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Identification of Novel Biomarkers from Supportive Microorganisms in TCE-dechlorinating Microbial Communities

By

Yujie Men

A dissertation submitted in partial satisfaction of the

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in

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in the

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of the

University of California, Berkeley

Committee in charge:

Professor Lisa Alvarez-Cohen, Chair
Professor David L. Sedlak
Professor Mary K. Firestone

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Identification of Novel Biomarkers from Supportive Microorganisms in TCE-
dechlorinating Microbial Communities

Abstract

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By

Yujie Men

Doctor of Philosophy in Engineering-Civil and Environmental Engineering

University of California, Berkeley

Professor Lisa Alvarez-Cohen, Chair

Chlorinated ethenes are toxic and carcinogenic compounds that have contaminated a large quantity of groundwater in the U.S. and other developed countries. In order to protect public health, *in situ* bioremediation via dehalorespiration by *Dehalococcoides* bacteria is a promising solution. The overall goal of this research is to understand the ecological relationship between *Dehalococcoides mccartyi* (Dhc) and supportive microorganisms in a community and identify novel biomarkers for monitoring TCE dechlorination activities during bioremediation. To accomplish these goals, traditional molecular and high throughput microarray techniques, as well as newly established analytical approaches were applied.

The first objective of this research was to characterize four Dhc-containing microbial communities enriched from contaminated groundwater under different cobalamin stress and methanogenic conditions. Microarray analyses targeting four Dhc genomes revealed a commonly shared core Dhc genome most similar to strain 195. Physiological characterization revealed that inhibited methanogensis optimized the dechlorination performance. Experimental evidence demonstrated the presence of Bacterial species providing corrinoids to Dhc. The dominance of closely related *Pelosinus* spp., *Dendrosporobacter* spp. and *Sporotalea* spp. and the significant effects of cobalamin addition and methanogenic inhibition on the distribution of *Clostridium* spp. in the communities suggest that species in these genera are potential corrinoid providers to Dhc.

In order to further target corrinoid production in enrichments without exogenous cobalamin, a detection method was established that successfully differentiates various corrinoid and lower ligand forms. Corrinoid and lower ligand profiles of different Dhc-containing enrichments indicate that cobalamin was the major corrinoid utilized by Dhc. Further evidence demonstrated that in enrichments without exogenous cobalamin, *p*-cresolylcobamide was produced, likely by *Pelosinus* spp., and then modified by Dhc into cobalamin in the presence of dimethylbenzimidazole (DMB) to support dechlorination reactions.

Differential gene expression between enrichments with and without exogenous cobalamin was investigated using microarrays targeting four sequenced Dhc genomes. Results suggest that *cobT* and *btuF* genes, the Nuo and Hym operons and the tryptophan operon could serve as biomarkers indicating cobalamin stress and corrinoid salvaging. Temporal global gene expression of the enrichment without exogenous cobalamin corroborates the importance of hydrogenases at an early stage of dechlorination and the close relation between TceA reductive dehalogenase and Hup hydrogenase.

Based on the knowledge obtained in the previous studies, defined consortia were constructed by growing Dhc strain 195 with potential supportive microorganisms: *Desulfovibrio vulgaris* Hildenborough, *Methanobacterium congolense*, and *Pelosinus fermentans* strain R7. Physiological, transcriptomic and proteomic analyses of strain 195 containing consortia provide strong evidence supporting the previous hypotheses that *Desulfovibrio* spp. play important roles in hydrogen transfer, *Pelosinus* spp. provide corrinoid forms to Dhc but not DMB, while methanogens do not contribute to Dhc cell growth or biological corrinoid generation in the presence of the other two species.

The significance of this research is that supportive microorganisms and related chemical and molecular biomarkers have been identified. And they may be useful for optimizing bioremediation processes by obtaining accurate feedback information from cells that are indicative of Dhc physiology. Knowledge developed in this research will aid practitioners to better design, monitor and optimize future *in situ* bioremediation systems.

To my family

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Acknowledgements

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The journey through graduate school definitely has its ups and downs, but I am blessed to have many trustworthy friends who stood by my side. The friendships of Chao Liu, Bing

1 Introduction and Objectives

1.1 Introduction

Trichloroethene (denoted TCE), which was widely used in cleaning and degreasing, due to its effectiveness, noncorrosivity, nonflammability, and ease of recycling, has been recognized as a common organic groundwater contaminant at hazardous waste sites for decades (McCarty, 1997; Doherty, 2000b; Seshadri *et al.*, 2005). Although TCE has been regulated since the late 1960s because of early improper disposal, as of 1997, TCE was reported to be present at 852 of 1430 National Priority List sites, making it one of the most pervasive groundwater pollutants at Superfund sites (Doherty, 2000b). Considering the potential health effects of TCE on human beings, the U.S. Environmental Protection Agency (USEPA) has established national primary drinking water regulations for TCE with maximum contaminant levels (MCLs) of 5µg/L. However, at many contaminated groundwater sites, TCE has a high frequency of concentrations greater than or near MCLs (Moran *et al.*, 2007). Thus, in order to maintain TCE under MCLs, remedial treatment is often required.

In the past, clean-up efforts for chlorinated ethenes relied heavily upon physical and chemical processes. However, gradually, bioremediation has become an acceptable alternative (Mackay and Cherry, 1989; Kohn *et al.*, 2005). Bioremediation takes advantage of microbial metabolism to transform contaminants from a harmful state to an innocuous state. It is more cost-effective with larger available capacities, since microorganisms are versatile and universally distributed. In aerobic environments, microorganisms can only metabolically oxidize less-chlorinated ethenes such as cis-dichloroethene (cis-DCE), and vinyl chloride (VC) to CO_2 (Coleman *et al.*, 2002b; Coleman *et al.*, 2002a; Mattes *et al.*, 2005), while microorganisms in anaerobic environment are able to metabolically degrade tetrachloroethene (PCE) and TCE to the innocuous end product ethene via a process termed dehalorespiration where the chlorinated ethenes serve as energetically favorable electron acceptors for energy generation (McCarty, 1997). Over the past two decades, tremendous progress has been made in understanding dehalorespiration. A large number of dehalorespiring bacterial strains have been successfully isolated and characterized, advancing our knowledge of chlorinated ethene bioremediation (Smidt and de Vos, 2004; Aulenta *et al.*, 2006).

So far, among the characterized dechlorinating isolates, only bacteria in the genus *Dehalococcoides* spp. (Dhc) are capable of complete reductive dechlorination from PCE and TCE to ethene. All organisms outside the Dhc genus only dechlorinate the higher chlorinated ethenes to DCE (Smidt and de Vos, 2004; Aulenta *et al.*, 2006). The first isolated Dhc strain was designated *Dehalococcoides mccartyi* strain 195, and there are now nine described strains (Maymó-Gatell *et al.*, 1997; Adrian *et al.*, 2000; Cupples *et al.*, 2003; He *et al.*, 2003b; He *et al.*, 2005; Sung *et al.*, 2006b; Cheng and He, 2009; Lee *et al.*, 2011), five of which have been fully sequenced (i.e. strain 195, CBDB1, BAV1, VS, and GT) (Kube *et al.*, 2005; Seshadri *et al.*, 2005; McMurdie *et al.*, 2009) (http://img.jgi.doe.gov). While nearly all Dhc strains are known to respire chlorinated ethenes, there are variations in their substrate range (Löffler and Edwards, 2006) and some can even respire a variety of anthropogenic organo-halide pollutants that have different chemical structures (Adrian *et al.*, 2000; Bunge *et al.*, 2003; Fennell *et al.*, 2004), making Dhc versatile organisms in bioremediation applications.

A number of field trials have now successfully established the link between the presence of Dhc populations and complete reductive dechlorination to ethene, while their absence has consistently resulted in the accumulation of DCEs (Ellis *et al.*, 2000; Hendrickson *et al.*, 2002; Major *et al.*, 2002; Lendvay *et al.*, 2003; Rahm *et al.*, 2006a). Since the 16S rRNA sequences are highly conserved among Dhc strains with different dechlorination abilities, misinterpretation of the dechlorination potential can result when relying only upon this gene for identification. Instead, the known functional reductive dehalogenase (RDase) genes (i.e. *pceA*, *tceA*, *vcrA*, *bvcA*) have been demonstrated to exhibit close correlation to dechlorination activities, thus serve as more effective biomarkers (Lee *et al.*, 2006; Ritalahti *et al.*, 2006; van der Zaan *et al.*, 2010).

Although Dhc are geographically widely distributed, they are usually not numerically dominant in environmental communities, thus the enhancement of Dhc growth is the goal in biostimulation and bioaugmentation applications. Laboratory-based studies have observed faster dechlorination and more stable and robust growth of Dhc when grown in mixed cultures than in isolation (Maymó-Gatell *et al.*, 1997; Duhamel *et al.*, 2004; He *et al.*, 2007). Consequently, improved understanding of the key players supporting the growth of Dhc and the relationship between Dhc and other organisms in a community will enable more effective bioremediation performance and enhanced prediction of success. Organic substrates, such as lactate, whey or solubilized vegetable oil, which are safer and more cost-effective than gaseous hydrogen, have been injected into many field sites to stimulate bioremediation (Ellis *et al.*, 2000; Lendvay *et al.*, 2003; Rahm *et al.*, 2006a). Therefore, fermenting microorganisms that provide hydrogen and acetate to support Dhc growth and dechlorination activities. Besides hydrogen and acetate, other growth requirements, such as corrinoid co-factors, might also be supplied by supportive microorganisms, which are essential for Dhc activities but cannot be biosynthesized *de novo* by Dhc (http://img.jgi.doe.gov). Microbial enrichments inoculated with groundwater and enriched under different conditions of interest will allow us to identify the 16S rRNA-based and function-based biomarkers indicative of dechlorination and growth activities of Dhc.

Previous studies with dechlorination cultures that focused on the effects of different electron donors (Freeborn *et al.*, 2005), potential competition for hydrogen among hydrogenotrophic microorganisms (Yang and McCarty, 1998), as well as transcription analysis of Dhc hydrogenase genes when grown with different hydrogen levels (Morris *et al.*, 2006), have increased our knowledge of interspecies hydrogen transfer in dechlorinating communities. However, no dechlorinating microbial community study has been carried out specifically focusing on corrinoid-providing organisms.

The limited knowledge of corrinoid-supplying organisms in these communities may be due to limitations in corrinoid detecting analytical techniques. That is, conventional biologically-based methods cannot differentiate corrinoid forms (Chanarin and Muir, 1982; Whitman and Wolfe, 1984; Dalbacke and Dahlquist, 1991; Kelleher and Broin, 1991). A recent developed method using liquid chromatography with mass spectrometry (LC/MS) makes it possible to identify all naturally occurring corrinoid forms individually

and to quantify them with low detection limits (Allen and Stabler, 2008). The establishment and optimization of corrinoid detecting methods will give us a better platform to identify organisms that provide corrinoids to Dhc when exogenous sources are limited. In addition, the availability of Dhc genomes and high throughput microarray technologies allow us to investigate the global responses of Dhc on a molecular level when grown with supportive organisms under various growth conditions. Together with other molecular tools in community structural and functional analysis, these approaches will give us better understanding of the relationship between Dhc and supportive organisms in dechlorinating communities.

Current bioremediation approaches are based upon empirical, rather than knowledge-based, approaches with trial-and-error methods dominating field practice (Lovley, 2003). Understanding the mechanisms by which Dhc interact with other community members in dechlorination processes will not only promote the identification of more comprehensive indicative biomarkers for monitoring and prediction purposes, but could also lead to improved bioremediation designs for more effective, well-controlled strategies for treatment of chlorinated ethenes.

1.2 Research objectives

The overall goal of this study is to identify novel biomarkers indicative of TCE dechlorination potential and activities from both *Dehalococcoides* and supportive microorganisms in dechlorinating communities. To accomplish this overall goal, the following objectives were pursued and accomplished.

1) Characterize physiological and genomic properties of *Dehalococcoides*-containing, TCE-dechlorinating microbial communities.
2) Identify diverse corrinoid and lower ligand forms produced in *Dehalococcoides*-containing microbial communities and the salvaging mechanisms utilized by *Dehalococcoides*.
3) Analyze differential gene expression under different cobalamin stresses and over time-courses of growth cycles.
4) Construct and characterize sustainable syntrophic growth of defined consortia containing *Dehalococcoides mccartyi* strain 195 with potential supportive microorganisms.

1.3 Dissertation Overview

This dissertation is organized into seven chapters. The background leading to the formation of the overall research goal and the specific objectives is outlined here in **Chapter 1**. Previously published literature that is relevant to the study of reductive dechlorination in *Dehalococcoides* spp. and biomarkers development is synthesized and presented in **Chapter 2**. The characterization of four TCE-dechlorinating microbial communities enriched under different cobalamin stress and methanogenic conditions are presented in **Chapter 3**. The identification and quantification of corrinoid and lower ligand species produced by supportive microorganisms in *Dehalococcoides*-containing microbial communities and the salvaging mechanisms by *Dehalococcoides* are presented

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in **Chapter 4**. This is the first time that corrinoid production and utilization has comprehensively been investigated in corrinoid-dependent dechlorinating communities. Potential biomarkers identified from the transcriptomes of *Dehalococcoides* genomes in dechlorinating communities under different cobalamin stress and growth phases are presented in **Chapter 5**. The characterization of syntrophic growth of strain 195 with supportive microorganisms in defined consortia along with whole-genome transcriptomic and proteomic analyses are presented in **Chapter 6**. Portions of this work have been published in Men *et al.*, 2012. Finally, in **Chapter 7**, the key findings of this research are summarized and directions for future research are proposed.

2 Literature Review

2.1 Chlorinated ethenes are common groundwater contaminants in the United States

Chlorinated ethenes are chemically stable organic molecules with a covalent carbon-carbon double bond as the backbone and chlorine or hydrogen atoms covalently bonded to each carbon (Figure 2.1). Because of their noncorrosivity, nonflammability, effectiveness and ease of recycling, they are widely used in many industrial fields, including dry-cleaning, degreasing, electronics, defense, chemical, rail, automotive, and food processing (Doherty, 2000b). For example, as of 2000, PCE was the cleaning solvent for nearly all of the approximately 30,000 dry cleaners and launderers in the United States (Doherty, 2000a).

Chlorinated ethenes have been regulated since the late 1960s because of environmental concerns (Doherty, 2000b). Given the evidence that VC is a human carcinogen, as well as the potential carcinogenicity of PCE and TCE, the use of, and exposure to, chlorinated ethenes is of significant concern for public health (Lee *et al.*, 2002). Considering the adverse health effects of chlorinated ethenes on human beings, the U.S. Environmental Protection Agency (USEPA) has established national primary drinking water regulations for PCE and TCE with maximum contaminant levels (MCLs) of 5µg/L (Doherty, 2000a; Moran *et al.*, 2007; USEPA, 2009).

