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Characterization of four TCE-dechlorinating microbial enrichments grown with different cobalamin stress and methanogenic conditions

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Abstract

To investigate the important supportive microorganisms responsible for TCE bioremediation under specific environmental conditions and their relationship with *Dehalococcoides* (Dhc), four stable and robust enrichment cultures were generated using contaminated groundwater. Enrichments were maintained under four different conditions exploring two parameters: high and low TCE amendments (resulting in inhibited and uninhibited methanogenic activity, respectively); and with and without vitamin B₁₂ amendment. Lactate was supplied as the electron donor. All enrichments were capable of reductively dechlorinating TCE to VC and ethene. The dechlorination rate and ethene generation were higher, and the proportion of electrons used for dechlorination increased when methanogenesis was inhibited. Biologically significant cobalamin biosynthesis was detected in the enrichments without B₁₂ amendment. Comparative genomics using a genus-wide microarray revealed a Dhc genome similar to that of strain 195 in all enrichments, a strain that lacks the major upstream corrin ring biosynthesis pathway. Seven other Bacterial OTUs were detected using clone libraries. OTUs closest to *Pelosinus*, *Dendrosporobacter*, and *Sporotalea* (PDS) were most dominant. The *Clostridium*-like OTU was most affected by B₁₂ amendment and active methanogenesis. Principal component analysis revealed that active methanogenesis, rather than vitamin B₁₂ limitation, exerted a greater effect on the community structures even though methanogens did not seem to play an essential role in providing corrinoids to Dhc. In contrast, acetogenic bacteria that were abundant in the enrichments, such as PDS and *Clostridium* sp., may be potential corrinoid providers for Dhc.

Keywords

Chlorinated solvents; *Dehalococcoides*; Reductive dechlorination; Corrinoid; Microbial community; Bioremediation

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Introduction

Trichloroethene (TCE) has been a common groundwater contaminant at hazardous waste sites for decades (Doherty 2000; Hendrickson et al. 2002; McCarty 1997) and is strictly regulated by the U.S. Environmental Protection Agency (USEPA) (Moran et al. 2007). Bioremediation of TCE by *Bacteria* capable of reductive dechlorination has been regarded as a more cost-effective and sustainable approach compared with other physical-chemical methods.

Thus far, *Dehalococcoides* (Dhc) strains are the only known *Bacteria* capable of reductive dechlorination of TCE to the innocuous end product ethene (Cupples et al. 2003; Cupples et al. 2004; He et al. 2005; He et al. 2003a; Maymó-Gatell et al. 1997). However, Dhc have very strict growth requirements, requiring hydrogen and acetate as electron donor and carbon source, and requiring exogenously supplied corrinoids such as vitamin B₁₂ (cyanocobalamin) as an essential cofactor for their reductive dehalogenases (RDases) (Maymó-Gatell et al. 1997; McMurdie et al. 2009; Seshadri et al. 2005). The growth of Dhc in isolation is relatively unreliable and slow with doubling times of 20–60 hours (Cupples et al. 2003; He et al. 2005; He et al. 2003b; Maymó-Gatell et al. 1997; Sung et al. 2006). In contrast, previous studies have shown that growth of Dhc in microbial enrichments or defined consortia is generally more rapid and robust (Duhamel and Edwards 2006; Duhamel et al. 2004; He et al. 2007; Maymó-Gatell et al. 1997; Men et al. 2012). This suggests that microorganisms other than Dhc play important roles in supporting Dhc in dechlorination processes.

Within Dhc-containing communities fed with fermentable organics, hydrogen and acetate are made available to Dhc by co-existing fermenting *Bacteria* such as *Desulfovibrio*, *Eubacterium*, *Clostridium*, *Acetobacterium*, *Citrobacter*, and *Spirochetes* (Duhamel and Edwards 2006; Freeborn et al. 2005; Lee et al. 2006; Richardson et al. 2002; Ritalahti and Löffler 2004). As an essential cofactor of RDases and other functionally important enzymes, vitamin B₁₂ has been exogenously amended in most of the previous laboratory-scale studies of dechlorinating enrichments (Cupples et al. 2003; Duhamel et al. 2004; Freeborn et al. 2005; Futamata et al. 2007; He et al. 2003a; Rowe et al. 2008). However, there have been reports of Dhc-containing enrichments where no exogenous vitamin B₁₂ was amended (Daprato et al. 2007; Gu et al. 2004), indicating that an alternate source of corrinoids is available in those communities. However, the specific organisms responsible for providing corrinoids in those communities are still unknown.

According to reported genomic data (<http://img.jgi.doe.gov/>), the five sequenced Dhc genomes (*i.e.*, CBDB1, BAV1, 195, VS and GT) do not have complete upstream corrin ring biosynthesis pathways, thus are not able to synthesize corrinoids *de novo*. However, a recent metagenomic study on an enrichment maintained with little exogenous vitamin B₁₂ (Brisson et al. 2012) reported that a nearly complete corrinoid biosynthesis pathway (except the non-essential *cbiJ* gene) has been identified in metagenomic contigs assigned to an unsequenced Dhc strain, although the functionality of this operon has not been confirmed. Despite the possibility for unsequenced Dhc strains to be capable of corrinoid biosynthesis, some fermentors, acetogens and methanogens that have often been observed within dechlorinating

communities (Cupples et al. 2003; Duhamel et al. 2004; Freeborn et al. 2005; He et al. 2005; He et al. 2003a; Rowe et al. 2008) are known to be able to synthesize corrinoids *de novo*, including *Clostridium* spp., *Desulfovibrio* spp., *Acetobacterium woodii*, and *Methanosarcina barkeri* (Guimaraes et al. 1994; Renz 1999; Stupperich et al. 1988), but their roles in supporting dechlorination remain elusive. Given the importance of cobalamin to the growth and energy generation of Dhc, and consequently to the potential success of TCE bioremediation, the aim of this study is to investigate the potential for *Bacteria* and methanogenic *Archaea* to supply corrinoids to Dhc within TCE-dechlorinating microbial communities.

