated in

CN-Cbl-free co-cultures [15]. *Geobacter sulfurreducens* did not support *Dehalococcoides* activity unless DMB was supplied to the growth medium suggesting that only the cobamides with specific types of lower ligands, such as DMB, meet *Dehalococcoides*' nutritional requirements.

In anoxic subsurface environments, *Dehalococcoides* are members of diverse microbial communities, and some populations synthesize cobamides with different lower α -ligands [16,17]. Cobalamin and the naturally occurring cobamides tested in this study are shown in figure 1. For example, 5'-hydroxybenzimidazolyl-cobamide (factor III) or adenosylcobamide (pseudo vitamin B₁₂) are typically produced by methanogenic Archaea as the cofactors for methyltransferases involved in methane formation [18]. Acetogenic Bacteria synthesize benzimidazolyl- or phenolyl-type cobamides required for specific enzymes of the Wood–Ljungdahl pathway [18]. Cobalamin (5',6'-dimethylbenzimidazolyl-cobamide) was identified as the corrinoid produced by *A. woodii*, phenolyl- and *p*-cresolyl-cobamide were purified from *S. ovata*, and 5'-methylbenzimidazolyl-cobamide was isolated from sulfate-reducers, such as *Desulfobacterium autotrophicum* and *Desulfobulbus propionicus* [19,20].

In this study, we investigated *Dehalococcoides* reductive dechlorination activity and growth during the co-cultivation with corrinoid-producing methanogenic Archaea and acetogenic Bacteria. Co-cultures established in CN-Cbl-free medium demonstrated interspecies cobamide transfer and identified the specific type(s) of corrinoid that *Dehalococcoides* can use. Knowledge of how *Dehalococcoides*' nutritional requirements are met in natural communities will reveal the ecophysiology of strictly organohalide-respiring, corrinoid-auxotrophic Chloroflexi. Understanding the bottlenecks (i.e.

controls) limiting the activity of organohalide-respiring Chloroflexi may lead to innovative engineering approaches that enhance reductive dechlorination rates and extents and enable more efficient bioremediation applications.

2. Methods

(a) Pure cultures and growth conditions

Dehalococcoides mccartyi strain BAV1 (ATCC BAA-2100), strain GT (ATCC BAA-2099) and strain FL2 (ATCC BAA-2098) were grown in 160 ml serum bottles containing 100 ml reduced, bicarbonate-buffered, defined mineral salts medium amended with acetate (5 mM) and a N₂-CO₂ (80/20, v/v) headspace [21–23]. Following autoclaving, the bottles were amended with 10 ml hydrogen and 25 µg l⁻¹ CN-Cbl as described [15]. Neat TCE (5 µl, 99%, Fisher, Pittsburgh, PA, USA) and *cis*-DCE (5 µl, 99.5%, Sigma-Aldrich-Fluka, St Louis, MO, USA) were added with a microlitre syringe (Hamilton, Reno, NV, USA). Following equilibration, the bottles received 3 per cent (v/v) inocula from dechlorinating cultures maintained under the same conditions. In addition, culture bottles amended with 1 µg l⁻¹ CN-Cbl were maintained to evaluate the effects of low CN-Cbl concentrations on *Dehalococcoides* growth yields and dechlorination activities. Pure cultures of *M. barkeri* strain Fusaro (DSM 804), *S. ovata* (DSM 2662) and *Sporomusa* sp. strain KB-1 (16S rRNA gene GenBank accession no. AY780559.1) were maintained in the same defined mineral salts medium except that acetate, CN-Cbl and chlorinated solvent additions were omitted. All culture vessels were incubated stationary, with the stoppers down, at 30°C in the dark.

(b) Co-cultures

Triplicate co-cultures containing one of the three *D. mccartyi* strains and either *M. barkeri* or one of the *Sporomusa* sp. were established in 160 ml vessels inside an anoxic chamber as described [15]. The vessels were amended with 5 mM acetate and contained a H₂-CO₂ (80/20, v/v) headspace. To avoid CN-Cbl carry-over, cells for inoculation were collected by centrifugation at 14 000g for 10 min at room temperature, and suspended in CN-Cbl-free medium prior to inoculation. The positive control inocula were prepared in the same way and inoculated into culture vessels amended with 25 µg l⁻¹ CN-Cbl. *Dehalococcoides mccartyi* pure cultures without CN-Cbl served as negative controls. Additional co-culture replicates received 10 µM DMB (more than 99% purity, Sigma-Aldrich-Fluka).

(c) DNA extraction and 16S rRNA gene quantification

Cells from *Dehalococcoides* pure cultures and co-cultures were harvested from 1 ml culture suspension as described [15,24]. Briefly, total genomic DNA was extracted using the MO BIO Soil DNA Isolation kit (MO BIO, Carlsbad, CA, USA), and a high efficiency bead ruptor homogenizer (Omni International, Kennesaw, GA, USA) following the manufacturer's protocols. Samples were processed with the bead ruptor homogenizer at a speed 3.25 m s⁻¹ for 5 min. Quantitative real-time PCR (qPCR) to enumerate *Dehalococcoides* 16S rRNA gene copies followed established protocols using primer set Dhc1200F/Dhc1271R and probe FAM-BHQ1 Dhc1240 [24].

(d) Corrinoid measurements

(i) Microbiological B₁₂ assay

Quantification of extracellular cobamides in pure and co-cultures was performed using a microbiological assay with *Lactobacillus delbrueckii* subsp. *lactis* (ATCC 7830) as the cobamide-auxotroph test organism following described procedures [15]. To detect intracellular cobamides, whole cells from 1.5 ml *S. ovata* or

Sporomusa sp. strain KB-1 cultures were collected by centrifugation at 14 000 g for 2 min, suspended in sterile water, and broken either by heating at 95°C for 15 min or by sonicating with a Branson 250 Sonifier Analog Cell Disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) for 15 min at 50 per cent duty cycle at an output of 60 W.