However, due to early improper disposal, as of 1997, TCE was reported as a contaminant at 852 of 1430 National Priority List sites, making it one of the most pervasively found pollutants at Superfund sites (Doherty, 2000b). At many contaminated groundwater sites, TCE has a high frequency of concentrations greater than or near MCLs (Moran *et al.*, 2007). In the 2004 U.S. National Priority List, chlorinated ethenes were by far the most common group of organic contaminants at sites prioritized for remediation (Lemming *et al.*, 2010). Therefore, in order to restore our groundwater resources and protect public health, effective remediation actions must be taken.

Tetrachloroethene (PCE)   Trichloroethene (TCE)

cis-Dichloroethene (*cis*-DCE)   trans-Dichloroethene (*trans*-DCE)   1,1-Dichloroethene (1,1-DCE)

Vinyl chloride (VC)
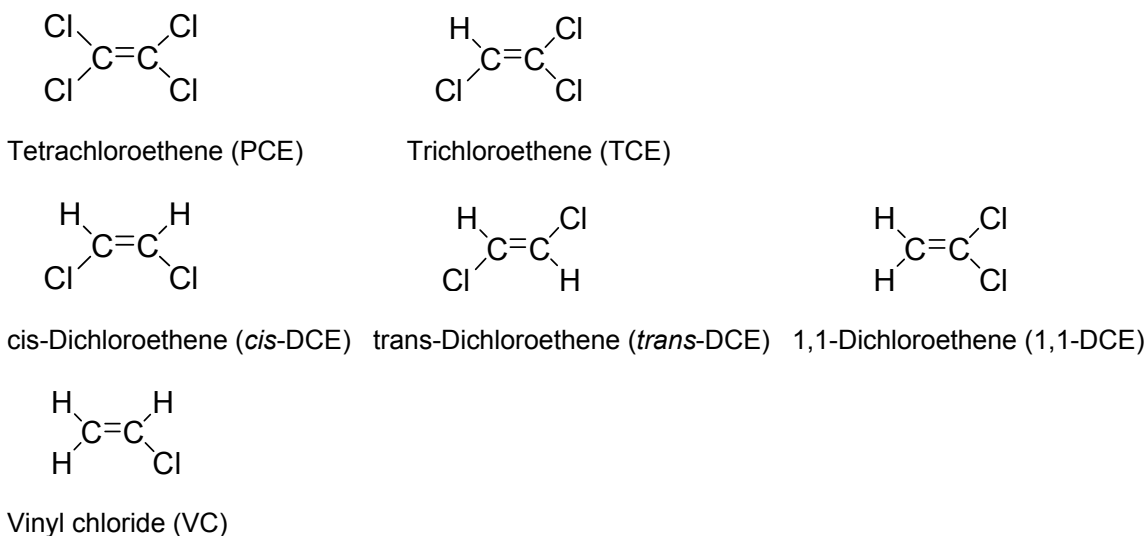
Figure 2.1 Chemical structures of chlorinated ethenes

2.2 *In-situ* bioremediation of chlorinated ethenes

Approaches that have been applied in chlorinated ethene remediation include ex-situ and in-situ, physical-chemical and biological processes. Given the relative low efficiency and high cost of physical-chemical *ex-situ* remediation techniques for TCE (He *et al.*, 2003b), along with the limited capacity and longevity issues of *in-situ* chemical remediation approaches such as permeable reactive barriers (PRB), *in-situ* bioremediation becomes a competitive alternative approach (Mackay and Cherry, 1989; Kohn *et al.*, 2005). In contrast with common physical-chemical treatment processes, *in-situ* bioremediation is more cost-effective with larger available capacities, since microorganisms are versatile and universally distributed.

In-situ bioremediation can be carried out either aerobically or anaerobically. Less chlorinated ethenes, such as VC (Coleman *et al.*, 2002b; Mattes *et al.*, 2005) and cis-DCE (Coleman *et al.*, 2002a) were reported to be able to serve as carbon and energy sources for some aerobic bacteria. TCE can be co-metabolized when a primary substrate such as methane, toluene or phenol is present (Alvarez-Cohen and Speitel, 2001). However, there is no evidence that higher-chlorinated ethenes (i.e. PCE and TCE) can be oxidized to CO_2 metabolically, whereas they could be reductively dechlorinated to less chlorinated ethenes or ethene under anaerobic conditions, typical conditions encountered in the subsurface where oxygen diffusion is limited.

In reductive dechlorination, PCE and TCE serve as electron acceptors, which take up one or more electrons as they are sequentially reduced to the less chlorinated ethenes DCEs, VC and ethene (Maymó-Gatell *et al.*, 1997). The stepwise reduction of chlorinated ethenes is an energetically favorable series of reactions. The estimated Gibbs free energies ($\Delta G°'$) for reductive dechlorination of chlorinated ethenes at pH 7 and room temperature range from -145 to -172 kJ/mol, corresponding to standard redox potentials (E_0') of +332 to +472 mV (Dolfing, 2000). The energetic process of reductive dechlorination is also called dehalorespiration. The reactions involved in reductive dechlorination are shown in Figure 2.2. Redox potentials (E_0') for reductive dechlorination are higher than sulfate reduction (-220 mV) and methanogenesis (-280 mV), but lower than nitrate (+740 mV) and iron (+760 mV) reduction (Madigan *et al.*, 2012), suggesting that thermodynamically, reductive dechlorination would typically occur in anaerobic environments under sulfate-reducing and methanogenic conditions.
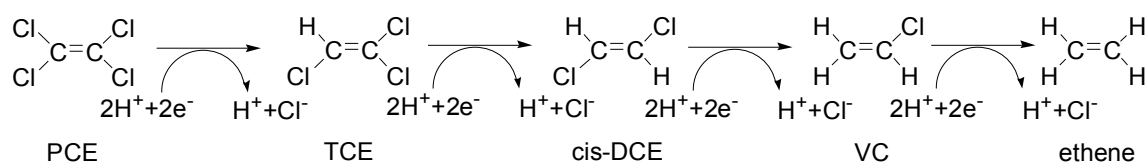
Figure 2.2 Reductive dechlorination pathways

Due to the complete dechlorination to ethene, energetic metabolism, as well as easy-to-achieve anaerobic conditions in contaminated groundwater plumes, anaerobic *in-situ*

bioremediation has become the preferred strategy in large-scale remediation applications of chlorinated ethenes. The microorganisms involved in those processes have also been extensively studied during the last two decades.

2.3 Microorganisms in reductive dechlorination

Many species of anaerobic bacteria have been identified to be capable of reductive dechlorination, which could be generally classified into four phyla: *Firmicutes*, ε-*Proteobacteria*, δ-*Proteobacteria*, and *Chloroflexi*.

Desulfitobacterium and *Dehalobacter* are the two main genera in the *Firmicutes* phylum where dehalorespiring organisms were identified. *Desulfitobacterium* spp. have versatile metabolisms, including the capabilities of using halogenated organic compounds, sulfite, metal, and humic acids as electron acceptors, and hydrogen, lactate, formate and pyruvate as electron donors (Villemur *et al.*, 2006). Dehalorespiring *Desulfitobacterium* species include *Desulfitobacterium* sp. strain Y51 (Suyama *et al.*, 2001), *Desulfitobacterium frappieri* TCE1 (Gerritse *et al.*, 1999), *Desulfitobacterium* sp strain PCE-S (Miller *et al.*, 1997), which are able to dechlorinate PCE or TCE to cDCE. *Desulfitobacterium* strain PCE1 can only dechlorinate PCE to TCE (Gerritse *et al.*, 1996). *Dehalobacter restrictus* is closely related to the *Desulfitobacterium* genus (Villemur *et al.*, 2006), but with more restricted metabolisms. *Dehalobacter* sp. solely use H_2 as electron donor, and PCE or TCE as electron acceptors with cDCE as the dechlorination product (Wild *et al.*, 1997; Holliger *et al.*, 1998a).

Dehalorespiring ε-*Proteobacteria* belong to the genus *Sulfurospirillum* (Luijten *et al.*, 2003). *Sulfurospirillum halorespirans* and *Sulfurospirillum multivorans* (formerly known as *Dehalospirillum multivorans*) are two isolated strains capable of reductive dechlorination of PCE or TCE to cDCE. Hydrogen and other organic acids such as lactate, pyruvate and formate can be used as electron donors (Miller *et al.*, 1996; Luijten *et al.*, 2003).

Desulfuromonas, *Desulfomonile*, *Geobacter* are the genera in δ-*Proteobacteria*, where bacteria capable of reductive dechlorination were isolated. *Desulfuromonas chloroethenica* strain TT4B (Krumholz, 1997), *Desulfuromonas michiganensis* strain BB1 and BRS1 (Krumholz, 1997), *Desulfomonile tiedjei* DCB-1 (Fathepure and Tiedje, 1994), *Geobacter lovleyi* (Sung *et al.*, 2006a) are typical strains capable of dechlorinating PCE or TCE to cDCE using various electron donors include hydrogen, lactate, acetate, pyruvate, *etc*.

Different from all the above organisms, strains of *Dehalococcoides mccartyi* (Dhc) in the phylum Chloroflexi are so far the only known bacteria that can reductively dechlorinate PCE or TCE beyond cDCE to the end innocuous product ethene (Löffler and Edwards, 2006). To date, nines strains of this species have been isolated: designated as strains 195 (Maymó-Gatell *et al.*, 1997), BAV1 (He *et al.*, 2003b), CBDB1 (Adrian *et al.*, 2000), VS (Müller *et al.*, 2004), GT (Sung *et al.*, 2006b), FL2 (He *et al.*, 2005), MB (Cheng and He, 2009), ANAS1 and ANAS2 (Lee *et al.*, 2011). Dhc are obligate anaerobic bacteria.

Besides chlorinated ethenes, other chlorinated organic compounds, such as chlorinated benzenes (Adrian *et al.*, 2000), phenols, furans (Fennell *et al.*, 2004), and dioxins (Bunge *et al.*, 2003), can also serve as the electron acceptors. Strain 195 is also capable of debrominating polybrominated biphenyl ethers (PBDEs), further expanding the versatility of Dhc. Therefore, Dhc are of great significance in the bioremediation of halogenated organic compounds and have become model organisms for studying reductive dechlorination.

Although Dhc have the versatility of using a wide electron acceptor range for reductive dechlorination, they are fastidious in their other nutrient requirements. They only use H_2 as electron donor, acetate as carbon source in the presence of CO_2, and cobalamin as an essential co-factor for the reductive dehalogenases which catalyze the dechlorinations (Seshadri *et al.*, 2005; Löffler and Edwards, 2006; He *et al.*, 2007). Further, the growth of Dhc isolates is somewhat unreliable and relatively slow with doubling times of 20-60 hours (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003a; He *et al.*, 2005; Sung *et al.*, 2006b). In contrast, previous studies have shown that growth of Dhc in microbial communities or defined consortia is generally more rapid and robust (Maymó-Gatell *et al.*, 1997; Duhamel *et al.*, 2004; Duhamel and Edwards, 2006; He *et al.*, 2007). The following two sections are devoted to review the current knowledge of the Dhc genus and the important roles played by other supportive organisms to enhance the dechlorination and growth of Dhc within Dhc-containing microbial communities.

2.4 Phylogeny, morphology, and physiology of *Dehalococcoides* spp.

2.4.1 Phylogeny

Strain 195, the first isolated Dhc strain, is grouped within the *Bacterial* domain but does not cluster within any of the known phylogenetic lines. However, based on maximum likelihood analysis of the 16S rRNA gene, it was placed on a branch that includes cyanobacteria and planctomycetes (Maymó-Gatell *et al.*, 1997). Due to its distinct place on the phylogenetic tree, "*Dehalococcoides*" was named by Maymó-Gatell and the co-workers as a novel genus. The following isolated strains were named under the species name "*Dehalococcoides mccartyi*". In addition, the Dhc genus is under the class "Dehalococcoidetes", which encompasses two other organohalide-respiring clusters represented by the isolate "*Dehalobium chlorocoercia*" strain DF-1 (AF393781) and *Dehalogenimonas lykanthroporepellens* (EU679418, ATCC BAA-1523 = JCM 15061) (Löffler *et al.*, 2012). Dhc is now placed in the group 2 of *Chloroflexi* (green non-sulfur) phylum (Hugenholtz *et al.*, 1998). The limited number of cultivated members of this phylum is an interesting group of diverse phenotypes, which include not only reductive dechlorinators (*Dehalococcoides mccartyi*), but also gliding, filamentous isolates that contain some form of bacteriochlorophyll frequently arranged in chlorosomes (*Chloroflexus*, *Oscillochloris*, *Chloronema*, and *Heliothrix*); gliding, filamentous, mesophilic, strict aerobic chemoheterotrophs (*Herpetosiphon*); a hyperthermophilic, irregular rod-shaped, nonmotile aerobic chemoheterotroph (*Thermomicrobium roseum*) (Rappe and Giovannoni, 2003). None of the cultivated members in the *Chloroflexi* phylum except Dhc are known to carry out reductive dechlorination and Dhc also do not exhibit many of the physiological traits of other members in the phylum. Chloroflexi 16S

rRNA gene sequences are found to be widespread in diverse environments such as ocean bacterioplankton, freshwater bacterioplankton, soils, sediments, and geothermal hot springs (Rappe and Giovannoni, 2003). By identifying more organisms in the *Chloroflexi* phylum, we will better understand whether dehalogenation is exclusive to the *Dehalococcoides* genus and the evolutionary history of this unique group of dehalorespiring bacteria.

16S rRNA genes of the isolated Dhc strains, together with a large number of environmental and mixed culture 16S rRNA gene clone sequences are highly conserved with a similarity greater than 98% (Hendrickson *et al.*, 2002). The tightly clustered group was further divided into three phylogenetic subgroups based on sequence signatures in the hypervariable V2 and V6 regions of the 16S rRNA gene: the "Cornell" subgroup includes strain 195 and strain MB (Cheng and He, 2009); the "Victoria" subgroup includes strain VS and the "Pinellas" subgroup comprises the other available Dhc isolates as well as most cultured Dhc strains identified in mixed cultures and the majority of the environmental clone sequences (Hendrickson *et al.*, 2002; Löffler *et al.*, 2012). The highly conserved 16S rRNA sequences among Dhc strains limits their use for predicting physiological characteristics among different members in this genus (Lovley, 2003; Löffler and Edwards, 2006).

2.4.2 Morphology

Dhc isolates are typically round, disc-shaped cells with 0.5-2 μm wide and 0.1-0.2 μm thick (Maymó-Gatell *et al.*, 1997; Adrian *et al.*, 2000; He *et al.*, 2003b; He *et al.*, 2005; Sung *et al.*, 2006b; Löffler *et al.*, 2012). Characteristic biconcave indentations on opposite flat sides of the cell were visible (Löffler *et al.*, 2012). Unusual cell surface features such as filamentous appendages were observed, the function of which is unclear but they may play a role in attachment of cells to each other or to surfaces (Löffler *et al.*, 2012). There's no evidence that the cells are motile. Growth on agar surfaces was not observed, either.