In this study, four TCE-dechlorinating microbial communities were enriched from contaminated groundwater using low or high initial TCE concentrations to enable or inhibit methanogenesis, and with or without the addition of vitamin B₁₂. Physiological characteristics were analyzed after 20 sub-culturing events to compare the dechlorination and growth of Dhc among these enrichments. A genus-wide microarray targeting four Dhc genomes was applied to analyze the Dhc genomes present in those enrichments. The presence of the Dhc up-stream corrin ring biosynthesis genes detected in the metagenome of another enrichment (Brisson et al. 2012) was also investigated. Finally, community structures were compared among enrichments grown under the different conditions, providing insight to potential corrinoid-providing species. This is the first study to investigate the roles of microbial community members in providing corrinoids to Dhc by comparing the physiology, genomic characteristics and community structures of dechlorinating communities enriched from environmental samples under different cobalamin stress.

Materials and methods

Chemicals

TCE, cis-dichloroethene (cDCE), and vinyl chloride (VC), were purchased from Sigma-Aldrich-Fluka (St. Louis, MO) or Supelco (Bellefonte, PA). Ethene was obtained from Alltech Associates, Inc. (Deerfield, IL). Vitamin B₁₂ was obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

Enrichment set-up and growth conditions

Groundwater from a TCE-contaminated site in New Jersey was withdrawn, stored in well-sealed aseptic bottles at 4°C, and sent overnight to University of California, Berkeley. Microorganisms in the groundwater were collected by filtering 200 mL groundwater through 0.22 µm filters. Filters were then placed into 160 mL serum bottles amended with 50 mL of filtered groundwater and 50 mL of autoclaved basal medium described previously (He et al. 2007). All steps were performed in an anaerobic chamber. The inoculated bottles were then flushed with N₂/CO₂ (90:10, vol/vol) and amended with 3.9 mmol lactate as electron donor, 2 µL TCE (~22 µmol) as electron acceptor, 0.5 mL vitamin solution (Wolin et al. 1963) containing 20 mg L⁻¹ vitamin B₁₂ (final concentration 100 µg L⁻¹) as supplemental nutrients. All inoculated bottles were incubated in the dark at 34°C without shaking. The bottle with the most rapid TCE dechlorination activity was re-amended with 22 µmol TCE

and 0.4 mmol lactate nine additional times (total TCE ~220 μmol). Then, after all TCE had been dechlorinated, the enrichment was sub-cultured (20%, vol/vol) into 80 mL fresh basal medium. After two subsequent sub-cultures under these conditions, sub-cultures were split into four enrichment conditions: (1) low initial TCE with B₁₂ (LoTCEB12) and (2) without B₁₂ (LoTCE); and (3) high initial TCE with B₁₂ (HiTCEB12) and (4) without B₁₂ (HiTCE). The high initial TCE conditions (~77 μmol) were applied to inhibit methanogenesis (Distefano et al. 1992; Men et al. 2012; Yu and Smith 2000) rather than the common inhibitor 2-bromoethanesulfonate (BES) due to its toxicity to Dhc (Löffler et al. 1997). Within each feeding cycle, the feeding regimes of lactate and TCE prior to sub-culturing were listed in Table 1. Subsequently, 5% (vol/vol) of each culture was inoculated into 95 mL fresh basal medium under the same feeding regime every 13–14 days. All experiments were carried out after 20 sub-culturing events with three biological replicates. Measurements of dechlorination, cell growth, organic acids, hydrogen, as well as total cobalamin were performed on at least two different generations, and the results were reproducible. Dechlorination, hydrogen, and organic acids are reported for one set of measurements. 16S rRNA gene copy numbers and cobalamin concentrations are averages of all measurements.

DNA isolation

For gene quantification, cells in 1.5 mL culture were collected by centrifuging at $15,000 \times g$, 4 °C for 10 min. Genomic DNA (gDNA) was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. For the construction of 16S rRNA gene cloning library and the microarray, nucleic acids (gDNA and RNA) were extracted from cell pellets collected from 45 mL culture using the phenol (pH = 8.0) chloroform method described previously (West et al. 2008). The gDNA was then separated from RNA using Allprep DNA/RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The purified gDNA was stored at –20°C prior to further use.

Quantitative PCR (qPCR)

To determine the presence and the quantity of Dhc 16S rRNA gene, as well as three RDase genes (*tceA*, *vcrA*, and *bvcA*), qPCR was applied using primer sets described in Online Resource 1. Each 20- μL reaction mixture contained 2.5 μL of sample or serially diluted standard, 10 μL 2 $\times$ Fast SYBR Green master mix (Applied Biosystems, Foster City, CA), and 0.625 μM of the forward and reverse primers. A linearized plasmid containing the Dhc 16S rRNA gene and all three RDase gene fragments (Holmes et al. 2006) was used as standard. Total Bacterial and Archaeal numbers were determined using universal Bacterial and Archaeal 16S rRNA gene primers (Online Resource 1), respectively. The gDNA of *Dehalococcoides mccartyi* (formerly designated *Dehalococcoides ethenogenes*) strain 195 and *Methanobacterium congolense* was used as standards for total *Bacteria* and *Archaea*, respectively. All qPCR standards were quantified by Nanodrop 3300 fluorometer according to the method from Nanodrop Technologies (Wilmington, DE).