(ii) Total cobamide extraction and purification

Total intracellular cobamides were extracted and purified using an established protocol with the following modifications [25]. *Sporomusa ovata* or *Sporomusa* sp. strain KB-1 cultures (1 litre each) grown with H₂/CO₂ were centrifuged at 15 000g for 15 min at 4°C. Following removal of the supernatants, each cell pellet was suspended in 5 ml deionized water, the pH was adjusted to 5–6 with 3 per cent (v/v) glacial acetic acid, and KCN (100 mM stock solution) was added to reach a final concentration of 10 mM. The suspensions were vigorously shaken, each transferred to sterile 50 ml plastic tubes and incubated for 20 min in a boiling water bath. Following centrifugation at 15 000g for 15 min, the supernatants were collected and the pellets were extracted a second time. The combined supernatants were mixed with 0.01 volumes of a 3 per cent acetic acid solution prior to loading onto a Sep-Pak C18 cartridge (Waters Corp, Milford, MA, USA), which had been equilibrated with 2 ml 100 per cent methanol and 40 ml deionized water. Following sample loading, the cartridge was washed with 10 ml deionized water (20 interstitial volumes) and 7.5 ml 10 per cent methanol (15 interstitial volumes) prior to the final elution step with 10 ml 50 per cent methanol (20 interstitial volumes). The slightly pink-coloured solution obtained from the final elution step was vacuum dried and the residues were suspended in 0.5 ml sterile, deionized water.

(iii) HPLC analysis of cyanocobamides

Cyanocobamides were analysed using an Agilent 1200 series HPLC system equipped with an Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm) and a diode array detector at a detection wavelength of 361 nm. Samples (20 µl each) were injected and separated at 30°C at a flow rate of 1.0 ml min⁻¹ using 0.1 per cent acetic acid in water (eluent A) and 0.1 per cent acetic acid in methanol (eluent B) as mobile phases. The initial ratio of 90 per cent A/10 per cent B was linearly decreased to 35 per cent A/65 per cent B over a 12 min time window followed by a 4 min hold, before equilibration with 90 per cent A. CN-Cbl was identified by comparing retention times and absorption spectra with authentic CN-Cbl standards (more than 98% purity, Fisher Scientific, Pittsburgh, PA, USA) dissolved in deionized water.

(e) Other analytical methods

Methane, ethene and chlorinated ethenes were quantified using an Agilent 7890 gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m × 0.32 mm × 1.8 µm) as described [22]. Acetate was analysed using an Agilent 1200 series HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). Samples were acidified with 1 M H₂SO₄ in a ratio of 19:1 (v/v) and separated with 4 mM aqueous H₂SO₄ as the mobile phase at a flow rate of 0.6 ml min⁻¹ and quantified using a UV detector set to 210 nm.

3. Results

(a) CN-Cbl-dependent reductive dechlorination and growth of *D. mccartyi* strains

In defined medium amended with 25 µg l⁻¹ CN-Cbl, strain BAV1 and strain GT cultures produced stoichiometric

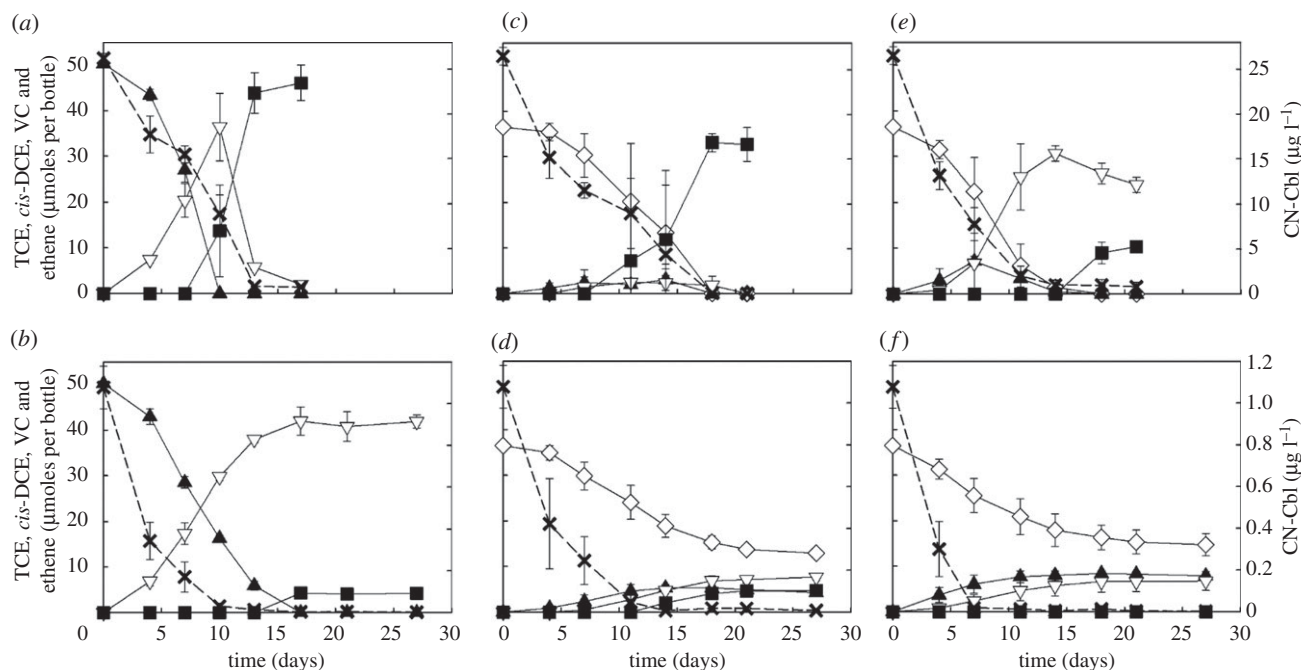


Figure 2. Dechlorination of TCE or *cis*-DCE by *Dehalococcoides* under CN-Cbl limiting and non-limiting conditions. Strain BAV1 cultures were supplied with *cis*-DCE as electron acceptor and (a) 25 $\mu\text{g l}^{-1}$ CN-Cbl or (b) 1 $\mu\text{g l}^{-1}$ CN-Cbl. Strain GT cultures were supplied with TCE as electron acceptor and (c) 25 $\mu\text{g l}^{-1}$ CN-Cbl or (d) 1 $\mu\text{g l}^{-1}$ CN-Cbl. Strain FL2 cultures were supplied with TCE as electron acceptor and (e) 25 $\mu\text{g l}^{-1}$ CN-Cbl or (f) 1 $\mu\text{g l}^{-1}$ CN-Cbl. Open diamond, TCE; filled triangle, *cis*-DCE; open inverted triangle, vinyl chloride; filled square, ethene; cross symbol, CN-Cbl. Error bars represent the standard deviations of triplicate samples and are not shown if they are smaller than the symbols.