One strategy employed to isolate Dhc is the application of the peptidoglycan biosynthesis inhibitors ampicillin and vancomycin (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003b). The continuous reductive dechlorination performance of Dhc in the presence of these antibiotics after several subsequent transfers suggests that the cells have an unusual cell wall structure. Staining tests on strain 195 cells confirmed the lack of a peptidoglycan cell wall and microscopy demonstrated the presence of an S-layer protein structure that is commonly found in many Archaea (Maymó-Gatell *et al.*, 1997). A 105-110 kDa protein encoded by DET1407 in strain 195 has been proposed to be an S-layer component (Morris *et al.*, 2006). The unusualness of the Dhc membrane is further revealed in phospholipid fatty acid (PLFA) analysis where little monounsaturated PLFA was found and instead, several novel furan fatty acids were present (White *et al.*, 2005).

2.4.3 Physiology

Although Dhc strains share highly conserved 16S rRNA sequences, they possess quite different dechlorination capacities in terms of the terminal electron acceptor chlorinated

ethenes. There are currently nine identified Dhc strains (strain 195, CBDB1, BAV1, VS, GT, FL2, MB, ANAS1 and ANAS2), each exhibits different metabolic and co-metabolic capabilities to reductively dechlorinate the chlorinated ethenes (Table 2.1). PCE, the most highly chlorinated ethene, is only used by strain CBDB1 and strain 195 as terminal electron acceptor for growth (Maymó-Gatell *et al.*, 1997; Löffler *et al.*, 2012). VC, the least chlorinated ethene, serves as the electron acceptor for strain BAV1, VS and GT (He *et al.*, 2003b; Müller *et al.*, 2004; Sung *et al.*, 2006b). TCE can be used as electron acceptor for the growth of eight strains (195, CBDB1, VS, GT, FL2, MB, ANAS1, and ANAS2), and cDCE for strain 195, BAV1, VS, GT, FL2, ANAS1 and ANAS2. When a chlorinated ethene cannot serve as a terminal electron acceptor for a Dhc strain, that compound can sometimes be co-metabolically dechlorinated. For example, VC is dechlorinated by strain 195 when the growth-supporting TCE is present and after a significant fraction of the amended TCE has been consumed (Maymó-Gatell *et al.*, 1997). So far, it is not clear why Dhc possess overlapping chlorinated ethene metabolism, but the presence of multiple Dhc strains in microbial communities is common and might be necessary to achieve complete reductive dechlorination of PCE or TCE to ethene. For example, in a long-maintained TCE dechlorinating enrichment (ANAS), there are two unsequenced Dhc strains, "ANAS1" and "ANAS2". ANAS1 is capable of dechlorinating TCE to VC metabolically, while ANAS2 is able to dechlorinate VC further down to ethene for growth. Therefore, with a combination of these two strains, this enrichment can reductively dechlorinate TCE completely to ethene (Lee *et al.*, 2011).

Based on the five sequenced Dhc genomes (195, CBDB1, BAV1, VS, and GT), there are more than 100 genes that are annotated as reductive dehalogenases (RDases) encoding genes (http://img.jgi.doe.gov). RDases were also identified from dechlorinators other than Dhc (Miller *et al.*, 1998; Villemur *et al.*, 2002). RDases are the proteins that catalyze the reductive dechlorination. So far, only three RDases have been purified and characterized from Dhc: a 51 kDa PCE-RDase and a 61 kDa TCE-RDase from strain 195 (Magnuson *et al.*, 1998); and a 62 kDa VC-RDase from strain VS (Müller *et al.*, 2004). The genes encoding these proteins are designated as *pceA*, *tceA*, and *vcrA*, respectively. Each of these RDases were purified from the membrane fraction of cell lysis, indicating that these enzymes are membrane-bound (Magnuson *et al.*, 1998; Müller *et al.*, 2004). In addition, the three RDase genes were found to be followed by a shorter open reading frame (ORF) downstream, which likely encodes a small integral membrane protein involved in the association of the RDase with the cytoplasmic membrane. These RDase anchoring protein encoding genes are designated as *pceB*, *tceB*, and *vcrB*, respectively (Magnuson *et al.*, 2000; Müller *et al.*, 2004).

Characterization of the purified RDases demonstrates their observed functions in the reductive-dechlorinating isolates, for example, PCE-RDase exhibits reductive dechlorination only with the substrate PCE, while TCE-RDase has a broader substrate range including TCE, cDCE, tDCE, 1,1-DCE and VC (Magnuson *et al.*, 1998; Magnuson *et al.*, 2000). However, the dechlorination rates of TCE-RDase for tDCE and VC were ten times slower than the other chlorinated ethenes, supporting the observation that tDCE and VC are co-metabolized by strain 195 (Magnuson *et al.*, 2000). VC-RDase reduces VC and all DCE isomers, but not PCE or TCE, at high rates (Müller *et al.*, 2004).

However, given that strain VS is able to dechlorinate TCE but not PCE (Cupples *et al.*, 2004), there must be other RDases in this strain that are capable of TCE reduction. Although no protein has been isolated or purified, a fourth VC-reducing RDase gene (*bvcA*) has been identified from strain BAV1 through transcriptional analysis.

Table 2.1 Metabolic and co-metabolic usage of chlorinated ethenes by Dhc

Strain	Metabolized chlorinated ethenes	Co-metabolized chlorinated ethenes	Major product	Known RDase genes involved
CBDB1	PCE,TCE	None	tDCE (*cis*-DCE)	N/A
195	PCE, TCE, cDCE, 1,1-DCE	*trans*-DCE, VC	VC, ethene	*pceA* (PCE →TCE), *tceA* (TCE→VC)
BAV1	cDCE, *trans*-DCE, 1, 1-DCE, VC	PCE, TCE	Ethene	*bvcA* (DCEs, VC → ethene)
VS	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	*vcrA* (DCEs, VC → ethene)
GT	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	*vcrA* (DCEs, VC → ethene)
FL2	TCE, *cis*-DCE, *trans*-DCE	PCE, VC	VC, ethene	*tceA* (TCE → VC)
MB	PCE, TCE	None	*Trans*-DCE	
ANAS1	TCE, *cis*-DCE, 1,1-DCE	VC	VC, ethene	*tceA* (TCE → VC)
ANAS2	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	*vcrA* (DCEs, VC → ethene)

N/A: not available.
Reference: Löffler *et al.*, 2012.

All RDases require Co (I) corrinoids as essential cofactors for proper function, except the chlorobenzene-reducing RDase identified from *Desulfomonile tiedjei* DCB-1, which was likely to be a heme protein (Ni *et al.*, 1995). The growth of all Dhc isolates requires the addition of exogenous cyanocobalamin (vitamin B$_{12}$) (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003a; He *et al.*, 2005; Sung *et al.*, 2006b), a typical form of corrinoid, while the TCE-RDase in *Sulfurospirillum multivorans* contains nor-pseudo B$_{12}$ as the cofactor (Krautler *et al.*, 2003). Besides RDases, corrinoids are also cofactors for enzymes that facilitate carbon skeleton rearrangements and methyl group transfers (Banerjee and Ragsdale, 2003). Genome annotations of five sequenced Dhc isolates reveal that they possess genes encoding two additional types of corrinoid-dependent enzymes beyond RDases, ribonucleotide reductases (RNRs) and corrinoid iron sulfur proteins (CFeSP), totaling 13-40 corrinoid-dependent enzymes in each genome (Kube *et al.*, 2005; Seshadri *et al.*, 2005; McMurdie *et al.*, 2009). This number of putative corrinoid-dependent enzymes is considerably higher than those found in other bacteria (Zhang *et al.*, 2009). In strain 195, gene expression and proteomic analyses demonstrated that transcripts and proteins corresponding to the RDase gene *tceA*, two of the three RNRs gene *nrdJ* paralogs, and both of the CFeSP genes are expressed (Johnson *et al.*, 2008; Johnson *et al.*, 2009). These observations indicate that corrinoid cofactors play important roles in dehalorespiring and other metabolic processes in Dhc. However, despite their dependence on corrinoid cofactors for central metabolic processes, none of

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the Dhc isolates reported to date can synthesize corrinoids *de novo*, and thus they are completely reliant on corrinoids produced by other microbes in their environment (Seshadri *et al.*, 2005; McMurdie *et al.*, 2009). A possible exception was found in a recent metagenomic analysis of a TCE-dechlorinating enrichment in which putative genes for the complete corrinoid biosynthesis pathway were identified in strain ANAS2 (Brission *et al.*, 2012). However, the functionality of this pathway has not yet been demonstrated.

Due to their strict nutrient requirements, the growth of Dhc isolates is unreliable and relatively slow with doubling times of 20-60 hours (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003a; He *et al.*, 2005; Sung *et al.*, 2006b). Yields of the isolates range from 6.3×10^7 to 3.1×10^8 cells per μmole of chloride released, with densities from 10^7 to 10^8 cells/mL (Löffler *et al.*, 2012). In addition, the growth of Dhc may be uncoupled from the dechlorination, suggesting that there might be other factors that limit their growth (Johnson *et al.*, 2008). Compared with Dhc isolates, previous studies have shown that growth of Dhc in microbial communities is generally more rapid and robust (Maymó-Gatell *et al.*, 1997; Duhamel *et al.*, 2004; Duhamel and Edwards, 2006; He *et al.*, 2007), which leads to our interests in studying the roles of supportive microorganisms in Dhc-containing communities and their ecological inter-relationships.

2.5 Microbial ecology of *Dehalococcoides*-containing microbial communities

Laboratory enrichment cultures (Richardson *et al.*, 2002; Gu *et al.*, 2004; Freeborn *et al.*, 2005; Yang *et al.*, 2005) and contaminated field sites (Dojka *et al.*, 1998; Hohnstock-Ashe *et al.*, 2001; Lowe *et al.*, 2002; Macbeth *et al.*, 2004) have been studied in order to better understand the benefits of growing Dhc syntrophically. Basically, three factors must be made available to Dhc for successful reductive dechlorination and growth, including hydrogen as electron donor, acetate as carbon source, and cobalamin as a cofactor of RDases.

2.5.1 Supportive microorganisms in hydrogen and acetate availability

Fermentation is a common process used to stimulate remediation of chlorinated ethene contaminated groundwater, soil or sediments, where excess fermentable organics were injected as substrates, while certain electron acceptors such as oxygen, sulfate, and Fe (III) are limited. A wide variety of microbial communities are maintained on fermentable organics such as lactate, methanol, propionate, butyrate, and whey, with hydrogen and acetate generated as fermentation products that can be utilized by Dhc (Duhamel *et al.*, 2004; Gu *et al.*, 2004; Freeborn *et al.*, 2005; Daprato *et al.*, 2007; Rowe *et al.*, 2008). Phylogenetic analyses of Dhc-containing microbial communities revealed organisms that are able to carry out fermentation including species from the genera *Clostridium*, *Eubacterium*, *Desulfovibrio*, *Bacteroidetes*, *Syntrophus*, *Acetobacterium*, *Spirochaetes*, and *Syntrophobacter* (Gu *et al.*, 2004; Duhamel and Edwards, 2006; Daprato *et al.*, 2007; Rowe *et al.*, 2008).

One possible reason for the robust growth and faster dechlorination of Dhc when growing in communities with these fermentors is interspecies hydrogen transfer. The

dehalorespiring bacterium *Desulfitobacterium frappieri* TCE1 was first reported to grow syntrophically with sulfate-reducing bacterium *Desulfovibrio fructosivorans* under sulfate limited conditions using fructose as the electron donor. Hydrogen required in the reductive dechlorination is provided by *D. fructosivorans* through fructose fermentation and interspecies hydrogen transfer between these two species (Drzyzga *et al.*, 2001). He and co-workers have successfully grown strain 195 with *Desulfovibrio desulfuricans* using lactate as electron donor during a single sub-culturing. The cell density of strain 195 in the co-culture was 1.5 times greater than that of the isolate (He *et al.*, 2007).

Although the mechanisms for hydrogen exchange between Dhc and other organisms have not yet been elucidated, interspecies hydrogen transfer between *Desulfovibrio* and other organisms has been well-studied. *Desulfovibrio vulgaris* is a representative sulfate-reducing bacterium. In the absence of sulfate, *Desulfovibrio vulgaris* and sulfate-reducing microorganisms in general can ferment organic acids and alcohols, producing hydrogen, acetate and carbon dioxide. The standard Gibbs free energy ($\Delta G^{o'}$) of lactate fermentation to hydrogen and acetate is very low -8.8 kJ/mol (Walker *et al.*, 2009), which suggests that the this reaction is relatively thermodynamically unfavorable. However, by forming syntrophic associations with hydrogen-consuming populations, this reaction is promoted. Typical hydrogen-consuming organisms that grow syntrophically with *Desulfovibrio* are methanogens (McInerney and Bryant, 1981; Stolyar *et al.*, 2007; Walker *et al.*, 2009). Comparative transcriptional analysis of a model sulfate-reducing microbe, *Desulfovibrio vulgaris* Hildenborough, grown with a hydrogenotrophic methanogen, *Methanococcus maripaludis* strain S2, demonstrated that during syntrophic growth on lactate, numerous genes involved in electron transfer and energy generation were up-regulated in *D. vulgaris* compared with their expression in sulfate-limited monocultures. In particular, genes encoding the putative membrane-bound Coo hydrogenase, two periplasmic hydrogenases (Hyd and Hyn), and the well-characterized high-molecular-weight cytochrome (Hmc) were among the most highly expressed and up-regulated genes. Additionally, a predicted operon containing genes involved in lactate transport and oxidation exhibited up-regulation, further suggesting an alternative pathway for electrons derived from lactate oxidation during syntrophic growth. Mutations in a subset of genes coding for Coo, Hmc, Hyd, and Hyn impaired or severely limited syntrophic growth but had little effect on growth via sulfate respiration (Walker *et al.*, 2009).

2.5.2 Supportive microorganisms in cobalamin availability

Cobalamin (vitamin B_{12}) is an important co-factor of RDases as well as two classes of enzymes: isomerases and methyltransferases (Banerjee and Ragsdale, 2003; Brown, 2005). Cobalamin belongs to the corrinoid family with many other naturally occurring corrinoid forms. Corrinoids have a cobalt-centered corrin ring with an upper (axial) ligand and a lower (axial) ligand. Cyanide (–CN) group is the stable chemical form of the upper ligand, whereas deoxyadenosyl group and methyl group are the two biological forms of the upper ligands, which are unstable and can be replaced by –CN. In general, it is the lower ligand that determines the various functional forms of corrinoids (See 4.1). Cobalamin is a corrinoid form with dimethylbenzimidazole (DMB) as the lower ligand.

Although corrinoid co-factors are required by animals, human beings, as well as a certain portion of prokaryotes, only a small portion of those prokaryotes (some Bacteria and Archaea) that require corrinoids are capable of *de novo* corrinoid synthesis (Martens *et al.*, 2002; Ryzhkova, 2003). The rest of the corrinoid-dependent microorganisms, like Dhc, rely on either exogenous corrinoids or corrinoid-synthesizing microorganisms.