Genus-wide microarray analysis

One μg of gDNA was applied on the custom-designed microarray (Affymetrix, Santa Clara, CA) targeting four sequenced Dhc genomes: strain CBDB1, BAV1, 195 and VS, as well as 348 outside genes, as described elsewhere (Lee et al. 2011). Biologically triplicated samples

from three parallel bottles of each enrichment were generated. The microarray was processed according to the instructions given in section 3 of the Affymetrix GeneChip Expression Analysis technical manual (Affymetrix, Santa Clara, CA). Data was analyzed using Affymetrix GeneChip software and the MAS5 algorithm. Each microarray was normalized by scaling the signal intensities of the positive control spike-mix to a target signal intensity of 2500 to allow comparison between microarrays. A gene was considered “present” in a culture if the probe set across all three replicated samples had signal intensities greater than 140 and *p* values less than 0.05.

Bacterial 16S rRNA gene clone library construction

Bacterial 16S rRNA genes were amplified using the universal Bacterial 16S rRNA gene primers, 8F and 1492R (Online Resource 1). Two μL of the gDNA sample was used in a 50- μL PCR reaction containing 5 μL 10 $\times$ Ex Taq buffer, 0.8 mM dNTP mix, 0.2 μM of each primer, and 1.25 U Ex Taq DNA polymerase (TaKaRa Bio Inc., Madison, WI). Four annealing temperatures (*i.e.*, 48°C, 50°C, 53°C and 58°C) were used in order to reach a higher recovery of all Bacterial 16S rRNA genes present in the communities. The combination of all PCR products under different annealing temperatures was used for the clone library construction, following manufacturer’s instructions for TOPO TA cloning kit (with the pCR2.1-TOPO vector) (Invitrogen, Carlsbad, CA). For each clone library, approximately 100 clones were picked, re-amplified using M13F and M13R primers (Online Resource 1), and then sequenced by a DNA sequencing facility (University of California, Berkeley, CA; <http://mcb.berkeley.edu/barker/dnaseq/>). The megablast algorithm in BLAST software (GenBank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) was applied to determine the closest cultured microorganism for each operational taxonomic unit (OTU). Identified OTUs were quantified using primer sets designed based on the sequenced 16S rRNA genes (Online Resource 1). PCR products of plasmid inserts corresponding to each 16S rRNA gene were used as standards. Principal component analysis (PCA) was carried out by StatistiXL software, using 16S rRNA gene copy numbers of OTUs over a time course of 13–14 days as input.

Amplification of up-stream cobalamin biosynthesis genes by PCR

The same PCR procedure was used as described above with an annealing temperature of 60 °C using primers targeting contigs classified as *cbiD*, *cbiE/cbiT*, *cbiC* and *cobN* (Online Resource 1). PCR products were visualized on a 0.8% agarose gel under BIO-RAD Gel Doc 2000 imaging system (BIO-RAD, Hercules, CA). gDNA of an unsequenced Dhc strain isolate, ANAS2, which possesses those genes served as the positive control (Brisson et al. 2012; Lee et al. 2011).

Analytical methods

Chlorinated ethenes, ethene and methane were measured by loading 100 μL headspace samples on an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) and a 30-m J&W capillary column with a 0.32-mm inside diameter (Agilent Technologies, Santa Clara, CA). Detector and injector temperatures were set at 250 and 220 °C, respectively. A gradient temperature program ramps the oven temperature from 45 to 200 °C within 4 min, and holds at 200 °C for 1min. Hydrogen in the headspace was

measured by gas chromatography using a reductive gas detector (Trace Analytical, Menlo Pak, CA) as described previously (Freeborn et al. 2005).

Organic acids were measured by high performance liquid chromatography (HPLC) with a photodiode array (PDA) detector (set at 210 nm) (Waters, Milford, MA). Chromatographic separation using an Aminex HPX-87H ion exclusion organic acid analysis column (300 × 7.8 mm, Bio-Rad, Hercules, CA) was performed with 5 mM aqueous H₂SO₄ at a flow rate of 0.6 mL min⁻¹ and a temperature of 30 °C. Sample preparation and quantification using an external standard calibration curve were performed as previously described (He et al. 2007).

For cobalamin measurement, cell pellets in 200–300 mL culture were first separated from supernatant by centrifugation at 15,000g, 4 °C for 10 min. The supernatant was concentrated by solid phase extraction using a C₁₈ Sep-Pak cartridge, eluted in 3 mL methanol.

Cobalamin from cell pellets and the concentrated supernatant were extracted, respectively by potassium cyanide as described elsewhere (Siebert et al. 2002; Stupperich et al. 1986) with a modified incubation temperature at 60 °C for 1.5 hours. Intra- and extra-cellular cobalamin were then measured by the same HPLC system described above with an Agilent Eclipse Plus C₁₈ column, 1.8 µm, 3.0 × 50 mm (Agilent Technologies, Santa Clara, CA). Gradient elution at 0.5 mL min⁻¹ was used with initial solvent 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 PDA detector was set at 360 nm. Commercial vitamin B₁₂ was used as the standard (Sigma-Aldrich-Fluka, St. Louis, MO). The sum of intra- and extra-cellular cobalamin concentrations are reported as total cobalamin concentration in the unit of µg per liter of culture.

NCBI accession numbers

The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers of JQ004083-JQ004090, KC517355-KC517356. The microarray data in this study (GSE33530) was deposited in the National Center of Biotechnology Information Gene Expression Omnibus database.