amounts of ethene from $50.3 \pm 0.4 \mu\text{moles}$ of *cis*-DCE (figure 2a) and $36.5 \pm 0.9 \mu\text{moles}$ of TCE (figure 2c) provided as electron acceptor, respectively. Strain FL2 cultures produced a mixture of VC and ethene (23.9 ± 1.7 and $10.3 \pm 0.9 \mu\text{moles}$, respectively) during a 3-week incubation period (figure 2e). Cultures that received 1 $\mu\text{g l}^{-1}$ CN-Cbl exhibited lower dechlorination rates and incomplete dechlorination. Strain BAV1 cultures dechlorinated *cis*-DCE ($50.3 \pm 0.4 \mu\text{moles}$) to VC ($42.0 \pm 3.1 \mu\text{moles}$) and small amounts of ethene ($4.3 \pm 0.4 \mu\text{moles}$) by day 17. VC remained the major dechlorination product ($90.8 \pm 0.1\%$) after 27 days and ethene accounted for $9.2 \pm 0.1\%$ (figure 2b). In strain GT cultures, more than one third of the initial TCE amount ($36.5 \pm 0.9 \mu\text{moles}$) remained after 27 days of incubation, and *cis*-DCE ($4.3 \pm 0.2 \mu\text{moles}$), VC ($7.7 \pm 0.7 \mu\text{moles}$) and ethene ($4.7 \pm 1.1 \mu\text{moles}$) were detected (figure 2d). TCE was dechlorinated at a rate of $9.0 \pm 1.1 \mu\text{moles l}^{-1} \text{d}^{-1}$ or about 2.2-fold slower compared with the TCE dechlorination rates ($19.6 \pm 0.2 \mu\text{moles l}^{-1} \text{d}^{-1}$) observed in strain GT cultures that received 25 $\mu\text{g l}^{-1}$ CN-Cbl (figures 2c,d). Similarly, strain FL2 cultures dechlorinated TCE at lower rates (i.e. 9.8 ± 2.3 versus $22.2 \pm 0.8 \mu\text{moles l}^{-1} \text{d}^{-1}$ in medium amended with 25 $\mu\text{g l}^{-1}$ CN-Cbl), and after a 4-week incubation period, $40.3 \pm 6.8\%$ of the initial TCE was still present with *cis*-DCE ($7.9 \pm 0.4 \mu\text{moles}$) and VC ($6.8 \pm 2.1 \mu\text{moles}$) as the dechlorination products but no ethene was formed (figure 2f).

CN-Cbl concentrations in the medium decreased concomitantly with reductive dechlorination and *Dehalococcoides* growth, and the initial CN-Cbl concentration of 1 $\mu\text{g l}^{-1}$ declined to below 20 ng l^{-1} during the 18-day incubation period (figure 2b,d,f). By day 27, reductive dechlorination of the remaining chlorinated ethenes ceased, and the CN-Cbl concentrations in strain GT, strain FL2 and strain BAV1 cultures were $7.7 \pm 2.0 \text{ ng l}^{-1}$, below 5 ng l^{-1} (i.e. below

the quantification limit of the microbiological B₁₂ assay), and below 2 ng l^{-1} (i.e. below the detection limit), respectively. CN-Cbl was depleted at higher rates in the 25 $\mu\text{g l}^{-1}$ CN-Cbl-amended *Dehalococcoides* cultures. At day 17, when BAV1 cultures had completely dechlorinated *cis*-DCE to ethene, CN-Cbl concentrations dropped to $0.7 \pm 0.1 \mu\text{g l}^{-1}$, averaging an uptake rate of $1500 \text{ ng l}^{-1} \text{d}^{-1}$ versus $63 \text{ ng l}^{-1} \text{d}^{-1}$ in the BAV1 cultures amended with 1 $\mu\text{g l}^{-1}$ CN-Cbl (figure 2a,b). Strain GT and strain FL2 cultures depleted CN-Cbl from an initial amount of $26.5 \pm 1.0 \mu\text{g l}^{-1}$ to 0.03 ± 0.03 and $1.0 \pm 0.7 \mu\text{g l}^{-1}$, respectively, at 25- and 24-fold higher rates compared with those rates observed in the corresponding cultures amended with 1 $\mu\text{g l}^{-1}$ CN-Cbl (figure 2c-f).

qPCR analysis demonstrated that limiting amounts of 1 $\mu\text{g l}^{-1}$ CN-Cbl affected *Dehalococcoides* growth yields, which were calculated as described [22]. For example, $1.4 \pm 0.8 \times 10^7$ BAV1 cells per μmole of Cl^- released were obtained in cultures amended with 1 $\mu\text{g l}^{-1}$ CN-Cbl, whereas $1.3 \pm 0.1 \times 10^8$ BAV1 cells per μmole of Cl^- released (a 9.1-fold increase) were measured in cultures amended with 25 $\mu\text{g l}^{-1}$ CN-Cbl (table 1). Similar results were observed in strain GT cultures with an average 2.8-fold higher growth yield in medium amended with 25 $\mu\text{g l}^{-1}$ CN-Cbl (table 1).

(b) Cobamide production in methanogenic and acetogenic cultures

During a 43-day incubation period, extracellular cobamide concentrations gradually increased to $4.2 \pm 0.1 \mu\text{g l}^{-1}$ in *M. barkeri* strain Fusaro cultures concomitantly with methane formation (figure 3a). No extracellular cobamides were detected in the acetate-producing *S. ovata* (figure 3b) and *Sporomusa* sp. strain KB-1 cultures, which was unexpected because *Sporomusa* spp. are known cobamide producers

Table 1. Comparison of *Dehalococcoides mccartyi* growth yields in axenic and in co-cultures amended with DMB.