The corrinoid-dependent isomerases found in bacteria play important roles in fermentation pathways (Banerjee and Ragsdale, 2003), suggesting that certain fermenting bacteria may synthesize corrinoids *de novo*. Among the fermenters detected in dechlorinating communities, certain *Clostridium*, *Desulfovibrio*, *Acetobacterium*, are reported to produce different corrinoid species (Stupperich *et al.*, 1988b; Guimaraes *et al.*, 1994). Current available genomic information (http://www.ncbi.nlm.nih.gov/) shows that some species in these genera possess complete or near-complete corrinoid biosynthesis pathways. Although the five known Dhc genomes do not have up-stream corrinoid biosynthesis pathways, they possess corrinoid transport and salvaging pathways, including *cbiZ*-like genes. *cbiZ* encodes the cobinamide amidohydrolase enzyme, which is involved in corrinoid remodeling system. It uses pseudo-cobalamin as substrate, which has adenine as the lower ligand, and converts it to cobalamin by cleaving off adenine and attaching DMB such as observed in *Rhodobacter sphaeroides* grown on acetate (Gray and Escalante-Semerena, 2009a). Similarly, in the presence of DMB, strain 195 was also able to convert 6 other corrinoid forms to cobalamin, its more favorable form (Yi *et al.*, accepted). Due to analytical limitations, the corrinoid forms produced in dechlorinating microbial communities still remain unknown and will be one of the main questions we address in these studies. Understanding the ecological relationship between Dhc and other supportive microorganisms is of great significance in the practice of *in-situ* bioremediation.

2.5.3 Archaeal species in Dhc-containing communities

In addition to Bacterial species, mostly fermenters, studies also showed the presence of Archaeal populations in Dhc-containing communities, mostly methanogens (Vogel and McCarty, 1985; Fathepure and Boyd, 1988; Richardson *et al.*, 2002; Macbeth *et al.*, 2004). The roles played by methanogens in these communities are still inconclusive. On one hand, some classes of methanogens are known to synthesize corrinoids, such as *Methanosarcina barkeri* (Pol *et al.*, 1982), thus they have a potential to provide corrinoids to Dhc. On the other hand, hydrogenotrophic methanogens compete with Dhc for hydrogen (Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Yang and McCarty, 1998). Although studies have been conducted to evaluate the link between methanogens and Dhc (Freedman and Gossett, 1989; Fennell *et al.*, 1997; Booker and Pavlostathis, 2000; Heimann *et al.*, 2006), more research is needed to elucidate the balance between benefits and drawbacks of methanogens in Dhc-containing communities.

2.6 Methods for monitoring and assessing Dhc-containing microbial communities

Microbial communities are complex systems with various microbial species and biological activities. Therefore, reliable, systematic and high-throughput techniques are useful to better monitor and assess Dhc-containing microbial communities. Advanced

molecular and analytical approaches are now available to assist in assessment efforts of these communities and some of these technologies are applicable in chlorinated ethene bioremediation.

2.6.1 Molecular approaches

16S rRNA gene based methods

Common phylogenetic analyses use 16S rRNA gene based tools, including clone libraries, denatured gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP), high-throughput 16S rRNA-based microarray (phylochip), as well as quantitative PCR (qPCR) (Brodie *et al.*, 2006; DeSantis *et al.*, 2007; Drenovsky *et al.*, 2008). Applying these techniques with genomic DNA or RNA enables the analysis of community structures and addresses the questions of identity and overall activity of community members. Moreover, qPCR could provide quantitative information of detected species. Fluorescence in situ hybridization (FISH) is used as a culture-independent method to study environmental communities without PCR bias associated with other 16S rRNA gene based molecular methods (Su *et al.*, 2012). Similarly, in order to better investigate non-dominant, hard-to-culture microorganisms within a complex community, such as Dhc, and enhance the resolution of genomic analysis, fluorescence-activated cell sorting (FACS) techniques integrated with whole genome amplification (WGA) have been developed recently (Kalyuzhnaya *et al.*, 2006; Podar *et al.*, 2007; Rodrigue *et al.*, 2009). FACS-WGA treated samples could then be queried using whole-genome microarrays for further genomic analysis.

Given that Dhc strains with different dechlorinating capabilities share highly conserved 16S rRNA gene sequences, the use of 16S rRNA gene as a biomarker indicating dechlorination activities is limited. Consequently, the detection of Dhc 16S rRNA genes alone could result in misinterpretation if the detected Dhc do not possess the metabolic capacities of interest. Therefore, targeting genes that are specific to functions of interest is necessary to effectively monitor the activities of Dhc and other microorganisms in the community.

Functional gene based methods

In Dhc-containing communities, RDase genes (*pceA*, *tceA*, *vcrA*, *bvcA*) have been typically used as functional gene based biomarkers indicative of dechlorination activities (Lee *et al.*, 2006). Given that the presence of a gene does not necessarily mean that it is actively functioning, RNA samples can also be quantified to investigate the transcriptional level of the functional genes of interest. High-throughput technologies applied to investigate gene expression include whole genome microarrays (Johnson *et al.*, 2008; Lee *et al.*, 2011), as well as functional gene arrays (FGA) such as GeoChip techniques (He *et al.*, 2012b). Microarrays targeting the whole genome of strain 195 and four genomes of sequenced Dhc strains (i.e. strain 195, CBDB1, BAV1, VS) have successfully been applied to enrichment cultures, defined consortia, and isolated cultures to investigate the effects of conditions such as growth phase, B_{12} level, and the presence

of other syntrophic microorganisms on the dechlorination and growth rates of Dhc (Johnson *et al.*, 2008; West *et al.*, 2008; Johnson *et al.*, 2009; Hug *et al.*, 2011; Lee *et al.*, 2011).

FGAs such as the GeoChip have been developed and applied for analyzing the functional diversity, composition, structure, and dynamics of microbial communities from different habitats, such as aquatic ecosystems, soils, contaminated sites, extreme environments, and bioreactors (He *et al.*, 2012b). Targeted functional genes on the GeoChip include key genes involved in biogeochemical cycling of carbon, nitrogen, sulfur, phosphorus and metals, virulence and antibiotic resistance, biodegradation of environmental contaminants, and stress responses (He *et al.*, 2012a). Various GeoChip-based FGA studies have demonstrated that FGAs are a robust, powerful, high-throughput tool to specifically, sensitively and quantitatively profile microbial communities and link their composition and structure with environmental factors and ecosystem functioning (Taroncher-Oldenburg *et al.*, 2003; Miller *et al.*, 2008; Hazen *et al.*, 2010; McGrath *et al.*, 2010; Lu *et al.*, 2012). The application of FGA to dechlorinating communities is ongoing, in order to comprehensively understand communities as a whole.

Metagenomics by next generation sequencing (NGS) technologies

Microarray technologies mentioned above only target known genomes or functional gene sequences and will not detect those that have not yet been detected and therefore do not have corresponding probes on the array. The recent technological advances in next generation sequencing (NGS) have brought the field closer to the goal of reconstructing all genomes within a community by generating high throughput sequencing at much lower costs (Scholz *et al.*, 2012; Su *et al.*, 2012). Typical NGS platforms used for metagenomics and metatranscriptomics include PCR-based 454 pyrosequencing, Illumina, SOLiD, Ion Torrent, as well as single-molecule, non-PCR sequencing technologies, HeliScope and SMRT (Shokralla *et al.*, 2012). 454 pyrosequencing provides long read length (Table 2.2) with relatively short run-time, and has been used for metagenome analyses. However, the advantages of Illumina and SOLiD, such as more reads, higher sequencing output per run, lower cost per megabase sequencing output, as well as accurate sequencing of homopolymer regions, makes these "short-read" technologies the most well-suited for deep-coverage sequencing and analysis of shotgun metagenomes (Desai *et al.*, 2012; Scholz *et al.*, 2012). However, the short reads make read-based analyses difficult, incomplete, or even impossible, especially in situations where no reference sequence is available to align, assign and annotate the short sequences (Shokralla *et al.*, 2012). Therefore, new methods are required to analyze Illumina (or SOLiD)-sequenced metagenomes. Various novel algorithms are being developed to process millions to billions of very short reads for sequence alignment, assembly, as well as read annotation (Flicek and Birney, 2009). There are two general types of analyses for metagenomics: 1) assembling the reads into contigs, and performing taxonomic classification and functional assignments; and 2) read-based reconstruction of the functional and taxonomic components of the metagenome. Besides 454 pyrosequencing, which has been applied to study microbial communities in marine, freshwater, and soil systems (Ficetola *et al.*, 2008; Williamson *et al.*, 2008; Nacke *et al.*, 2011), Illumina platforms have also been successfully utilized for metagenome analysis of a full-scale

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microbial community carrying out enhanced biological phosphorus removal (Albertsen *et al.*, 2012). However, potential sources of error, including sequencing artifacts and taxonomic misidentification, should be taken into consideration when using short-read NGS tools to discover the biodiversity of environmental samples (Degnan and Ochman, 2011).

Table 2.2 Comparison of currently available next-generation sequencing technologies

NGS technologies	Platform	Read length (bp)	Max. number of reads/run	Sequencing output/run	Run time
PCR-based	Roche 454	400-800	$(1\text{-}10) \times 10^5$	35-700 Mb	10-23h
	Illumina HiSeq	100-200	$(3\text{-}6) \times 10^9$	$\leq$ 270-600 Gb	8.5-11d
	Illumina MiSeq	100-150	7×10^6	$\leq$ 1-2 Gb	19-27h
	SOLiD	35-75	$2.4\text{-}6 \times 10^9$	100-250 Gb	4-8d
	Ion Torrent	100-200	$1\text{-}11 \times 10^6$	$\geq$ (10-1000) Mb	3.5-5.5h
Non-PCR	HeliScope	30-35	1×10^9	~20-28 Gb	$\leq$ 1d
	SMRT	$\geq$ 1500	50×10^3	~60-75 Mb	0.5h

Reference: Shokralla *et al.*, 2012

2.6.2 Analytical approaches

Corrinoids and lower ligand detection

Since corrinoids play important roles in reductive dechlorination, the detection of corrinoids and their corresponding lower ligands in laboratory cultures and field sites is required in order to elucidate the production and utilization of these co-factors. Conventional detection methods include microbiological assays, HPLC/UV, and LC/MS. Microbiological assays are sensitive, but are semi-quantitative, only providing a concentration of all usable corrinoids without differentiating their forms. Corrinoid species that cannot be used by the test microorganisms will be excluded in the results. HPLC/UV method can differentiate corrinoid species according to retention time of specific forms. However, due to the similarity in chemical structures of certain corrinoids, the retention times tend to be so close that complete separation is difficult to achieve. In addition, the detection limit of the HPLC/UV method is high (> 0.1 mg/L, c.a 74nM) (Whitman and Wolfe, 1984; Dalbacke and Dahlquist, 1991; Guggisberg *et al.*, 2012), which is more suitable for vitamin B_{12} detection in the food industry or clinical and pharmaceutical fields, but not for reductive dechlorination at bioremediation field sites where corrinoid concentration might be rather low (< 1 µg/L). Recently, Allen and co-workers reported a new LC/MS method that can differentiate 13 corrinoid species with a lower detection limit (Allen and Stabler, 2008). The ability to quantify different corrinoid species at low levels allows us to investigate corrinoid profiles of corrinoid-dependent Dhc-containing microbial communities, as well as the mechanisms employed by Dhc for corrinoid salvaging and remodeling.

Stable carbon isotope technologies

Biological reactions in subsurface aquifers are often accompanied and masked by physical processes such as dissolution, volatilization and sorption, making it difficult to

attribute changes in chlorinated ethene concentrations to biological conversion. One promising solution to overcome this problem is the quantification of shifts in the stable carbon isotope ratios of the chlorinated ethenes to evaluate the extent of biodegradation (Meckenstock *et al.*, 2004). Previous research has shown that physical processes cause minor or negligible shifts in isotope ratio (Huang *et al.*, 1999; Slater *et al.*, 2001), while biological conversion can cause significant changes in the isotope ratios of chlorinated ethenes (Hunkeler *et al.*, 1999; Bloom *et al.*, 2000; Lollar *et al.*, 2001; Nijenhuis *et al.*, 2005). Thus, fractionation of the stable carbon isotope of chlorinated ethenes can provide evidence that changes in chlorinated ethene concentrations are due to biological reactions. A number of laboratory studies have applied compound-specific carbon isotope analysis to measure kinetic isotope fractionation during reductive dechlorination in laboratory enrichment cultures, field samples and isolates (Hunkeler *et al.*, 1999; Bloom *et al.*, 2000; Lollar *et al.*, 2001; Slater *et al.*, 2001; Nijenhuis *et al.*, 2005; Lee *et al.*, 2007).

Besides stable carbon isotope fractionation, which uses naturally labeled stable carbon isotope, artificially-labeled stable carbon isotopes can also be used in metabolomic analyses (Zamboni *et al.*, 2009; Feng *et al.*, 2010a), as well as protein-based or nucleic acids-based stable isotope probing (SIP) (Radajewski *et al.*, 2000; Dumont and Murrell, 2005; Drenovsky *et al.*, 2008). ^{13}C-labeled substrates have been used to study metabolic pathways in different microorganisms (Tang *et al.*, 2009; Feng *et al.*, 2010b; Chen *et al.*, 2011). Central metabolic pathways of *D. mccartyi* strain 195 have been investigated using ^{13}C-labeled acetate and carbon dioxide as carbon sources. Results demonstrate that strain 195 contains complete amino acid biosynthesis pathways, even though current genome annotation suggests that several of these pathways are incomplete. The tricarboxylic acid cycle of strain 195 is confirmed to be branched. There is also evidence showing that strain 195 may contain an undocumented citrate synthase (Tang *et al.*, 2009).

Another useful application of stable carbon isotope labeling is stable isotope probing (SIP). The coupling of molecular biological methods with stable isotope abundance in biomarkers has provided a culture-independent method of linking the identity of Bacteria with their function in the environment. DNA and rRNA are informative taxonomic biomarkers, the use of which has the potential to identify a wider range of Bacteria with a greater degree of confidence. ^{13}C-DNA, produced during the growth of metabolically distinct microbial groups on a ^{13}C-enriched carbon source, can be resolved from ^{12}C-DNA by density-gradient centrifugation. DNA isolated from the target group of microorganisms can be characterized taxonomically and functionally by combining SIP with other emerging technologies such as microarrays and metagenomics. Among other functions, DNA-SIP has been applied in studying methanotrophs and methylotrophs, as these microorganisms are known to utilize single-carbon compounds as their sole carbon source (Radajewski *et al.*, 2000; Morris *et al.*, 2002). DNA-SIP has also been successfully applied to determine the primary member of a complex community that is responsible for naphthalene catabolism *in situ* (Jeon *et al.*, 2003). In addition, RNA-SIP was used to identify Bacteria that degrade phenol in an aerobic industrial bioreactor (Manefield *et al.*, 2002). SIPs thus offer a powerful technique for identifying microorganisms that are actively involved in specific metabolic processes, such as reductive dechlorination, under conditions that approach those occurring *in situ*.