Results

Physiological characteristics of the four enrichment cultures

TCE was dechlorinated to VC and ethene in all four enrichments whether or not B₁₂ was amended or methanogenesis was inhibited over 20 sub-culturing events. For the methanogenic cultures, the total 220 µmol TCE was dechlorinated to VC and ethene within each 14-day subculturing event, while the non-methanogenic cultures took less than 10 days (Fig. 1a–d). Dechlorination of subsequent doses of TCE became more rapid after the first dose of TCE was dechlorinated. HiTCEB12 and HiTCE exhibited the greatest generation of ethene with 65 ± 0.5% and 36 ± 1% of the total TCE, while LoTCEB12 and LoTCE generated only 5.5 ± 1.4% and 3.0 ± 0.5% of the total TCE, respectively. Dhc 16S rRNA genes were detected in all four cultures and among the three tested RDase genes (*i.e.*, *tceA*, *vcrA*, *bvcA*), only *tceA* was detected. The copy numbers of Dhc 16S rRNA genes and *tceA*

were closely correlated (Fig. 1a-d), suggesting that the dominant Dhc strain in the enrichments carries the *tceA* gene. The cell yields of Dhc in all cultures were similar, with LoTCEB12, LoTCE, HiTCEB12 and HiTCE exhibiting $(2.3 \pm 0.1) \times 10^7$, $(2.7 \pm 0.2) \times 10^7$, $(3.3 \pm 0.3) \times 10^7$, $(2.3 \pm 0.7) \times 10^7$ copies per $\mu\text{mol Cl}^-$ released, respectively. All enrichments had similar densities of total Bacterial 16S rRNA gene prior to each sub-culturing event, in the range of $2\text{--}5 \times 10^8$ copies per mL. Cultures with active methanogenesis contained $\sim 10^6$ copies per mL of Archaeal 16S rRNA genes, while the non-methanogenic cultures contained $\sim 10^5$ copies per mL (Fig. 2).

In all enrichments, lactate was fermented to hydrogen, propionate and acetate, with small amounts of butyrate generated only in HiTCE and HiTCEB12 (Table 2). Methane generation was detected only in LoTCEB12 and LoTCE as expected, at continually increasing concentrations, whereas HiTCEB12 and HiTCE did not generate detectable methane (Fig. 1). Aqueous hydrogen reached a peak of $1\text{--}1.5 \mu\text{M}$ in the first day for the methanogenic cultures, and then remained below $0.2 \mu\text{M}$ afterwards (Fig. 1a and 1b). In contrast, aqueous hydrogen remained above $0.5 \mu\text{M}$ for the non-methanogenic cultures throughout the incubation period (Fig. 1c and 1d). The electron equivalents (eeq) involved in dechlorination, methanogenesis, and other redox reactions like homoacetogenesis were measured or calculated from the stoichiometry of three possible lactate fermentation reactions (Table 2), taking into consideration the electrons remaining in the acetate, propionate, butyrate and hydrogen present at the end of the subculturing event. For methanogenic cultures (LoTCE and LoTCEB12), a total of $5.6\text{--}6.8 \text{ mmol eeq}$ in the form of hydrogen were generated from lactate fermentation, 12–15% of which contributed to dechlorination, 35% to methanogenesis, and the rest to other processes, including biosynthesis and potentially, homoacetogenesis. In the non-methanogenic cultures, a total of 4.6 mmol eeq was generated, 21–22% of which was involved in dechlorination. The inhibition of methanogenesis increased the proportion of electron flow to dechlorination. The fermentation of lactate in methanogenic cultures resulted in a propionate to acetate ratio of around 1:1, while the ratio was 1.5:1 in the non-methanogenic cultures. Based on the stoichiometry of biosynthesis (Table 2) and the assumption that most bacteria are rod shaped with a size of $(0.6\text{--}1) \mu\text{m} \times (2\text{--}7) \mu\text{m}$ (Moe et al. 2012), 0.2 g of dry weight per gram of wet weight (Cupples et al. 2003), and the total bacteria is 5×10^8 cells per mL, we estimate that $0.1\text{--}1.5 \text{ mmol}$ acetate would be consumed per bottle for synthesis of biomass (Online Resource 2). However, in methanogenic cultures, close to 100% of the fermented lactate was recovered in products, therefore, homoacetogenesis must have generated some acetate from hydrogen and CO_2 , offsetting the amount used in biosynthesis (Table 2). The eeq for acetate is 8 eeq per mol , so the total eeq consumed in homoacetogenesis should be $0.8\text{--}12.0 \text{ mmol}$, which is included in the eeq consumed in other redox reactions (Table 2). In the non-methanogenic cultures, about 87% lactate eeq were recovered in fermentation products, indicating that acetate generated from homoacetogenesis was less than what was consumed in biosynthesis.

Genomic characteristics of Dhc in the four enrichment cultures

In order to query the gene content of the unsequenced Dhc strains in the enrichments, gDNA from the cultures was analyzed using the Dhc genus-wide microarray. Data indicate that the

Dhc in all four enrichments share a genome most similar to strain 195 (Online Resource 3), and that none of the 348 non-Dhc RDase or other targeted genes were detected. In all, about 80% of genes in strain 195 were detected in the cultures, and 85% of the non-detected genes from strain 195 are located within its high plasticity regions (HPRs) or integrated elements (IEs). For the 9 IEs defined in the strain 195 genome, only 13 out of 29 genes in IE (I) were detected, while genes in the other eight IEs were entirely missing. Only 5 of 101 Dhc RDase-encoding genes targeted by the microarray (DET0079 (*tceA*), DET0088, DET0173, DET0180, and DET1545) were detected in the four enrichments, all from strain 195.