culture	CN-Cbl ($\mu\text{g l}^{-1}$)	DMB ^a	<i>D. mccartyi</i> 16S rRNA gene copies per ml			growth yield (cells per $\mu\text{mole Cl}^{-}$ released)
			inoculum	final	fold increase ^e	
axenic cultures						
BAV1	1	—	$6.3 \pm 2.0 \times 10^6$	$1.3 \pm 0.4 \times 10^{7b}$	2.1	$1.4 \pm 0.8 \times 10^7$
	25	—	$6.3 \pm 2.0 \times 10^6$	$1.3 \pm 0.2 \times 10^{8c}$	20.3	$1.3 \pm 0.1 \times 10^8$
GT	1	—	$4.0 \pm 0.5 \times 10^6$	$2.1 \pm 0.3 \times 10^{7b}$	5.4	$5.3 \pm 1.3 \times 10^7$
	25	—	$4.0 \pm 0.5 \times 10^6$	$1.5 \pm 0.1 \times 10^{8c}$	36.5	$1.5 \pm 0.3 \times 10^8$
FL2	1	—	$2.4 \pm 0.3 \times 10^6$	$2.3 \pm 0.7 \times 10^{6b}$	1.0	— ^f
	25	—	$2.4 \pm 0.3 \times 10^6$	$3.7 \pm 1.1 \times 10^{7c}$	15.3	$4.4 \pm 1.5 \times 10^7$
co-cultures						
BAV1/KB-1	—	+	$6.3 \pm 2.0 \times 10^6$	$1.9 \pm 0.1 \times 10^{8d}$	29.8	$2.1 \pm 0.2 \times 10^8$
BAV1/Fusaro	—	+	$6.3 \pm 2.0 \times 10^6$	$2.0 \pm 0.1 \times 10^{8c}$	30.9	$2.1 \pm 0.2 \times 10^8$
GT/KB-1	—	+	$4.0 \pm 0.5 \times 10^6$	$2.1 \pm 0.3 \times 10^{8d}$	51.5	$2.1 \pm 0.3 \times 10^8$
GT/Fusaro	—	+	$4.0 \pm 0.5 \times 10^6$	$1.6 \pm 0 \times 10^{8c}$	41.0	$1.8 \pm 0.1 \times 10^8$
FL2/KB-1	—	+	$2.4 \pm 0.3 \times 10^6$	$9.5 \pm 1.6 \times 10^{7d}$	39.6	$9.0 \pm 1.4 \times 10^7$
FL2/Fusaro	—	+	$2.4 \pm 0.3 \times 10^6$	$9.0 \pm 2.7 \times 10^{7c}$	37.3	$9.8 \pm 2.1 \times 10^7$

^aDMB supplied at 10 μM .^bBiomass collected at day 27.^cBiomass collected at day 21.^dBiomass collected at day 17.^eData averaged from triplicates.^fNo growth occurred.

when grown via H_2/CO_2 reductive acetogenesis. Cobamides were also not detected in *Sporomusa* cell lysates suggesting that *Sporomusa* produced cobamide(s), to which the indicator organism *L. delbrueckii* subsp. *lactis* did not respond (i.e. the cobamide cannot be detected and quantified with the microbiological B_{12} assay).

(c) Reductive dechlorination in co-cultures

In *D. mccartyi* strain BAV1 and *M. barkeri* strain Fusaro co-cultures amended with 25 $\mu\text{g l}^{-1}$ CN-Cbl, reductive dechlorination and methane formation occurred concomitantly. Strain BAV1 dechlorinated 53.1 ± 0.6 μmoles of *cis*-DCE to stoichiometric amounts of ethene with concomitant methane formation (86.9 ± 15.9 μmoles) by strain Fusaro within 25 days (figure 4a). In BAV1/Fusaro co-cultures not amended with CN-Cbl, methane was formed (225.2 ± 26.2 μmoles per bottle) and maximum amounts of 3.7 ± 0.2 $\mu\text{g l}^{-1}$ cobamide were measured as CN-Cbl equivalents using the microbiological B_{12} assay in the culture suspensions after 30 days (figure 4b). Only negligible reductive dechlorination activity occurred in these co-cultures, and less than 12 per cent of the initial amount of *cis*-DCE was dechlorinated to VC (6.1 ± 0.1 μmoles). Negative controls (i.e. strain BAV1 cultures without CN-Cbl) produced 9.8 ± 0.9 μmoles of VC (figure 4c).

Dehalococcoides/S. ovata co-cultures amended with 25 $\mu\text{g l}^{-1}$ CN-Cbl exhibited acetate formation concomitantly with complete reductive dechlorination of 65.9 ± 1.7 μmoles of *cis*-DCE to ethene (figure 4d), indicating growth of both populations. Both *S. ovata* and *Sporomusa* sp. strain KB-1 grew

in co-cultures lacking CN-Cbl but extracellular cobamides were not detected using the microbiological B_{12} assay, and only 10.6 ± 3.6 and 10.4 ± 0.5 μmoles of VC were produced in BAV1/*S. ovata* and BAV1/*Sporomusa* sp. strain KB-1 co-cultures, respectively (figure 4e,f). These findings demonstrate that *Sporomusa* sp. were unable to support BAV1 reductive dechlorination activity. Activities of *D. mccartyi* strains GT and FL2 were also tested in the co-cultures with *M. barkeri* strain Fusaro, *S. ovata* and *Sporomusa* sp. strain KB-1. Only insignificant dechlorination of TCE to *cis*-DCE (less than 0.5% of the initial amount of TCE) occurred in these co-cultures (data not shown).

(d) Guided biosynthesis of cobalamin in *Sporomusa*

The CN-Cbl standard (5 mg l^{-1}) eluted at a retention time of 6.94 min (figure 5a). HPLC separation of cyanocobamides extracted from *Sporomusa* sp. strain KB-1 (figure 5b) and *S. ovata* (data not shown) cells collected from cultures not amended with DMB yielded two major peaks with retention times of 9.80 and 11.25 min, matching previous reports that two types of cobamides, phenolyl-cobamide and *p*-cresolyl-cobamide, were synthesized by *S. ovata* [26]. The addition of 10 μM DMB to the culture medium did not affect growth, and *Sporomusa* sp. strain KB-1 cultures reached similar OD 600 nm values of 0.040–0.041 (data not shown). Distinct cyanocobamide patterns were observed in *Sporomusa* sp. strain KB-1 cultures amended with DMB. The peak areas corresponding to phenolyl-cobamide and *p*-cresolyl-cobamide both decreased by about 80 per cent, and a major peak with a retention time of 6.94 min, identical to that of