2.7 Summary

Chlorinated ethenes contamination is an environmental problem that has been identified for decades. Dhc are the key microorganisms in the bioremediation of chlorinated ethenes. Since the first Dhc strain was isolated more than a decade ago, we have made significant progress in understanding the physiology, biochemistry, genetics and ecology of this group of organisms. We have also applied Dhc-containing cultures in field scale operations with some success. Despite these achievements, much remains to be learned about this unique group of organisms both from a fundamental biology point of view and from an applied microbiology and engineering point of view. For example, we still have limited knowledge of many pathways directly or indirectly related to dechlorination; exploring these pathways will likely provide us with clues to further optimize metabolism and avoid bottlenecks in growth. We still lack a systematic recognition of roles played by supportive microorganisms in terms of the exchange of hydrogen, corrinoid co-factors and other important nutrients. Understanding the ecological relationships among a microbial community may help optimize the application of bioremediation. Parallel with laboratory investigations, we also need to continue to optimize the subsurface monitoring techniques so that we no longer approach the field environment as a black box.

3 *Characterization of Four TCE-Dechlorinating Microbial Enrichments Grown with Different Cobalamin Stress and Methanogenic Conditions

3.1 Introduction

Trichloroethene (TCE) has been a common groundwater contaminant at hazardous waste sites for decades (McCarty, 1997; Doherty, 2000a; Hendrickson *et al.*, 2002) 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 competitive 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 (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003a; Cupples *et al.*, 2004; He *et al.*, 2005). 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 B_{12} (cyanocobalamin) as an essential co-factor for their reductive dehalogenases (RDases) (Maymó-Gatell *et al.*, 1997; Seshadri *et al.*, 2005; McMurdie *et al.*, 2009). The growth of Dhc in isolation is somewhat unreliable and relatively slow with doubling times of 20-60 hours (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003b; He *et al.*, 2005; Sung *et al.*, 2006b). In contrast, previous studies have shown that growth of Dhc in microbial enrichments or defined consortia is generally more rapid and robust (Maymó-Gatell *et al.*, 1997; Duhamel *et al.*, 2004; Duhamel and Edwards, 2006; He *et al.*, 2007; 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 fermentors such as *Desulfovibrio*, *Eubacterium*, *Clostridium*, *Acetobacterium*, *Citrobacter,* and *Spirochetes* (Richardson *et al.*, 2002; Ritalahti and Löffler, 2004; Freeborn *et al.*, 2005; Duhamel and Edwards, 2006; Lee *et al.*, 2006; Men *et al.*, 2012). As an essential co-factor of RDases as well as other functionally important enzymes, vitamin B_{12} has been exogenously amended in most of the previous laboratory studies of dechlorinating enrichments (Cupples *et al.*, 2003; He *et al.*, 2003a; Duhamel *et al.*, 2004; Freeborn *et al.*, 2005; Futamata *et al.*, 2007; Rowe *et al.*, 2008). However, there have been reports of Dhc-containing enrichments where no exogenous vitamin B_{12} was amended (Gu *et al.*, 2004; Daprato *et al.*, 2007), indicating that an alternate source of corrinoids is available in those communities. However, the specific organisms responsible for providing corrinoids 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 cobalamin biosynthesis pathways, thus are not able to synthesize cobalamin *de novo*. However, a recent metagenomic study on an enrichment maintained with little exogenous vitamin B_{12} (Brission *et al.*, 2012) reported that a nearly complete cobalamin biosynthesis pathway (except the non-essential *cbiJ* gene) has been identified in a Dhc strain, although the functionality of this operon has not been confirmed. On the other hand, some fermentors, acetogens and methanogens often observed within dechlorinating communities (Cupples *et al.*, 2003; He *et al.*, 2003a; Duhamel *et al.*, 2004; Freeborn *et al.*,

2005; He *et al.*, 2005; Rowe *et al.*, 2008) are able to synthesize cobalamin *de novo*, including *Clostridium* spp., *Desulfovibrio* spp., *Acetobacterium woodii*, and *Methanosarcina barkeri* (Stupperich *et al.*, 1988b; Guimaraes *et al.*, 1994; Renz, 1999), 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, it is worthwhile to investigate the potential for supportive microorganisms 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 exogenous vitamin B_{12}. Physiological characteristics were analyzed 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 cobalamin biosynthesis genes detected in the metagenome of another enrichment (Brission *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 supportive microorganisms by comparing the physiology, genomic characteristics and community structures of dechlorinating communities enriched from environmental samples under different cobalamin stress.

3.2 Methods

3.2.1 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 B_{12} was obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

3.2.2 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 UC 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, the composition of which was described previously (He *et al.*, 2007). All steps were performed in an anaerobic chamber. The inoculated bottles were then flushed with N_2/CO_2 (90:10, vol/vol) and amended with 38 mM 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 B_{12} (final concentration 100 μg/L, ca. 74 nM) 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 reamended with 22 μmol TCE and 4 mM lactate 9 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: using low initial TCE (~ 22 μmol) to allow methanogenesis: 1) with B_{12} (LoTCEB12) and 2) without B_{12} (LoTCE); using high initial TCE (~ 77 μmol) rather than common methanogen inhibitor 2-bromoethanesulfonate (BES) due to its toxicity to Dhc (Löffler *et al.*, 1997), to inhibit methanogenesis (Distefano *et al.*, 1992; Yu and Smith, 2000; Men *et al.*, 2012): 3) with B_{12} (HiTCEB12) and 4) without B_{12} (HiTCE) . Within each feeding cycle, the feeding regimen of lactate, TCE prior to sub-culturing were listed in Table 3.1. Subsequently, 5% (vol/vol) of each culture was inoculated into 95 mL fresh basal medium under the same feeding regimen 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 repeatable. 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.

Table 3.1 Enrichment conditions of the four TCE-dechlorinating microbial communities

Enrichments	Lactate (mmol)		TCE (μmol)		Vitamin B_{12} (μg/L)
	Feeding regimen	Total	Feeding regimen	Total	One-time addition
LoTCEB12	3.8, .4, .4, .4, .2	**5.3**	22, 55, 55, 55, 33	**220**	100
LoTCE	3.8, .4, .4, .4, .2	**5.3**	22, 55, 55, 55, 33	**220**	0
HiTCEB12	3.8, .4, .5, .5	**5.3**	77, 77, 66	**220**	100
HiTCE	3.8, .4, .5, .5	**5.3**	77, 77, 66	**220**	0

3.2.3 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. External calibration curves were generated using serum bottles containing anaerobic medium with known amounts of each compound. Headspace concentrations (C_G) were converted to total mass of chlorinated ethenes (M) in vials using Henry's constants ($k_H^{cc} = \dfrac{C_G}{C_L}$) (Table 3.2) according to a material balance: $M = C_G V_G + \dfrac{C_G}{k_H^{cc}} V_L$, where V_G and V_L are the headspace (60 mL) and liquid (100 mL) volumes in the serum bottles, respectively.

Hydrogen in the headspace was measured by gas chromatography using a reductive gas detector (RGD) (Trace Analytical, Menlo Pak, CA). The linear range of the instrument was up to a concentration of 7.7 μM (Freeborn *et al.*, 2005). Calibration bottles with known amounts of H_2 were used for standard curves as described above, and samples that exceeded the upper linear limit were diluted in N_2-purged tubes prior to analysis.

Organic acids were measured by high performance liquid chromatography (HPLC) with a UV detector (set at 210 nm) (Waters, Milford, MA). The eluent was 5 mM aqueous H_2SO_4, which was pumped at a flow rate of 0.6 mL/min through an Aminex HPX-87H ion exclusion organic acid analysis column (300 by 7.8 mm; Bio-Rad, Hercules, CA).

Total cobalamin from 200-300 mL culture was extracted by adding 20 mg KCN per gram of cell pellets and incubating in 60 ºC water bath for 1.5 hr. Cobalamin concentrations were then measured by LC/MS/MS. Commercial vitamin B_{12} was used to establish standard curves. More method details are described in Chapter 4 (4.2.2).

Table 3.2 Physical characteristics of different compounds measured by GC

Compounds	MW	Density 20°C (g/L)	Henry's law Constant 34°C (C_G/C_L)	Reference
TCE	131.4	1464.20	0.591	Gossett, 1987
cDCE	96.95	1283.70	0.216	Gossett, 1987
VC	62.5	2.6	1.42	Gossett, 1987
Ethene	28.05	1.16	10.25	Sander, 1999
Methane	16.04	0.719	34.03	Sander, 1999
H_2	2.02	0.0837	53.54	Sander, 1999

3.2.4 DNA isolation

For gene quantification, Cells in 1.5 mL culture were collected by centrifuging at 15,000 × 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. The cell pellets amended with 1 g 100-μm-diameter zirconia-silica beads (Biospec Products, Bartlesville, OK) were resuspended in 250 μL lysis buffer (50mM sodium acetate, 10mM EDTA; pH 5.1), 100 μL 10% sodium dodecyl sulfate, 1.0 ml pH 8.0 buffer-equilibrated phenol (Sigma-Aldrich, St. Louis, MO). The cells were lysed by heating them to 65°C for 2 min, bead beating them with a Mini Bead Beater (Biospec Products) for 2 min, incubating them for 8 min at 65°C, and bead beating them for an additional 2 min. Cellular debris was collected by centrifugation (14,000 × g for 5 min at 4°C), and the aqueous lysate was transferred to a new, DNase-free microcentrifuge tube. The aqueous lysate was extracted two times with 1 volume of phenol (pH 8.0)-chloroform-isoamylalcohol (25:24:1, v/v) (Sigma-Aldrich, St. Louis, MO), and one time with 1 volume of chloroform-isoamylalcohol (24:1, v/v). DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of 100% isopropanol. The precipitate was collected by centrifugation (21,000 × g for 30 min at 4°C), washed once with 70% ethanol, and resuspended in 50 μL of nuclease-free water. 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.

3.2.5 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 Table S1.   Each 20-μL reaction mixture contained 2.5 μL of sample or serially diluted standard, 10 μL 2 × 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 genes 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 (Table 3.3), respectively. The gDNA of *Dehalococcoides mccartyi* strain 195 (formerly designated *Dehalococcoides ethenogenes*) 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).

3.2.6 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 by Lee *et al*. (Lee *et al.*, 2011). Biological triplicate analyses were performed for each culture. 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 replicate samples had signal intensities greater than 140 and *p* values less than 0.05.

3.2.7 Bacterial 16S rRNA gene clone library construction

Bacterial 16S rRNA genes were amplified using the universal Bacterial 16S rRNA gene primers, 8F and 1492R (Table 3.2). Two μL of the gDNA sample was used in a 50-μL PCR reaction containing 5 μL 10 × 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 up, re-amplified using M13F and M13R primers (Table 3.3), and then sequenced by a DNA sequencing facility (University of California, Berkeley, Berkeley, CA; http://mcb.berkeley.edu/barker/dnaseq/).

Table 3.3 Primers used for PCR, qPCR and clone library construction

Target gene (Accession No.)	Primer sets	Sequence	Reference
Dehalococcoides sp. 16S rRNA gene (CP000027)	Forward	5'-GGTAATACGTAGGGAAGCAAGCG	(Holmes *et al.*, 2006; Lee *et al.*, 2008)
	Reverse	5'-CCGGTTAAGCCGGGAAATT	
tceA gene (AF228507)	Forward	5'-ATCCAGATTATGACCCTGGTGAA	
	Reverse	5'-GCGGCATATATTAGGGCATCTT	
vcrA gene (AY322364)	Forward	5'-CTCGGCTACCGAACGGATT	
	Reverse	5'-GGGCAGGAGGATTGACACAT	
bvcA gene (AY563562)	Forward	5'-GGTGCCGCGACTTCAGTT	
	Reverse	5'-TCGGCACTAGCAGCAGAAATT	
cobN [a]	Forward	5'-TGCACATCGGCAAGCACGGT	This study
	Reverse	5'-TGGCCGCTGAGTCCTCCTCA	
cbiD [a]	Forward	5'-ACCGCCAGCCTCAGGGTTGA	This study
	Reverse	5'-ACAGCCGCCATGGCACACAG	
cbiE [a]	Forward	5'-CCGCCGGATTATGCCGCCTT	This study
	Reverse	5'-TTGAGCGGGCTTAGGCGGGT	
cbiC [a]	Forward	5'-CGCCGTTGTCCGCCAGCTTA	This study
	Reverse	5'-TTTCACCCGCCGCTTCTGCC	
Univ. Bacterial 16S rRNA gene (qPCR)	318F	5'-TCCTACGGGAGGCAGCAG	
	745R	5'GGACTACYVGAGTATCTAATYCTGTT	
Univ. Archaeal 16S rRNA gene (qPCR)	349F	5'-GYGCASCAGKCGMGAAW	(Takai and Horikoshi, 2000)
	806R	5'-GGACTACVSGGGTATCTAAT	
Univ. Bacterial 16S rRNA gene (PCR)	27F	5'-AGAGTTTGATYMTGGCTCAG	(Freeborn *et al.*, 2005; Frank *et al.*, 2008)
	1492R	5'-GCYTACCTTGTTACGACTT	
M13 on pCR2.1 TOPO vector	Forward	5'-GTAAAACGACGGCCAG	
	Reverse	5'-CAGGAAACAGCTATGAC	
*Desulfovibrio*_GW (JQ004087)	538F	5' GGGTGCAAGCGTTAATCGGAATCA	This study
	653R	5' TCAAGTCCAGCAGTATCAAGGGCA	
*Spirochaetes*_GW (JQ004088)	847F	5' TCTCCATAACTCAAGCCCAGCAGT	This study
	1045R	5' AATGCCCGCATGATGACATGAACC	
*Bacteroides*_GW (JQ004089)	771F	5' TCGCAATCGGAGTTCTGCGTGATA	This study
	868R	5' ATGCCGTTGAAACTGTCGGACTTG	
PDS [b]	1348F	5' AGGCCACCTTTAGTATCAARYCCA	This study
	1462R	5' AACGGGTGAGTAACGCGTAGACAA	
*Clostridium*_GW (JQ004086)	1213F	5' TGGTGGGCTGTTATCTCACCAACT	This study
	1319R	5' AATACCGCATGTCATTGCGAGAGG	
	probe	5' FAM-ATGCGGGCCCATCCTGTACCGAATAAAT-TAMRA	

[a] Primers were designed based on the metagenomic sequences of a TCE-dechlorinating microbial community (ANAS)
[b] This set of primers target *Pelosinus*_GW (JQ004084), *Dendrosporobacter*_GW (JQ004085) and *Sporotalea*_GW (JQ004090) due to high similarity in the three 16S rRNA gene sequences.

The BLAST software (GenBank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) was applied to determine the closest cultured microorganism for each OTU. Identified OTUs were quantified using primer sets designed based on the sequenced 16S rRNA genes (Table 3.2). 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 13-15 days as input.

3.2.8 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* (Table 3.2). 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, ANAS2, which possesses those genes served as the positive control (Brission *et al.*, 2012).