Most of the strain 195 genes involved in cobalamin uptake and salvaging were detected in all enrichments (Online Resource 4), including the duplicated operons for cobalamin lower ligand attachment (DET0657-0660/DET0691-0694) and cobalamin transport and uptake (DET0650-0652/DET0684-0686). In addition, two duplicated *cbiZ*-like genes (DET0653/0687) out of seven (DET0242, DET0249, DET0314/1165, DET0653/0687, and DET1556) predicted in strain 195 were detected (Online Resource 4). However, none of the cobalamin salvaging genes from the other three genomes targeted by the microarray were detected. Furthermore, the upstream corrin ring biosynthesis genes (*i.e.*, *cbiD*, *cbiE/cbiT*, *cbiC* and *cobN*) found in metagenomic contigs assigned to Dhc strain ANAS2 (Brisson et al. 2012) were not detected in the four enrichments by PCR amplification (Online Resource 5), indicating the inability of *de novo* cobalamin synthesis of the Dhc strains in those enrichments.

In addition, a total of 31 Dhc genes outside of the strain 195 genome were detected (Online Resource 6), including 29 genes belonging to strain VS and two to strain BAV1. Of the VS genes, the entire tryptophan operon (DhcVS_1251-1258) was detected in all four enrichments, while that operon from strain 195 (DET1481-1488) was absent. Another cluster of VS genes (DhcVS_1414-1417) which encode a transcriptional regulator (DhcVS_1415), ABC transporter (DhcVS_1416), Fe-S oxidoreductase (DhcVS_1417), and a hypothetical protein (DhcVS_1414) were also detected.

Microbial community structures of the four enrichments

Table 3 summarizes bacterial OTUs identified from clone libraries of the four enrichments. As expected, an OTU with 99% sequence similarity to strain 195 was detected. Besides Dhc, the next four commonly detected OTUs (denoted “*Pelosinus*_GW, *Dendrosporobacter*_GW, *Sporotalea*_GW and *Clostridium*_GW”) exhibited the highest sequence similarity to *Pelosinus* sp. UFO1, *Dendrosporobacter quercicolus*, *Sporotalea propionica* (recently renamed *Pelosinus propionicus*) (Moe et al. 2012) and *Clostridium propionicum*, which all belong to the *Firmicutes*. Consistent with what had been found by other researchers (Moe et al. 2012; Ray et al. 2010) with *Pelosinus*/*Sporotalea* species, the 16S rRNA gene sequences of clones closely related to *Pelosinus*/*Sporotalea* contained inserts that differed in length in variable region I, including a 118-bp longer insert (KC517355) and a 10-bp shorter insert (JQ004084) for the *Pelosinus*-like OTU, as well as a 113-bp longer insert (JQ004090) and a 7-bp shorter insert (KC517356) for the *Sporotalea*-like OTU. Subsequent similarity analysis and primer design was based on the sequences without inserts. OTUs with similarity to the

phyla of *δ-proteobacteria*, *Spirochaetes*, and *Bacteroidetes* were also detected (denoted “*Desulfovibrio_GW*, *Spirochaetes_GW* and *Bacteroides_GW*”).

Copy numbers of each OTU were quantified by qPCR (Fig. 3). The greatest variation between cultures occurred with *Clostridium_GW*, whose copy number was 5 times higher in LoTCE ($\sim 1 \times 10^7$ copies per 10^8 copies of *Dhc_GW*) compared with LoTCEB12 ($\sim 2 \times 10^6$ copies per 10^8 copies of *Dhc_GW*). In the HiTCE enrichments, normalized copy numbers of *Clostridium_GW* were one order of magnitude higher than LoTCE. *Desulfovibrio_GW* did not exhibit significant difference between enrichments with B₁₂ and without, whereas it was 5–6 times lower in non-methanogenic enrichments than methanogenic ones. *Pelosinus_GW*, *Dendrosporobacter_GW*, and *Sporotalea_GW* (PDS) were the most dominant in all enrichments, with the highest numbers observed in LoTCEB12. In contrast, *Bacteroides_GW* exhibited relatively lower numbers in the enrichments with the methanogenic cultures, only about one fifth as high as the non-methanogenic cultures.

Principal component analysis (PCA) was applied to 16S rRNA gene copy numbers of different OTUs in the four enrichments collected over a time course of 13–14 days (Fig. 4). Different time points of the four enrichments clustered into two groups distinguished by the presence of methanogenesis, indicating that this characteristic exerted more influence on community structure than B₁₂ amendment.

Total cobalamin in the four enrichments

Concentrations of 3–4 $\mu\text{g L}^{-1}$ cobalamin were detected in the enrichments without B₁₂ amendment, indicating that community members generate cobalamin or other corrinoid species that *Dhc* could potentially use (Online Resource 7). Interestingly, in the enrichments with 100 $\mu\text{g L}^{-1}$ exogenous cobalamin, only 12 $\mu\text{g L}^{-1}$ and 3 $\mu\text{g L}^{-1}$ were recovered in LoTCEB12 and HiTCEB12, respectively (Online Resource 7), indicating a significant consumption of the added B₁₂ in these enrichments.