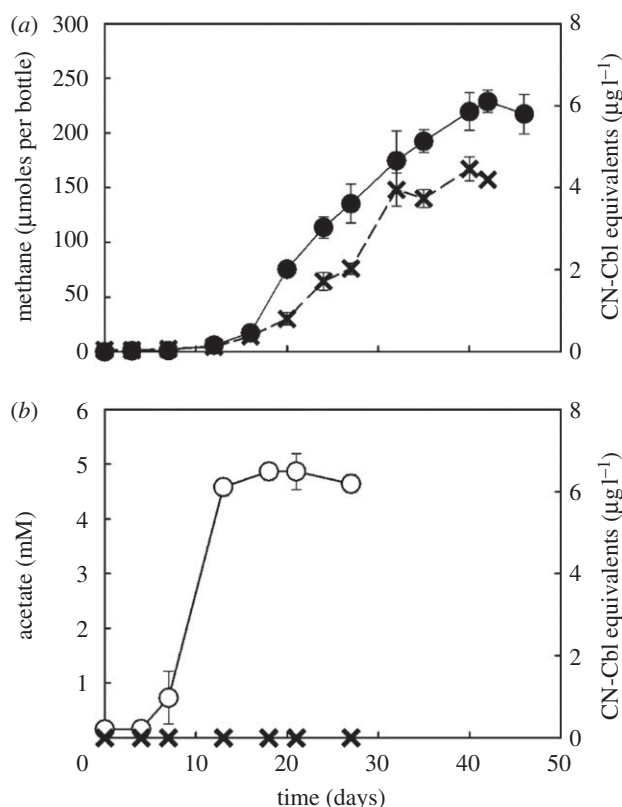


Figure 3. Extracellular cobamide production by *Methanosarcina barkeri* strain Fusaro and *Sporomusa ovata* during autotrophic growth with H_2/CO_2 . (a) Methane and cobamide production by *Methanosarcina barkeri* strain Fusaro and (b) acetate production and lack of measureable cobamide(s) in *Sporomusa ovata* cultures. Filled circle, methane; open circle, acetate, cross symbol, cobamide(s) reported as CN-Cbl equivalents. Error bars represent the standard deviations of triplicate samples and are not shown if they are smaller than the symbols.

authentic CN-Cbl, emerged (figure 5c). These findings provide evidence for guided biosynthesis [27], and cyanocobalamin was the major cobamide when 10 μM DMB was added to the growth medium.

(e) Recovery of *Dehalococcoides* dechlorination activity in DMB-amended co-cultures

The addition of 10 μM DMB to non-dechlorinating *Dehalococcoides*/*Sporomusa* sp. strain KB-1 co-cultures stimulated reductive dechlorination activity. DMB-amended BAV1/KB-1 and GT/KB-1 co-cultures completely dechlorinated *cis*-DCE ($52.1 \pm 0.5 \mu\text{moles}$) and TCE ($34.4 \pm 0.7 \mu\text{moles}$) to ethene, respectively (figure 6a,b). FL2/KB-1 co-cultures reduced the initial TCE amount to VC ($8.5 \pm 1.2 \mu\text{moles}$) and ethene ($28.5 \pm 2.0 \mu\text{moles}$) (figure 6c). Concomitant with *cis*-DCE or TCE dechlorination, extracellular cobamides measured using the microbiological B_{12} assay after 17 days of incubation gradually increased from below the detection limit (i.e. less than 2 ng l^{-1}) to 2.5 ± 0.4 , 0.9 ± 0.4 and $1.0 \pm 0.3 \mu\text{g l}^{-1}$ in strain BAV1/strain KB-1, strain GT/strain KB-1 and strain FL2/strain KB-1 co-cultures, respectively (figure 6a–c).

Guided cobalamin biosynthesis through lower ligand exchange with DMB was also observed in *Dehalococcoides*/*M. barkeri* strain Fusaro co-cultures (figure 6d–f). Methane formation occurred and extracellular cobamide concentrations

increased to $1.4\text{--}1.9 \mu\text{g l}^{-1}$. In addition, *cis*-DCE and TCE were completely dechlorinated to ethene in the strain BAV1 and strain GT co-cultures, respectively, and strain FL2 co-cultures dechlorinated the initial TCE amount ($34.4 \pm 0.7 \mu\text{moles}$) of VC ($16.0 \pm 4.1 \mu\text{moles}$) and ethene ($18.8 \pm 5.3 \mu\text{moles}$).

Negligible *Dehalococcoides* growth was observed in the co-cultures without DMB. In contrast, *Dehalococcoides* cell densities increased 29.8- to 51.5-fold in the DMB-amended co-cultures with *Sporomusa* sp. strain KB-1. Strain BAV1 cells increased from $6.3 \pm 2.0 \times 10^6 \text{ cells ml}^{-1}$ (cells introduced with the inoculum) to $1.9 \pm 0.1 \times 10^8 \text{ cells ml}^{-1}$, strain GT cells increased from $4.0 \pm 0.5 \times 10^6$ to $2.1 \pm 0.3 \times 10^8 \text{ cells ml}^{-1}$, and strain FL2 cells increased from $2.4 \pm 0.3 \times 10^6$ to $9.5 \pm 1.6 \times 10^7 \text{ cells ml}^{-1}$. In the DMB-amended *M. barkeri* strain Fusaro co-cultures, *D. mccartyi* strain BAV1, strain GT and strain FL2 cell densities increased 31-, 41- and 37-fold to reach $2.0 \pm 0.1 \times 10^8$, $1.6 \pm 0.0 \times 10^8$ and $9.0 \pm 2.7 \times 10^7 \text{ cells ml}^{-1}$, respectively (table 1).