3.2.9 NCBI accession numbers

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

3.3 Results

3.3.1 Physiological characteristics of the four enrichment cultures

TCE was dechlorinated to VC and ethene in all four enrichments. For the methanogenic cultures, the total 220 μmol TCE was dechlorinated to VC and ethene within 14 days, while for the nonmethanogenic ones it took 11 days (Figure 3.1A-D). Dechlorination of subsequent doses of TCE became more rapid after the first dose of TCE was dechlorinated.   HiTCEB12 and HiTCE exhibited the highest conversion from VC to ethene with a percentage of $65 \pm 0.5\%$ and $36 \pm 1\%$, while LoTCEB12 and LoTCE generated only $5.5 \pm 1.4\%$ and $3.0 \pm 0.5\%$, 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 genes and *tceA* were closely correlated, suggesting that the dominant Dhc strain in the enrichments carries the *tceA* gene. The cell yields of Dhc in LoTCEB12, LoTCE, HiTCEB12 and HiTCE were $(2.3 \pm 0.1) \times 10^7$, $(2.7 \pm 0.2) \times 10^7$, $(3.3 \pm 0.3) \times 10^7$, $(2.3 \pm 0.7) \times 10^7$ copies per μmol Cl⁻ released, respectively.   All enrichments had similar total Bacterial 16S rRNA gene concentrations prior to each subculturing event, in the range of $2\text{-}5 \times 10^8$ copies per mL. Methanogenic cultures contained $\sim 10^6$ copies per mL of Archaeal 16S rRNA genes, while $\sim 10^5$ copies per mL for non-methanogenic cultures (Figure 3.2).
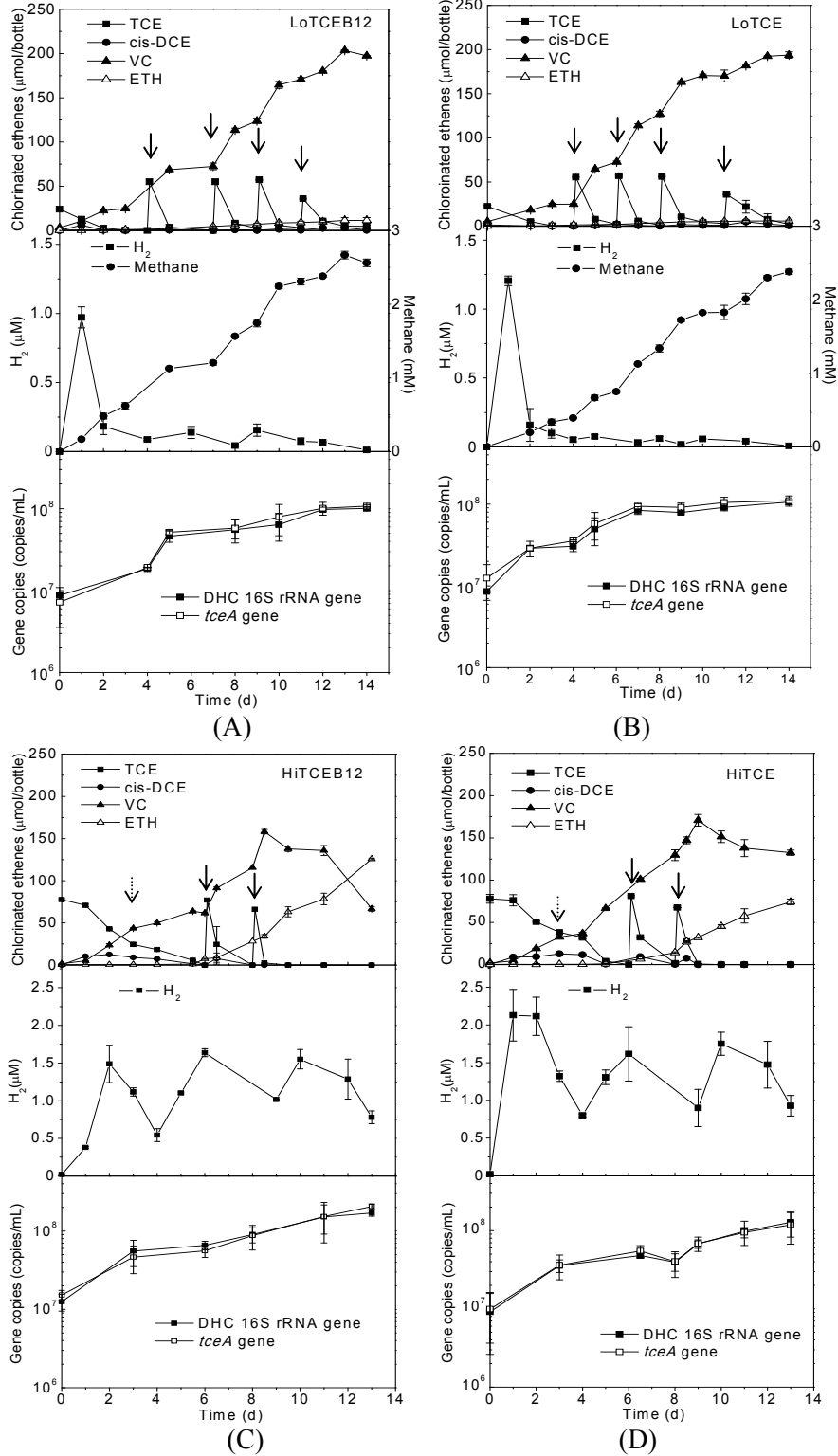
Figure 3.1 Dechlorination, methane generation, aqueous hydrogen and Dhc growth in enrichments: (A)LoTCEB12; (B)LoTCE; (C)HiTCEB12; and (D)HiTCE. ↓indicates the amendment of lactate and TCE, ⤋ indicates the amendment of lactate alone. Note: the aqueous H$_2$ concentration scales vary among plots and have different units from methane.
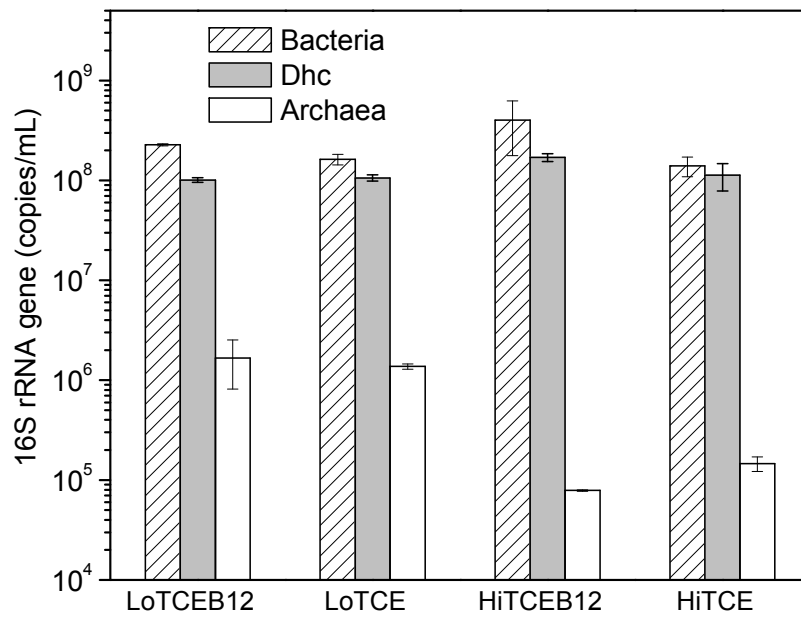
Figure 3.2 Total Bacteria, Dhc and Archaea in the four enrichments as measured by 16S rRNA gene copy numbers.

Table 3.4 Electron balance of the four enrichments

Enrichments	Lactate added (mmol)	Propionate (mmol)	Acetate (mmol)	Butyrate (mmol)	Organic acids recovered[a] (%)	eeq (mmol)				
						Total available[b]	Dechlorination	Methanogenesis	H_2	Other redox reactions
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	~0	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	~0	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	0.006	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	0.006	3.6 ± 1.1

[a] Values are calculated from known lactate amendment, measured organic acids concentrations and stoichiometry based on the lactate fermentation equations below.
[b] Values are calculated from lactate fermentation to acetate and hydrogen as shown below.

	Reaction	Reference
Lactate fermentations	$CH_3CHOHCOO^- + H_2O \rightarrow CH_3COO^- + 2H_2 + CO_2$ (lactate) (acetate)	Walker *et al*., 2009;
	$3CH_3CHOHCOO^- \rightarrow 2CH_3CH_2COO^- + CH_3COO^- + CO_2 + H_2O$ (lactate) (propionate) (acetate)	Madigan *et al*., 2012
	$CH_3CHOHCOO^- + CH_3COO^- + H^+ \rightarrow CH_3CH_2CH_2COO^- + CO_2 + H_2O$ (lactate) (acetate) (butyrate)	Muñoz-Tamayo *et al*., 2011
Hydrogenotrophic reactions Dechlorination	$C_2HCl_3 + 2H_2 \rightarrow C_2H_3Cl + 2H^+ + 2Cl^-$ (TCE) (VC) $C_2HCl_3 + 3H_2 \rightarrow C_2H_4 + 3H^+ + 3Cl^-$ (TCE) (Ethene)	Maymó-Gatell *et al*., 1997
Methanogenesis	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Methane)	Walker *et al*., 2009
Homoacetogenesis	$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ (acetate)	Yang and McCarty, 1998
Biosynthesis	$1/8\ CH_3COO^- + 1/20\ NH_4^+ + 3/40\ CO_2 \rightarrow 3/40\ HCO_3^- + 3/40\ H_2O + 1/20\ C_5H_7O_2N$ (acetate) (cells)	Rittman and McCarty, 2001

In all enrichments, lactate was fermented to hydrogen, propionate and acetate, with small amounts of butyrate generated only in HiTCE and HiTCEB12 (Table 3.4). Methane generation was detected only in LoTCEB12 and LoTCE as expected, at continually increasing concentrations, whereas HiTCEB12 and HiTCE did not generate detectable methane. Aqueous hydrogen reached a peakof 1-1.5 µM in the first day for the methanogenic cultures, and then remained below 0.2 µM afterwards (Figure 3.1A & B). In contrast, aqueous hydrogen remained above 0.5 µM for the non-methanogenic cultures throughout the incubation period (Figure 3.1C & D). 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 3.4), taking into consideration the acetate, propionate, butyrate and hydrogen present at the end of the experiment. For methanogenic cultures (LoTCE and LoTCEb12), a total of 5.6-6.8 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 4.6-4.7 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 to ratio of around 1:1, while the ratio was 1.5:1 in the non-methanogenic cultures. Based on the stoichiometry of biosynthesis (Table 3.4) and the assumption that most bacteria are rod shaped with a size of (0.6-1) µm × (2-7) µm (Moe *et al.*, 2011), 0.2 g of dry weight per gram of wet weight (Cupples *et al.*, 2003), and the total bacteria is 5×10^8 cells/mL we estimate that 0.1-1.2 mmol acetate would be consumed per bottle for synthesis of biomass. However, in methanogenic cultures, close to 100% of the fermented lactate was recovered in products, therefore, homoacetogenesis must be generating some acetate from hydrogen and CO_2, offsetting the amount used in biosynthesis (Table 3.4). In the non-methanogenic cultures, about 87% lactate was recovered in fermentation products, indicating that acetate generated from homoacetogenesis was less than what was consumed in biosynthesis.

3.3.2 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. Microarray data show that the Dhc in all four enrichments share a genome most similar to strain 195 (Figure 3.3), 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 (DET0079 (*tceA*), DET0088, DET0173, DET0180, and DET1545) targeted by the microarray were detected in the four enrichments, all from strain 195.
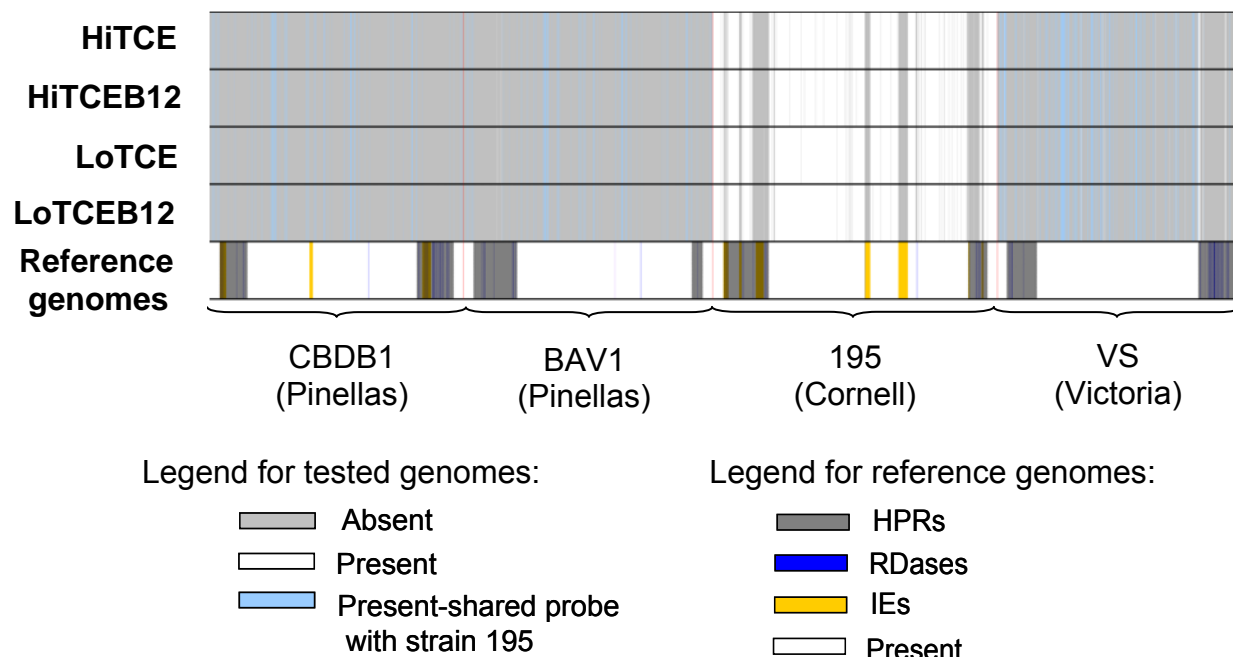
Figure 3.3 Linear representation of the four Dhc genomes that are tiled on the array with gDNA of different enrichment cultures and isolated Dhc strains. Each row represents a sample as indicated on the y-axis. The gray and white colors in the corresponding samples represent genes that are absent and present, respectively, light blue indicates genes outside of the strain 195 genome that are present due to sharing a probe set with genes in strain 195. The last row indicates the four Dhc strains and the genome annotation with orange indicating designated integrated elements (IE), and dark gray indicating high plasticity regions (HPR).

Most of the strain 195 genes involved in cobalamin uptake and salvaging were detected in all enrichments (Table 3.5), including the duplicated operons for cobalamin lower ligand attachment (DET0657-0660/DET0691-0694) and cobalamin transport and uptake (DET0650-0652/DET0684-0686). In addition, seven *cbiZ*-like genes (i.e. DET0242, DET0249, DET0314/1165, DET0653/0687, DET1556) predicted in strain 195 were detected (Table 3.5). However, none of the cobalamin salvaging genes from the other three genomes targeted by the microarray was detected. Furthermore, the up-stream cobalamin biosynthesis genes (i.e. *cbiD*, *cbiE*/*cbiT*, *cbiC* and *cobN*) found in another un-sequenced Dhc strain ANAS2 (Brission *et al.*, 2012) were not detected in the four cultures by PCR amplification (Figure 3.4), confirming the inability of *de novo* cobalamin synthesis of the Dhc strains in the enrichments.