Discussion

The aim of this study was to investigate the ecological roles played by the co-existing Bacteria and methanogenic *Archaea* within *Dhc*-containing dechlorinating enrichments in terms of interspecies transfer and utilization of the required corrinoid cofactor. In order to achieve this goal, four stable and robust TCE-dechlorinating microbial communities were enriched over 20 sub-culturing events from contaminated groundwater inocula with the same amounts of lactate and TCE as electron donor and acceptor, respectively, but with different conditions of methanogenesis and vitamin B₁₂ amendment. Dechlorination was observed in all enrichments. Non-methanogenic cultures generated significantly more ethene than methanogenic cultures, although similar cell yields were observed in all enrichments. Compared with other enrichment cultures, *Dhc* cell yields in this study ($2.3\text{--}3.3 \times 10^7$ copies per $\mu\text{mol Cl}^-$ released) were slightly higher than ANAS (1.4×10^7 copies per $\mu\text{mol Cl}^-$ released) (Holmes et al. 2006), but lower than two other highly enriched cultures KB-1 (36×10^7 copies per $\mu\text{mol Cl}^-$ released) (Duhamel et al. 2004) and VS (58×10^7 copies per $\mu\text{mol Cl}^-$ released) (Cupples et al. 2004). The relatively lower cell yields in this study is likely due

to the ability of Dhc to uncouple growth from TCE degradation after the dechlorination of multiple doses of TCE (Johnson et al. 2008; Maymó-Gatell et al. 1997).

Hydrogen generated from lactate fermentation by bacteria such as *Desulfovibrio* (Walker et al. 2009), *Syntrophomonas* (Sieber et al. 2010), and *Clostridium* (Wu et al. 2012) serves as e⁻ donor for hydrogenotrophic processes such as dechlorination, methanogenesis, and homoacetogenesis in the TCE-dechlorinating enrichments. The inhibition of methanogenesis by high TCE concentrations increased the electron flow to dechlorination by an average of 7%. Since aqueous hydrogen concentrations (7–12 nM) in methanogenic cultures were above the reported threshold for Dhc (1–3 nM) (Yang and McCarty 1998), Dhc in the methanogenic enrichments were not under hydrogen limitation. The co-existence of methanogenesis and homoacetogenesis did not adversely affect TCE dechlorination or Dhc growth. However, aqueous hydrogen in the non-methanogenic enrichments remained 10 times higher than in methanogenic enrichments, which might account for the higher ethene production in those cultures. It is interesting that with more available hydrogen, the production of more acetate from homoacetogenesis is not observed in the non-methanogenic enrichments. On the contrary, less acetate was detected in non-methanogenic enrichments. This suggests that the homoacetogenesis might be inhibited by high concentrations of TCE, although little has been reported on the effects of TCE on homoacetogens.

Due to the effective hydrogen-consuming activity of hydrogenotrophic methanogens, they are commonly found to be involved in syntrophic relationships with thermodynamically unfavorable organisms, such as *Desulfovibrio* (Men et al. 2012; Walker et al. 2009) and *Syntrophomonas* (Sieber et al. 2010). Although results from this study indicate that methanogens did not benefit Dhc by enhancing dechlorination activity or cell growth, or by providing critical corrinoids, PCA analysis indicated that methanogenic activity affected community structures more significantly than supplying exogenous vitamin B₁₂. Among the 7 tracked non-Dhc OTUs, the numbers of *Desulfovibrio*_GW and PDS decreased as methanogenesis was inhibited, while the cell numbers of the other OTUs increased. Since *Desulfovibrio* sp. is known to ferment lactate to acetate and hydrogen without propionate generation (Men et al. 2012), its decrease in non-methanogenic enrichments might also account for the decrease in acetate production.

All four enrichments contained Dhc with the same core genome and identified functional RDase gene (*tceA*). This is reasonable since all enrichments were derived from the same original groundwater inoculum. Since the Dhc strains in the four enrichments lack the upstream corrin ring biosynthesis genes, they are incapable of biosynthesizing cobalamin *de novo*. However, they possess all of the strain 195 genes involved in the lower ligand attachment (*cobCDSTU*) as well as duplicated *cbiZ*-like genes that are involved in cobalamin salvaging pathways in *Archaea* and in *Rhodobacter sphaeroides* strain 2.4.1, the function of which is to cleave off the lower ligands of corrinoids (Gray and Escalante-Semerena 2009). A recent study demonstrated that in addition to directly using functional corrinoid forms provided by other members within a microbial community, Dhc strains are also capable of modifying nonfunctional corrinoid forms by replacing the original lower ligand with the functional lower ligands required for dechlorination (Yi et al. 2012).

In enrichments without exogenous vitamin B₁₂, 3–4 µg L⁻¹ cobalamin was detected, which is above the reported minimum requirement needed to sustain strain 195 (1 µg L⁻¹) (He et al. 2007), suggesting biologically significant amounts of corrinoids were produced by other microorganisms in the communities to support Dhc growth. Although the cobalamin detected in the enrichments without exogenous B₁₂ must have originated from other organisms, given that Dhc is able to modify other corrinoids into cobalamin (Yi et al. 2012), it is possible that the cobalamin in B₁₂-unamended enrichments was formed from the modification of other corrinoid species by Dhc. However the exact corrinoid forms produced by supportive microorganisms remain unclear. A similar symbiotic relationship between B₁₂-dependent algae and bacteria has previously been reported (Croft et al. 2005). The authors provided convincing evidence that the cobalamin required by B₁₂-dependent algae *Amphidinium operculatum* and *Porphyridium purpureum* was provided by *Halomonas* sp., a strain capable of *de novo* cobalamin synthesis. Moreover, enhanced growth and up-regulated B₁₂ biosynthesis in *Halomonas* sp. was observed in the presence of algal extracts.