4. Discussion

CN-Cbl is a required cofactor for the characterized *D. mccartyi* strains grown in axenic culture [2]. The responses of *D. mccartyi* strain 195 to different CN-Cbl concentrations in the medium revealed that maximum TCE dechlorination rates and growth yields required CN-Cbl of $25 \mu\text{g l}^{-1}$ or higher concentrations [13]. In cultures of *D. mccartyi* strains BAV1, GT and FL2 amended with $1 \mu\text{g l}^{-1}$ CN-Cbl, CN-Cbl was quickly depleted to below 10 ng l^{-1} , and TCE and *cis*-DCE dechlorination was incomplete. Ethene formation, about 2-fold higher dechlorination rates, and 2.8–9.1-fold increases in *Dehalococcoides* growth yields were observed in cultures that received $25 \mu\text{g l}^{-1}$ CN-Cbl. These findings demonstrate that *Dehalococcoides*' requirement for this essential cofactor must be understood to achieve and sustain desirable reductive dechlorination rates and endpoints at sites impacted with chloroorganic contaminants. Reductive dechlorination and growth of *D. mccartyi* strain BAV1 and strain FL2 were sustained in co-cultures with the PCE-to-*cis*-DCE dechlorinating species *G. lovleyi* strain SZ, which possesses the complete cobamide biosynthesis pathway [15]. The cobamide concentrations in these dechlorinating co-cultures ranged from 10 to 30 ng l^{-1} , suggesting that the cobamide flux may be the relevant factor to sustain *Dehalococcoides*' dechlorination activity in microbial communities. Genes encoding the prokaryotic BtuB/CD corrinoid uptake system, which belongs to the ABC-type (ATP-binding cassette) transporter family, were identified in all sequenced *Dehalococcoides* genomes [11]. *In vitro* experiments demonstrated that the outer membrane corrinoid receptor BtuB of *Escherichia coli* had a high affinity for CN-Cbl with a half-saturation concentration of 0.5 nM ($0.68 \mu\text{g l}^{-1}$) [28]. *Dehalococcoides* reductive dechlorination activity was sustained in *D. mccartyi*/*G. lovleyi* co-cultures with cobamide concentrations of $10\text{--}30 \text{ ng l}^{-1}$, suggesting that *D. mccartyi* strains also possess high affinity cobamide uptake systems.

A crucial question is the source of the corrinoid cofactor to sustain *Dehalococcoides* reductive dechlorination activity in the environment, particularly in chlorinated solvent-contaminated aquifers. The *Dehalococcoides*-containing consortium KB-1 used for bioaugmentation contains methanogens and

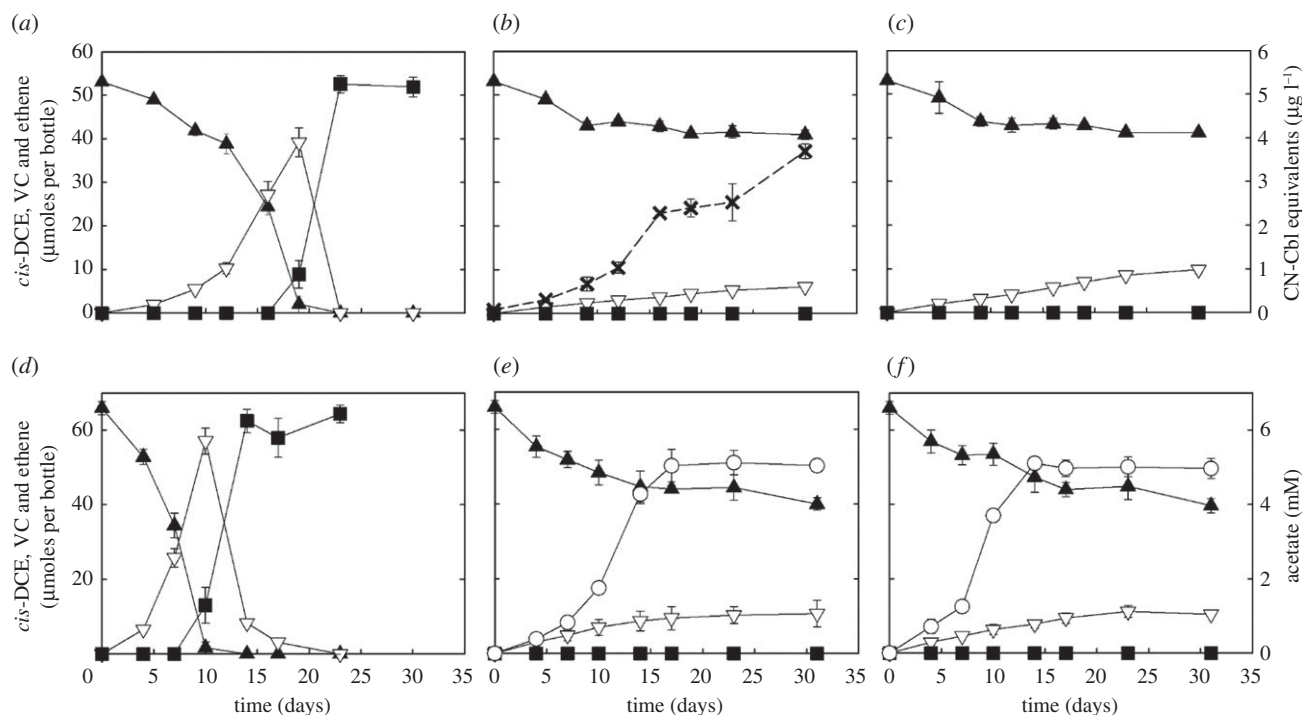


Figure 4. Dechlorination and extracellular cobamide production in *Dehalococcoides mccartyi* strain BAV1/*Methanosarcina barkeri* strain Fusaro and strain BAV1/*Sporomusa ovata* co-cultures. (a) Strain BAV1/*Methanosarcina barkeri* strain Fusaro co-culture amended with $25 \mu\text{g l}^{-1}$ CN-Cbl (positive control); (b) strain BAV1/strain Fusaro co-culture without CN-Cbl addition; (c) strain BAV1 culture without CN-Cbl (negative control); (d) Strain BAV1/*Sporomusa ovata* co-culture amended with $25 \mu\text{g l}^{-1}$ CN-Cbl (positive control); (e) strain BAV1/*Sporomusa ovata* co-culture without CN-Cbl addition; (f) strain BAV1/*Sporomusa* sp. strain KB-1 co-culture without CN-Cbl addition. Filled triangle, *cis*-DCE; open inverted triangle, vinyl chloride; filled square, ethene; open circle, acetate; cross symbol, cobamide(s) reported as CN-Cbl equivalents. Error bars represent the standard deviations of triplicate samples and are not shown if they are smaller than the symbols.