In addition, a total of 31 Dhc genes outside of the strain 195 genome were detected, 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.

Table 3.5 Genes related to cobalamin transport, uptake and salvaging systems in strain 195

| Locus tag	Genome	Description	Presence/absence (+/-)			
			LoTCEB12	LoTCE	HiTCEB12	HiTCE
DET0240	Strain 195	tetrapyrrole methylase family protein (*cbiE*)	-	-	-	-
DET0296	Strain 195	precorrin-6Y C5,15-methyltransferase, putative (*cbiE*)	+[*]	+[*]	-	-
DET0128	Strain 195	*cobB*	+	+	+	+
DET0245	Strain 195	*cobA*-1	-	-	-	-
DET1139	Strain 195	*cobA*-2	+	+	+	+
DET1224	Strain 195	*cobA*-3	+	+	+	+
DET0936	Strain 195	*cobQ*	+	+	+	+
DET0246	Strain 195	*cobD*-1	-	-	-	-
DET0654	Strain 195	*cobD*-2	+	+	+	+
DET0688	Strain 195	*cobD*-3	+	+	+	+
DET1138	Strain 195	*cobD*-4	+	+	+	+
DET0657/0691	Strain 195	*cobT*-1, -2	+	+	+	+
DET0658/0692	Strain 195	*cobS*-1, -2	+	+	+	+
DET0659/0693	Strain 195	*cobC*-1, -2	+	+	+	+
DET0660/0694	Strain 195	*cobU*-1, -2	+	+	+	+
DET0650/0684	Strain 195	ABC-type cobalamin/Fe^{3+}-siderophores transport systems, periplasmic binding protein, putative	+	+	+	+
DET0651/0685	Strain 195	ABC-type cobalamin/Fe^{3+}-siderophores transport systems, permease component	+	+	+	+
DET0652/0686	Strain 195	ABC-type cobalamin/Fe^{3+}-siderophores transport system, ATP-binding protein	+	+	+	+
DET0242	Strain 195	*cbiZ*-like	-	-	-	-
DET0249	Strain 195	*cbiZ*-like	-	-	-	-
DET0314/1165	Strain 195	*cbiZ*-like	-	-	-	-
DET0653/0687	Strain 195	*cbiZ*-like	+	+	+	+
DET1556	Strain 195	*cbiZ*-like	-	-	-	-

[*] signal intensity < 300
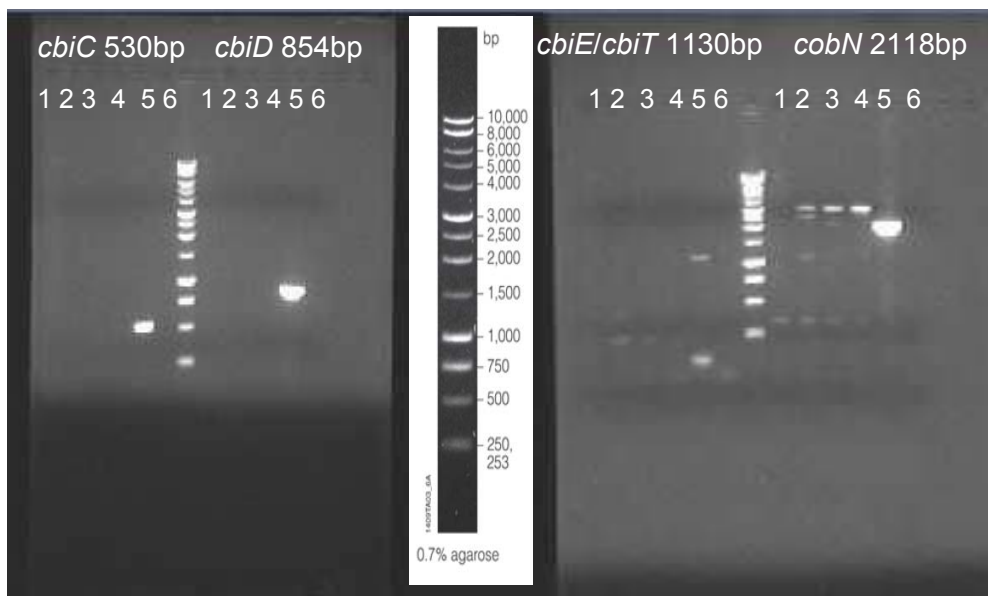
Figure 3.4 Amplification results for *cbiC*, *cbiD*, *cbiE*/*cbiT* and *cobN* (1: HiTCEB12; 2: HiTCE; 3: LoTCEB12; 4: LoTCE; 5: ANAS2; 6: Neg. Ctrl).

3.3.3 Microbial community structure of the four enrichments

Table 3.6 summarizes bacterial OTUs identified from clone libraries of the four enrichments. As expected, an OTU with 99% sequence similarity to Dhc 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., *Dendrosporobacter quercicolus*, *Sporotalea propionica* (recently renamed *Pelosinus propionica* (Moe *et al*., 2011)) and *Clostridium propionicum*, which all belong to the *Firmicutes*. Other than that, 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 (Figure 3.5). The copy numbers of non-Dhc OTUs were normalized to the Dhc_GW copy numbers in Figure 4 for comparison. The greatest variation between cultures occurred in *Clostridium*_GW, whose normalized copy number was 5 times higher in LoTCE($\sim 1 \times 10^7$ copies per10^8 copies of Dhc_GW) compared with LoTCEB12 ($\sim 2 \times 10^6$ copies per10^8 copies of Dhc_GW). In the HiTCE enrichments, normalized copy numbers of *Clostridium*_GWwere one order of magnitude higher than LoTCE. *Desulfovibrio*_GW did not exhibit significant difference between enrichmentswith B_{12} and without, however *Desulfovibrio*_GW 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.

Table 3.6 Summary of clone library results of the four enrichments

OTUs (NCBI accession number), closest cultivatable species	Maximum identity to the closest neighbor (query coverage)(%)	% of clones in each library			
		LoTCEB12	LoTCE	HiTCEB12	HiTCE
*Dehalococcoides*_GW (JQ004083), *Dehalococcoides ethenogenes* strain 195	99 (99)	8.5	4.2	11.4	16.5
*Pelosinus*_GW (JQ004084), *Pelosinus* sp. UFO1	93 (99)	27.7	59.7	40.4	24.3
*Dendrosporobacter*_GW(JQ004085), *Dendrosporobacter quercicolus* strain DSM 1736(T)	100 (89)	10.6	4.2	11.4	14.8
*Sporotalea*_GW (JQ004090), *Sporotalea propionica* strain TM1	91 (99)	7.4	2.8	3.5	0.88
*Clostridium*_GW (JQ004086), *Clostridium propionicum*	98 (99)	2.1	6.9	8.8	14.8
*Desulfovibrio*_GW (JQ004087), *Desulfovibrio oryzae*	99 (99)	19.1	2.8	N.D[1]	4.3
*Spirochaetes*_GW (JQ004088), *Spirochaetes bacterium* SA-8	99 (97)	3.2	N.D	1.8	4.3
*Bacteroides*_GW (JQ004089), *Bacteroides* sp. strain Z4	99 (99)	N.D	N.D	0.88	4.3
Total clones		94	72	114	115

[1] not detected
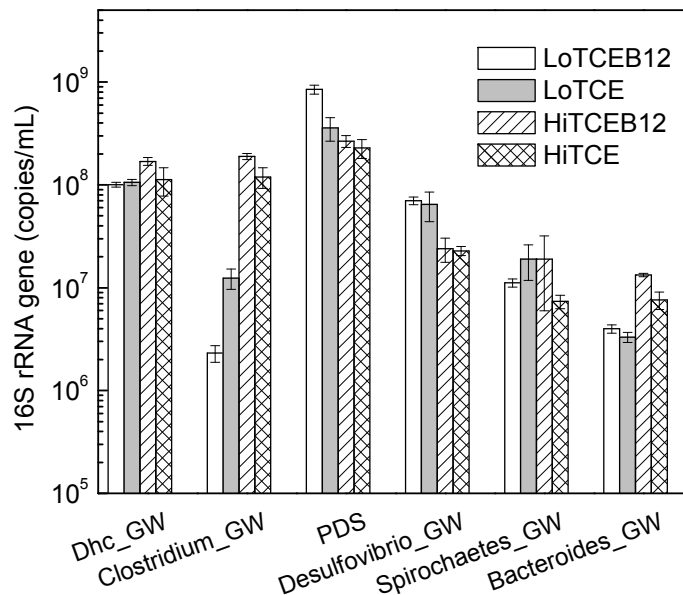
Figure 3.5 16S rRNA gene copy numbers of dominant OTUs in the four enrichments at the end of a feeding cycle (Note: PDS represents a sum of *Pelosinus*_GW, *Dendrosporobacter*_GW and *Sporotalea*_GW).

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 approximately 15 days (Figure 3.6). Different time points of the four enrichments clustered into two groups distinguished by the presence of methanogenesis, suggesting that this characteristic exerted more influence on community structure than B_{12} amendment.
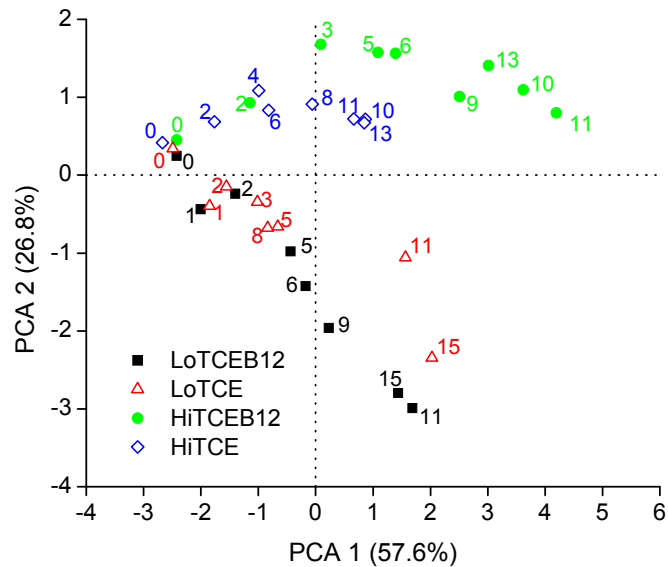

Figure 3.6 Principal component analysis (PCA) plot based on a time-course 16S rRNA gene copy numbers of 8 OTUs in the 4 enrichments (numbers next to each dot represents incubation days).

3.3.4 Total cobalamin in the four enrichments

Concentrations of 2-3 nM (ca. 3-4 µg/L) cobalamin were detected in the enrichments without B_{12} amendment, indicating that community members generate cobalamin that Dhc could potentially use (Figure 3.7). Interestingly, in the enrichments with 74 nM B_{12} added, only 9 nM (ca. 12 µg/L) and 2 nM (ca. 3 µg/L) were recovered in LoTCEB12 and HiTCEB12, respectively (Figure 3.7), indicating a significant consumption of B_{12} in these enrichments.
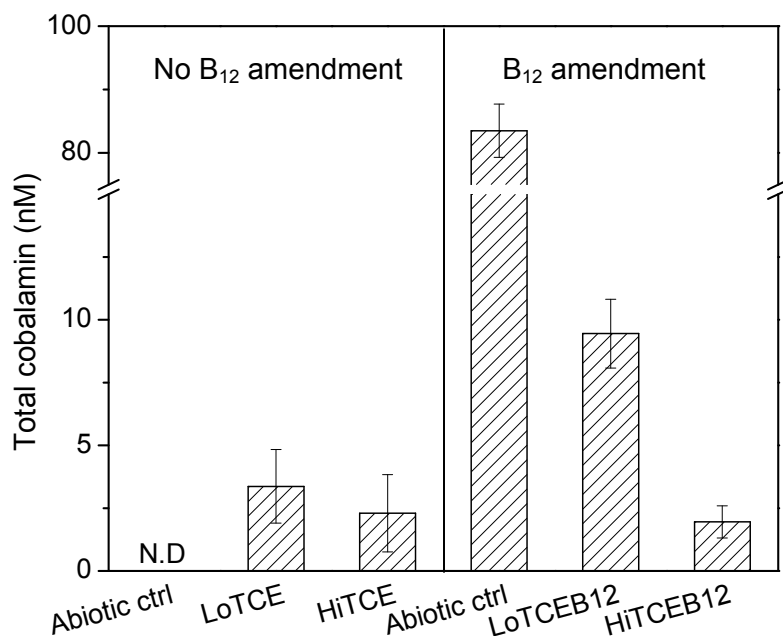
Figure 3.7 Total cobalamin in the four enrichments at the end of incubation
(N.D: not detected).

3.4 Discussion

The aim of this study was to investigate the ecological relationships between Dhc strains and the co-existing Bacteria and methanogenic Archaea within dechlorinating enrichments in terms of cross-feeding of required compounds including hydrogen, acetate and cobalamin. In order to achieve this goal, four stable and robust TCE-dechlorinating microbial communities were enriched 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 B_{12} amendment over 20 sub-culturing events. Dechlorination was observed in all enrichments and occurred slightly faster in non-methanogenic cultures. Non-methanogenic cultures also 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 (2.3-3.3 $\times$ 10^7 copies per μmol Cl$^-$ released) were slightly higher than ANAS (1.4 $\times$ 10^7 copies per μmol Cl$^-$ released) (Holmes $et\ al.$, 2006), but lower than two other well-studied cultures KB-1 (36 $\times$ 10^7 copies per μmol Cl$^-$ released) (Duhamel $et\ al.$, 2004) and VS (58 $\times$ 10^7 copies per μmol Cl$^-$ released) (Cupples $et\ al.$, 2004), perhaps due to the ability of Dhc to uncouple growth from degradation (Maymó-Gatell $et\ al.$, 1997; Johnson $et\ al.$, 2008).

Hydrogen generated from lactate fermentation by different fermentors, such as *Desulfovibrio* (Walker $et\ al.$, 2009), *Syntrophomonas* (Sieber $et\ al.$, 2010), and *Clostridium* (Wu $et\ al.$, 2012) serves as the 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%. Aqueous hydrogen concentrations (7-12 nM) in methanogenic cultures was near the reported threshold for methanogens (10-13 nM), while above that for Dhc (1-3 nM) (Yang and McCarty, 1998). In contrast, aqueous hydrogen in the non-methanogenic enrichments remained 10 times higher, which might account for the higher ethene production in those cultures.