In these dechlorinating enrichments, we observed stimulated growth of the *Clostridium*_GW OTU in the methanogenic enrichment without B₁₂ (LoTCE) compared with LoTCEB12. *Clostridium propionicum* is the species closest to *Clostridium*_GW, which can ferment acrylate or lactate to acetate and propionate with a 1:2 ratio (Janssen 1991). Although the corrinoids produced by *C. propionicum* are unknown, other *Clostridium* species are reported to be corrinoid producers. For example, *Moorella thermoacetica* is reported to produce an alternate corrinoid form, 5-methoxybenzimidazolylcobamide as well as cyanocobalamin, and *Clostridium formicoaceticum* can generate corrinoid 5-methoxy-6-methylbenzimidazolylcobamide (Renz 1999). In addition to *Clostridium* spp., two corrinoid forms, guanylcobamide and hypoxanthylcobamide are formed by *Desulfovibrio vulgaris* (Guimaraes et al. 1994). *Bacteroides* spp. are reported to have vitamin B₁₂-dependent fermentation pathways for glucose (Chen and Wolin 1981), while it is unknown whether they can synthesize corrinoids or not. However, no significant difference in growth was observed for OTUs closest to these two genera between enrichments with and without B₁₂.

Unlike *Clostridium*, *Desulfovibrio*, *Spirochaetes* and *Bacteroides*, genera of *Pelosinus*, *Dendrosporobacter* and *Sporotalea* (PDS) are not as often reported within dechlorinating microbial enrichments (Daprato et al. 2007; Freeborn et al. 2005; Rowe et al. 2008). *Pelosinus* spp. have been reported as metal-reducing, lactate-fermenting bacteria in lactate-amended enrichments (Mosher et al. 2012; Ray et al. 2011; Shelobolina et al. 2007). The most recent *Pelosinus* strain isolated from chlorinated solvent contaminated groundwater was *Pelosinus defluvii* sp. nov. (Moe et al. 2012). In the same study, *Sporotalea propionica* was renamed *Pelosinus propionicus* comb. nov. due to its genotypic and phenotypic similarities to that genus. The isolated *Pelosinus* spp. are not able to form acetate from H₂/CO₂ (Moe et al. 2012; Shelobolina et al. 2007), while *Dendrosporobacter* can grow chemoautotrophically on H₂/CO₂ (Strömpl et al. 2000), which might contribute to the observed homoacetogenesis. *Pelosinus*, *Sporotalea*, and *Dendrosporobacter* all belong to the *Sporomusa*–*Pectinatus*–*Selenomonas* phyletic group (Shelobolina et al. 2007; Strömpl et al. 2000). Another species within this group, *Sporomusa ovata* has been reported to produce *p*-cresolylcobamide, a form of corrinoid with a phenolic lower ligand (Stupperich et al. 1988). PDS copy numbers dominate the four enrichments, suggesting that they are playing

important roles in these communities, and possibly providing corrinoids, however, thus far, little information on PDS corrinoid production has been made available.

In summary, four TCE-dechlorinating microbial communities enriched from TCE-contaminated groundwater resulted in stable and robust dechlorination activity under different cobalamin stress and methanogenic conditions. Inhibition of methanogenesis resulted in higher dechlorination and ethene generation rates, as well as a greater proportion of electrons driving dechlorination. The Dhc genomes in the communities resemble strain 195 and do not contain genes for a complete corrinoid synthesis pathway, but do contain genes for corrinoid uptake, modification and salvaging, suggesting that Dhc takes advantage of corrinoids produced by other community members, possibly *Clostridium* and PDS species. Although methanogens were shown here not to be essential for supplying corrinoids to Dhc, their presence exerted more influence on community structure than B₁₂ amendment. This study improves our understanding of important microbial roles within dechlorinating communities that could improve our ability to identify biomarkers indicative of robust dechlorination activity during bioremediation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dechlorination, methane generation, aqueous hydrogen and Dhc growth in enrichments: (a) LoTCEB12; (b) LoTCE; (c) HiTCEB12; and (d) HiTCE. ↓ indicates the amendment of lactate and TCE, ↓↓ indicates the amendment of lactate alone. Note: the aqueous H₂ concentration scales vary among plots and have different units from methane

Fig. 2.
Total *Bacteria*, *Dhc* and *Archaea* in the four enrichments as measured by 16S rRNA gene copy numbers

Fig. 3.

16S rRNA gene copy numbers of dominant OTUs in the four enrichments at the end of a feeding cycle (Note: PDS represents a sum of *Pelosinus_GW*, *Dendrosporobacter_GW* and *Sporotalea_GW*)

Fig. 4. Principal component analysis (PCA) plot based on a time-course 16S rRNA gene copy numbers of 8 OTUs in the 4 enrichments (numbers next to each dot represents incubation days)

Table 1

Enrichment conditions of the four TCE-dechlorinating microbial communities

Enrichments	Lactate (mmol)		TCE (μmol)		Vitamin B ₁₂ (μg/L)	
	Feeding regime	Total	Feeding regime	Total	One-time addition	
LoTCEB12	3.9, 0.4, 0.4, 0.4, 0.2	5.3	22, 55, 55, 55, 33	220	100	
LoTCE	3.9, 0.4, 0.4, 0.4, 0.2	5.3	22, 55, 55, 55, 33	220	0	
HITCEB12	3.9, 0.4, 0.5, 0.5	5.3	77, 77, 66	220	100	
HITCE	3.9, 0.4, 0.5, 0.5	5.3	77, 77, 66	220	0	