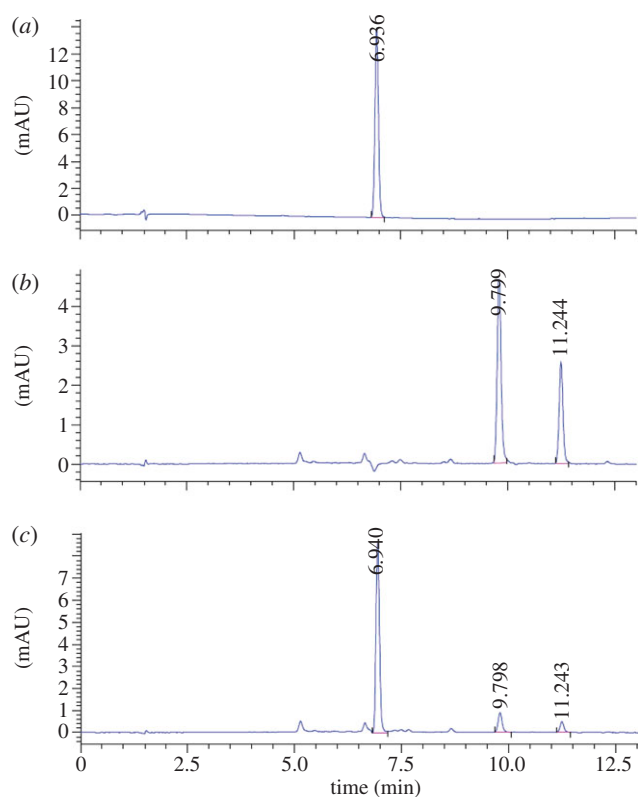


Figure 5. Demonstration of guided cobalamin biosynthesis. HPLC chromatograms of (a) authentic CN-Cbl standard (5 mg l^{-1} dissolved in water); (b) cyano-phenolyl and cyano-*p*-cresolyl cobamides extracted from 1 l of *Sporomusa* sp. strain KB-1 culture grown autotrophically with H_2/CO_2 ; and (c) cyanocobalamin extracted from 1 litre of *Sporomusa* sp. KB-1 culture grown autotrophically with H_2/CO_2 and the addition of $10 \mu\text{M}$ DMB. mAU = milliabsorbance units. (Online version in colour.)

CO_2 -reducing acetogens [29]. Both methanogenic Archaea and acetogenic Bacteria are known to synthesize cobamides with different lower ligands (figure 1). 5'-Hydroxybenzimidazolyl-cobamide (factor III) and adenylyl-cobamide (pseudo vitamin B_{12}) are the predominant cobamides isolated from methanogenic Archaea [30], and factor III was identified in *M. barkeri* cultures [31]. In *Dehalococcoides* co-cultures with *M. barkeri* strain Fusaro, more than $4 \mu\text{g l}^{-1}$ CN-Cbl equivalents, presumably factor III, were quantified in culture suspensions; however, only negligible reductive dechlorination activity, similar to negative control cultures, was observed, indicating that factor III cannot fulfil *Dehalococcoides*' corrinoid requirement. These findings are supported by a previous study that reported that *D. mccartyi* strain 195 reductive dechlorination activity was not enhanced in co-cultures with *Methanobacterium congolense* [14]. Pure culture studies with *D. mccartyi* strain 195 corroborated that purified factor III and phenolic cobamides did not support reductive dechlorination, and growth only occurred when cobamides containing benzimidazolyl types of lower ligands were available [32]. Although the specific cobamides produced by *Methanobacterium congolense* and many other methanogens have not been thoroughly explored, the data reported here confirm that at least the 5'-hydroxybenzimidazolyl-cobamide produced by some methanogens cannot fulfil the *D. mccartyi* corrinoid cofactor requirement.

Cobamides containing a phenolic-type lower ligand, i.e. phenolyl- and *p*-cresolyl-cobamide, were first purified from *S. ovata*, and were found in other Bacteria capable of H_2/CO_2 reductive acetogenesis [26]. *Sporomusa* sp. strain KB-1 was isolated from the dechlorinating KB-1 consortium and shares 98.5 per cent sequence identity with the 16S rRNA gene of *S. ovata*

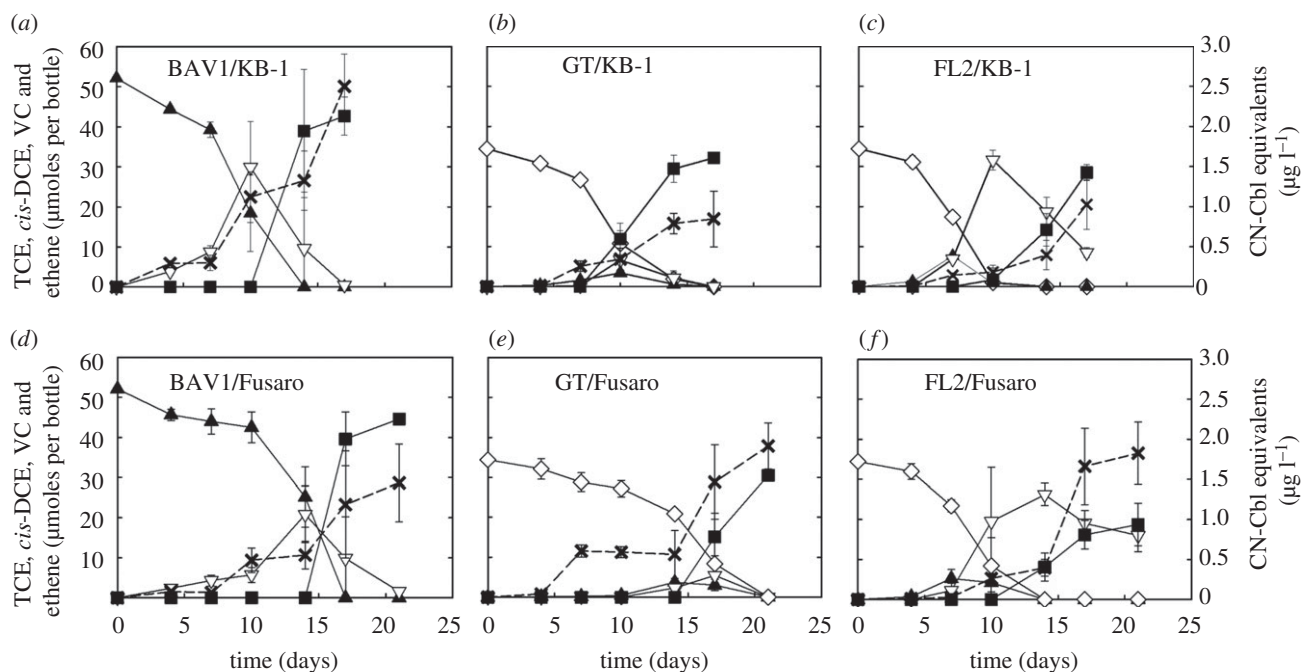


Figure 6. Reductive dechlorination activity and extracellular cobamide production in *Dehalococcoides/Sporomusa* sp. strain KB-1 and *Dehalococcoides/Methanosarcina barkeri* strain Fusaro co-cultures amended with 10 μM DMB. (a) BAV1/KB-1 co-cultures; (b) GT/KB-1 co-cultures; (c) FL2/KB-1 co-cultures; (d) BAV1/Fusaro co-cultures; (e) GT/Fusaro co-cultures and (f) FL2/Fusaro co-cultures. Open diamond, TCE; filled triangle, *cis*-DCE; open inverted triangle, vinyl chloride; filled square, ethene; cross symbol, cobamide(s) reported as CN-Cbl equivalents. Error bars represent the standard deviations of triplicate samples and are not shown if they are smaller than the symbols.

(AJ279800.1). Both *Sporomusa* isolates produced phenolyl- and *p*-cresolyl-cobamide, suggesting that they represent the characteristic cobamides of the genus *Sporomusa*.

The test organism for the microbiological B₁₂ assay, *L. delbrueckii*, did not respond to *S. ovata* or *Sporomusa* sp. strain KB-1 culture suspensions or cell lysates, indicating that the cobamides with phenolic lower ligands cannot fulfil the corrinoid cofactor requirement of *L. delbrueckii*. Many prokaryotes, including *D. mccartyi* strains and *L. delbrueckii*, use adenosyl-cobalamin-dependent class II ribonucleotide reductases (RNRs), which catalyse the conversion of ribonucleotides to deoxyribonucleotides [11]. Since *D. mccartyi* genomes harbour multiple copies of class II RNR genes, it is possible that the lack of dechlorination activity and *Dehalococcoides* growth in the *Sporomusa* co-cultures was not only caused by non-functional RDases but also because of non-functional RNRs.

Interestingly, *Dehalococcoides* dechlorination activity and growth occurred in the DMB-amended co-cultures. In the presence of 10 μM DMB, CN-Cbl was the predominant corrinoid extracted from *Sporomusa* sp. strain KB-1 cultures, indicating that guided biosynthesis allows *Sporomusa* sp. to generate a corrinoid cofactor that fulfils *Dehalococcoides*' nutritional requirements. Similar observations were made in *D. mccartyi*/G. *sulfurreducens* co-cultures that were amended with DMB [15], suggesting that guided biosynthesis may be a relevant process for supplying *D. mccartyi* strains with the required cobalamin cofactor.

In DMB-amended *Sporomusa* sp. strain KB-1 co-cultures with each of the *D. mccartyi* strains BAV1, GT and FL2, the dechlorinators attained population sizes of $2.1 \pm 0.2 \times 10^8$, $2.1 \pm 0.3 \times 10^8$ and $9.0 \pm 1.4 \times 10^7$ cells per μmole of chloride released, respectively. These cell numbers were 1.7-, 1.4- and 2.2-fold higher compared with axenic cultures that received 25 $\mu\text{g l}^{-1}$ CN-Cbl. *Dehalococcoides mccartyi* strains

are known to grow better in mixed cultures, presumably because community members supply *Dehalococcoides* with unknown growth factors. Hence, it is possible that *M. barkeri* strain Fusaro and the *Sporomusa* isolates provided the *D. mccartyi* strains with not yet identified compounds that enhance growth yields. Another possible explanation for the lower growth yields observed with CN-Cbl is the cell's energetic burden associated with the replacement of the upper cyanide ligand [33].

The microbial synthesis of non-native cobamides in the presence of excess non-native lower ligand is known as guided biosynthesis [17,27,34]. Guided biosynthesis has been demonstrated with model organisms such as *Propionibacterium shermanii*, *E. coli* and *Streptomyces griseus* to obtain desired cobamides [17]. Factor III synthesized by *Methanobacterium thermoautotrophicum* was replaced by a cobalamin in medium amended with DMB without affecting methane formation and growth [35]. Synthesis of a cobalamin in the presence of DMB has also been demonstrated in *M. barkeri* cultures [36]. The co-culture experiments with *D. mccartyi* strains demonstrated that the addition of DMB to the medium enabled guided biosynthesis and the formation of a cobalamin by *M. barkeri* strain Fusaro and *Sporomusa* sp. strain KB-1. *Sporomusa* sp. strain KB-1 replaced the phenolic cobamides with a cobalamin without apparent effects on its own growth suggesting that cobalamin can replace the phenolyl- and *p*-cresolyl-cobamides during growth with H₂/CO₂.

Survival of corrinoid auxotrophs such as *Dehalococcoides* relies on cobamides and corrinoid precursors (e.g. cobinamides) produced and released by corrinoid-synthesizing Archaea and Bacteria. It is currently unclear to what extent and under what conditions microorganisms capable of de novo corrinoid synthesis produce and release cobamides and/or corrin ring precursors to the surrounding medium. While methanogens and acetogens readily release cobamides

into the medium, at least under laboratory cultivation conditions, the formation of cobalamin requires DMB. Key questions are the origin of DMB in anoxic environments (i.e. who is synthesizing DMB?), and whether the availability of DMB limits cobalamin biosynthesis and *Dehalococcoides* activity. The biosynthesis pathway(s) of DMB under anoxic conditions has remained elusive, and the flux of DMB in subsurface environments has not been evaluated. While our experiments have demonstrated cobalamin production through guided biosynthesis, the relevance of this process in natural microbial communities has yet to be demonstrated. All *D. mccartyi* strains harbour *cobT*, *cobC* and *cobS* genes implicated in the cobalamin assembly from DMB and cobinamide [37], suggesting *Dehalococcoides* has the ability to assemble cobalamin if the precursors are available. Independently of whether *Dehalococcoides* or other microbes perform the final cobalamin assembly, the sources and fluxes of its precursors, in particular DMB, need to be understood in natural and disturbed microbial communities to

identify *Dehalococcoides*' nutritional limitations and potentially predict and manipulate reductive dechlorination activity. Limited dechlorination activity of *Dehalococcoides* and related corrinoid-auxotroph, organohalide-respiring *Chloroflexi* (e.g. *Dehalogenimonas* [38,39], 'Dehalobium' [40]) is regarded as a major constraint to achieve detoxification at sites contaminated with chlorinated contaminants. Hence, elucidating the nutritional requirements and ecophysiology of organohalide-respiring *Chloroflexi* in natural and contaminated environments will help develop predictive understanding and new strategies for stimulating *in situ* reductive dechlorination activity.

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