Table 2

Electron balance of the four enrichments

eeq (mmol)										
Enrichments	Lactate added (mmol)	Propionate (mmol)	Acetate (mmol)	Butyrate (mmol)	Organic acids recovered ^a (%)	Total available ^b	Dechlorination ^c	Methanogenesis	H ₂	Other redox reactions ^d
LoTCEB12	5.3 ± 0.18	2.6 ± 0.12	2.7 ± 0.11	0	100 ± 5	5.6 ± 1.0	0.86 ± 0.02	2.0 ± 0.04	8.2×10 ⁻⁵	2.7 ± 1.0
LoTCE	5.3 ± 0.18	2.4 ± 0.08	2.8 ± 0.04	0	98 ± 4	6.8 ± 0.9	0.82 ± 0.02	1.9 ± 0.03	4.9×10 ⁻⁵	4.1 ± 0.9
HiTCEB12	5.3 ± 0.18	2.7 ± 0.05	1.8 ± 0.09	0.1	87 ± 3	4.6 ± 0.8	1.02 ± 0.01	0	5.2×10 ⁻³	3.6 ± 0.8
HiTCE	5.3 ± 0.18	2.7 ± 0.13	1.8 ± 0.11	0.09	87 ± 5	4.6 ± 1.1	0.98 ± 0.02	0	6.0×10 ⁻³	3.6 ± 1.1

^aValues are calculated from known lactate amendment, measured organic acids concentrations at the end of the enrichment period (i.e. the last time point from Figure 1) and stoichiometry based on the lactate fermentation equations below.

^bValues are calculated from lactate fermentation to acetate and hydrogen as shown below.

^cValues are calculated from known TCE amendment, measured chloroethenes and ethene concentrations at the end of the enrichment period in Figure 1 and stoichiometry based on the reductive dechlorination equations below.

^dValues are calculated by subtracting eeq remained in H₂ and consumed in dechlorination and methanogenesis from the total available eeq.

Reaction		Reference
Lactate fermentations	$\text{CH}_3\text{CHOHCOO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{CO}_2$ <div>(lactate) (acetate)</div>	(Walker et al. 2009) (Madigan et al. 2012) (Muñoz-Tamayo et al. 2011)
	$3\text{CH}_3\text{CHOHCOO}^- \rightarrow 2\text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{COO}^- + \text{CO}_2 + \text{H}_2\text{O}$ <div>(lactate) (propionate) (acetate)</div>	
	$\text{CH}_3\text{CHOHCOO}^- + \text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + \text{CO}_2 + \text{H}_2\text{O}$ <div>(lactate) (acetate) (butyrate)</div>	
Hydrogenotrophic reactions	Dechlorination	(Maymó-Gatell et al. 1997)
	$\text{C}_2\text{HCl}_3 + 2\text{H}_2 \rightarrow \text{C}_2\text{H}_3\text{Cl} + 2\text{H}^+ + 2\text{Cl}^-$ <div>(TCE) (VC)</div>	
	$\text{C}_2\text{HCl}_3 + 3\text{H}_2 \rightarrow \text{C}_2\text{H}_4 + 3\text{H}^+ + 3\text{Cl}^-$ <div>(TCE) (Ethene)</div>	
Methanogenesis	$\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$ <div>(Methane)</div>	(Walker et al. 2009)

Reference	Reaction
(Rittmann and McCarty 2001)	Homoacetogenesis $2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2\text{O}$ (acetate)
	Biosynthesis $\frac{1}{8}\text{CH}_3\text{COO}^- + \frac{1}{20}\text{NH}_4^+ + \frac{3}{40}\text{CO}_2 \rightarrow \frac{3}{40}\text{HCO}_3^- + \frac{3}{40}\text{H}_2\text{O} + \frac{1}{20}\text{C}_5\text{H}_7\text{O}_2\text{N}$ (acetate) (cells)

Table 3

Summary of clone library results of the four enrichments

OTU, closest cultivatable species (NCBI accession numbers)	Maximum percent identity to the closest neighbor (% coverage)	% of clones in each library				
		LoTCEB12	LoTCE	HITCEB12	HITCE	HITCE
<i>Dehalococcoides</i> _GW (JQ004083), <i>Dehalococcoides mccartyi</i> strain 195 (CP0000027.1)	99 (99)	8.5	4.2	11.4	16.5	
<i>Pelosinus</i> _GW (JQ004084/KC517355) ¹ , <i>Pelosinus</i> sp. UFO1 (DQ295866)	93 (99)	27.7	59.7	40.4	24.3	
<i>Dendrosporobacter</i> _GW (JQ004085), <i>Dendrosporobacter quercicolus</i> strain DSM 1736 (NR_041949)	94 (89)	10.6	4.2	11.4	14.8	
<i>Sporotalea</i> _GW (KC517356/JQ004090) ¹ , <i>Sporotalea propionica</i> strain TM1 (FN689723)	91 (99)	7.4	2.8	3.5	0.88	
<i>Clostridium</i> _GW (JQ004086), <i>Clostridium propionicum</i> (AB649276)	98 (99)	2.1	6.9	8.8	14.8	
<i>Desulfovibrio</i> _GW (JQ004087), <i>Desulfovibrio onyzae</i> (AF273083)	99 (99)	19.1	2.8	N.D. ²	4.3	
<i>Spirochaetes</i> _GW (JQ004088), <i>Spirochaetes bacterium</i> SA-8 (AY695839)	99 (97)	3.2	N.D	1.8	4.3	
<i>Bacteroides</i> _GW (JQ004089), <i>Bacteroides</i> sp. strain Z4 (AY949860)	99 (99)	N.D	N.D	0.88	4.3	
Total clones		94	72	114	115	

¹Two lengths of sequences were obtained, one with a shorter insert (10 bp for *Pelosinus*_GW, 7 bp for *Sporotalea*_GW), and the second with a longer insert (118 bp for *Pelosinus*_GW and 113bp for *Sporotalea*_GW).

²N.D: not detected