Due to the powerful hydrogen-consuming activity of hydrogenotrophic methanogens, they are commonly found to be involved in syntrophic relationships with thermodynamically unfavorable organisms, such as *Desulfovibrio* (Walker *et al.*, 2009) and *Syntrophomonas* (Sieber *et al.*, 2010). Although results in this study indicate that methanogens do not exert positive effects on the dechlorination activity, cell growth of Dhc or the corrinoid supply, PCA analysis indicated that methanogenic activity does affect community structures more significantly than exogenous vitamin B_{12}. Among the 8 tracked OTUs, the numbers of *Desulfovibrio*_GW and PDS decreased as methanogenesis was inhibited, while the cell numbers of the other OTUs increased. Similarly, a previous study conducted with co-cultures and tri-cultures containing Dhc also reported less growth of a *Desulfovibrio* strain when growing with Dhc alone compared to growth with Dhc and a methanogen (Men *et al.*, 2012).

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 up-stream cobalamin biosynthesis genes, they are incapable of biosynthesizing cobalamin *de novo*. However, they possess all of the strain 195 genes (*cobCDSTU*) involved in the lower ligand attachment 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, 2009b). A recent study demonstrated that in addition to directly using 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.*, accepted).

In enrichments without exogenous vitamin B_{12}, 3-4 µg/L cobalamin was detected, which is above the reported minimum requirement for strain 195 (1 µg/L) (He *et al.*, 2007), indicating a production of sufficient corrinoids by other microorganisms to support Dhc growth. A similar syntrophy between B_{12}-dependent algae and bacteria has previously been reported (Croft *et al.*, 2005). The authors provided convincing evidence that the cobalamin required by B_{12}-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 B_{12} biosynthesis in *Halomonas* sp. was observed in the presence of algal extracts.

In these dechlorinating enrichments, we observed stimulated growth of *Clostridium*_GW in the methanogenic enrichment without B_{12} (LoTCE) compared with LoTCEB12.

Clostridium thermoaceticum 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). *Clostridium* species are known to possess complete up-stream corrinoid biosynthesis pathways (http://www.genome.jp/kegg/pathway.html) (Banerjee, 1997; Banerjee and Ragsdale, 2003), suggesting that they may be supplying corrinoids to Dhc. In addition to *Clostridium* spp., sequenced *Desulfovibrio* spp. and *Bacteroides* spp. genomes also have partial up-stream corrinoid biosynthesis pathways (http://www.genome.jp/kegg/pathway.html). Two corrinoid forms, guanylcobamide and hypoxanthylcobamide are formed by *Desulfovibrio vulgaris* (Guimaraes *et al.*, 1994). *Bacteroides* spp. are reported to have vitamin B_{12}-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 B_{12}.

Different from *Clostridium*, *Desulfovibrio*, *Spirochaetes* and *Bacteroides*, genera of *Pelosinus*, *Dendrosporobacter* and *Sporotalea* (PDS) are not as often reported within dechlorinating microbial enrichments (Freeborn *et al.*, 2005; Daprato *et al.*, 2007; Rowe *et al.*, 2008). *Pelosinus* spp. have been reported as metal-reducing fermentors in lactate-amended enrichments (Shelobolina *et al.*, 2007; Ray *et al.*, 2011; Mosher *et al.*, 2012). The most recent *Pelosinus* strain isolated from chlorinated solvent contaminated groundwater was *Pelosinus defluvii* sp. nov. (Moe *et al.*, 2011). 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 H_2/CO_2 (Moe *et al.*, 2011), while *Dendrosporobacter* can grow chemoautotrophically on H_2/CO_2 (Strömpl *et al.*, 2000). *Pelosinus*, *Sporotalea*, and *Dendrosporobacter* all belong to the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Strömpl *et al.*, 2000; Shelobolina *et al.*, 2007). 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.*, 1988b). Since PDS copy numbers dominate the four enrichments, it is possible that they also contribute to providing corrinoids to the community, however little information on PDS corrinoid production is 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 that may be encountered in the environment. 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 cobalamin synthesis pathway, but do contain genes for corrinoid uptake, modification and salvaging, suggesting that Dhc takes advantage of corrinoids produced by other bacterial members, most likely *Clostridium* and PDS species. PCA analysis of the four enrichments suggested that the presence of methanogens exerted more influence on community structure than B_{12} amendment, impacting both the distribution of the corrinoid-providing and hydrogen-producing bacterial species.

Improved understanding of the roles played by different dechlorinating community members under different potential environmental conditions will improve our abilities to identify biomarkers indicative of robust dechlorination activity. In addition, quantification of corrinoid co-factors in dechlorinating communities and genes related to their biosynthesis and salvaging pathways could serve as useful monitoring and diagnostic biomarkers for effective biodegradation of chlorinated solvents.

3.5 Acknowledgements

4 [*]Identification of Diverse Corrinoid and Lower Ligand Species in *Dehalococcoides*-Containing Microbial Communities

4.1 Introduction

Cobalamin (vitamin B_{12}) is an important co-factor of three classes of enzymes: isomerases, methyltransferases, and reductive dehalogenases (RDases) (Banerjee and Ragsdale, 2003; Brown, 2005). As one form of cobamide, cobalamin belongs to the corrinoid family with ten other naturally occurring forms (Figure 4.1). The structure of a complete corrinoid (i.e., a cobamide) has a cobalt-centered corrin ring with an upper (axial) ligand and a lower (axial) ligand (Figure 4.1). Cyanide (–CN) group is the stable chemical form of the upper ligand, whereas the deoxyadenosyl and methyl groups are the two biological forms of upper ligands, which are unstable and are easily interchanged with –CN. Basically, it is the lower ligand part that determines the various cobamide forms as shown in Figure 4.1. Although corrinoid co-factors are required by animals, humans, and a certain portion of prokaryotes, only a small portion of those prokaryotes (some Bacteria and Archaea) are capable of *de novo* corrinoid synthesis (Martens *et al.*, 2002; Ryzhkova, 2003). The rest of the corrinoid-dependent microorganisms rely on either exogenous corrinoids or corrinoid-synthesizing microorganisms. One typical example is *Dehalococcoides mccartyi* (Dhc), a bacterium that requires corrinoid co-factors for its reductive dechlorination respiration processes.

Chlorinated ethenes, such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) are commonly-found groundwater contaminants in the U.S. (Moran *et al.*, 2007). So far, Dhc are the only known bacteria capable of completely dechlorinating PCE/TCE to the innocuous product ethene (Maymó-Gatell *et al.*, 1997; He *et al.*, 2003b; Cupples *et al.*, 2004). Cobalamin serves as the co-factor of reductive dehalogenases (RDases), the enzymes that catalyze reductive dechlorination reactions, not only in Dhc (Maymó-Gatell *et al.*, 1997; Muller *et al.*, 2004; Seshadri *et al.*, 2005), but also other dechlorinators such as *Dehalobacter restrictus* (Maillard *et al.*, 2003), *Dehalosprillum multivorans* (Siebert *et al.*, 2002). However, genomic analysis of the five sequenced Dhc strains: CBDB1 (Kube *et al.*, 2005), 195 (Seshadri *et al.*, 2005), BAV1, VS and GT (http://img.jgi.doe.gov/) indicates that none of them contains complete up-stream cobalamin biosynthesis pathways, rendering Dhc incapable of synthesizing cobalamin *de novo*. Therefore, commercially-available cobalamin (i.e. cyanocobalamin, also known as vitamin B_{12}) is added into either laboratory-scale cultures or in-situ bioremediation sites as a growth factor to enhance dechlorination performance (Lesage *et al.*, 1996; Richardson *et al.*, 2002; He *et al.*, 2003b; Cupples *et al.*, 2004; Duhamel *et al.*, 2004). However, our latest study on *D. mccartyi* strain 195 indicates that besides cobalamin, [5-MeBza]Cba and [5-OMeBza]Cba are also functional corrinoid forms for strain 195 grown in isolation.

More robust growth and faster dechlorination of Dhc have been observed in microbial communities rather than in isolation (Duhamel *et al.*, 2004; Duhamel and Edwards, 2006; Men *et al.*, 2012). One possible reason is that Dhc can take advantage of corrinoids produced by other community members. Although Dhc cannot biosynthesize cobalamin or other functional cobamides *de novo*, genomic information and previous pure culture studies indicate that they possess down-stream corrinoid modification genes (*cobP/U*, *cbiZ*), as well as genes encoding ABC-type transporters (*BtuCDF*) (Seshadri *et al.*, 2005;

Gray and Escalante-Semerena, 2009b) that enable them to scavenge other forms of corrinoids including cobinamide from the environment, and to transform them into functional forms such as cobalamin by up-taking and attaching dimethylbenzimidazole (DMB), the corresponding lower ligand of cobalamin. In our most recent study, strain 195 was able to modify non-functional corrinoid species to cobalamin in the presence of DMB (Yi *et al.*, accepted) with dechlorination performance and cell growth similar to that of cells growing with exogenous cobalamin.

Some microorganisms commonly found in Dhc-containing communities, for example, fermentors like *Clostridium* and *Desulfovibrio*, and acetogens like *Acetobacterium*, as well as methanogens (Richardson *et al.*, 2002; Freeborn *et al.*, 2005; Rowe *et al.*, 2008) are also known to biosynthesize corrinoids (Stupperich *et al.*, 1988a; Guimaraes *et al.*, 1994; Renz, 1999). One recent study generated and characterized methanogenic and non-methanogenic Dhc-containing enrichments, which were both capable of reductively dechlorinating TCE to VC and ethene without exogenous vitamin B_{12} in defined medium (Men *et al.*, submitted) (Chapter 3). This study reveals that the Dhc species in those enrichments lack complete *de novo* up-stream corrinoid biosynthesis pathways and obtain corrinoids from other Bacterial rather than Archaeal community members. Besides *Clostridium* sp. and *Desulfovibrio* sp., possible corrinoid producers that are also commonly found in other Dhc-containing communities, two other dominant but infrequently reported genera closest to *Pelosinus* sp. and *Dendrosporobacter* sp. were detected in those enrichments, although their corrinoid productivity is yet unknown (Men *et al.*, submitted).

Due to limitations of analytical techniques for corrinoids, little is known about specific corrinoid forms, concentrations as well as their associated functions in corrinoid-dependent microbial communities. Few studies have specifically focused on the characterization and quantification of corrinoid co-factors in microbial communities, and none of them focused on well characterized dechlorinating enrichment communities. Therefore, this is the first study to successfully characterize and quantify various corrinoid and lower ligand forms in Dhc-containing microbial communities enriched under different cobalamin stress (Richardson *et al.*, 2002; Men *et al.*, submitted). Moreover, by analyzing an enrichment with inhibited Dhc growth, we were able to study the effects of Dhc presence on the corrinoid forms produced by other supportive microorganisms within those communities. This is the first attempt to elucidate the relationship between corrinoid-dependent microorganisms without *de novo* synthesis pathways and corrinoid-synthesizing microorganisms in communities.

4.2 Methods

4.2.1 Materials

Corrinoids and lower ligand bases
13 corrinoid species targeted in this study are listed in Figure 4.1, together with the associated lower ligand bases. 5 of the listed lower ligand species were also targeted, which are Bza, 5-MeBza, 5-OMeBza, 5-OHBza, and DMB. Cobalamin, Cbi, and 4 lower ligands (except 5-OHBza) were purchased from Sigma Chemical Co. (St Louis, MO).

Cobyric acid was abiologically synthesized using cobalamin, all other corrinoid forms and 5-OHBza were biologically synthesized according to the biosynthesis methods in section **4.2.2**.

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

Enrichments and growth conditions
ANAS subcultures investigated in this study were inoculated from ANAS, which was enriched from contaminated soil obtained from Alameda Naval Air Station and has been functionally stable for over ten years, with the ability to reductively dechlorinate TCE completely to ethene. ANAS was characterized in previous studies (Richardson *et al.*, 2002; Freeborn *et al.*, 2005; Lee *et al.*, 2006). In 160-mL serum bottles, 95 mL defined basal medium as previously described (He *et al.*, 2007) was added, with 60 mL N_2/CO_2 (90:10) headspace. Lactate (final concentration 48 mM) was amended as both the carbon source and electron donor, 2 μL TCE (ca. 0.2 mM, final concentration) was supplied as the terminal electron acceptor, as well as 0.5 mL Wolin vitamin stock (Wolin *et al.*, 1963) with a final concentration of 100 μg/L vitamin B_{12} as the co-factor. 5% of ANAS culture (5 mL) was inoculated into 95 mL medium. After all TCE is dechlorinated to ethene, 5% of ANAS subculture was transferred into fresh medium using the same condition. In addition, another batch-culture was constructed by inoculating 5% ANAS subculture into 95 mL the same medium but without the addition of vitamin B_{12}. We use the term "SANASB12" and "SANAS" for ANAS subcultures grown with and without vitamin B_{12}, respectively (Table 4.1). Experiments were carried out after 5 times of subculturing for SANASB12 and 3 times of subculturing for SANAS. Both SANASB12 and SANAS were able to completely dechlorinate TCE to ethene.

The four groundwater enrichments (LoTCEB12, LoTCE, HiTCEB12 and HiTCE) used in this study were previously described in chapter 3. They were capable of dechlorinating TCE to VC and ethene. They were enriched over more than 40 subculturing events under each of the four enrichment conditions (Table 4.1): 1) with low initial TCE concentration (to sustain methanogenesis), with vitamin B_{12} (LoTCEB12); 2) with low initial TCE concentration, without B_{12} (LoTCE); 3) with high initial TCE concentration (to inhibit methanogenesis), with B_{12} (HiTCEB12); 4) with high initial TCE concentration, without B_{12} (HiTCE). In order to investigate the role of cobalamin-providing microorganisms, another two cultures were further constructed from HiTCE and HiTCEB12 using the same growth condition, but with no TCE added (denoted "NoTCE", "NoTCEB12", respectively) (Table 4.1). Experiments were carried out after approximately 40 subculturing events for LoTCE, LoTCEB12, HiTCE and HiTCEB12, and 6 subculturing events for NoTCE and NoTCEB12.

Benzimidazoles

Purines

Phenolics

5, 6-dimethylbenzimidazole
Cobalamin

Benzimidazole
[Bza]Cba

Adenine
[Ade]Cba

Phenol
[Phe]Cba

5-Methylbenzimidazole
[5-MeBza]Cba

5-hydroxybenzimidazole
[5-OHBza]Cba

2-Methyladenine
[2-MeAde]Cba

p-Cresol
[p-Cre]Cba

5-Methoxybenzimidazole
[5-OMeBza]Cba

5-Methoxy, 6-methylbenzimidazole
[5-OMe, 6-MeBza]Cba

2-Methylthioadenine
[2-SMeAde]Cba

Figure 4.1 Structures of corrinoid and lower ligand species together with abbreviated designation. Lower ligand name italicized. Cby: cobyric acid; Cbi: cobinamide; Cba: cobamide.

Table 4.1 Conditions of different enrichments and experimental cultures

Enrichments	Lactate (mmol)		TCE (μmol)		Vitamin B$_{12}$ (μg/L)	Dechlorination products	Methane produced
	Feeding regimen[a]	Total	Feeding regimen[a]	Total	One-time addition		
SANAS	48	48	22	22	0	Ethene	+
SANASB12	48	48	22	22	100	Ethene	+
LoTCE	38, 4, 4, 4, 2	52	22, 55, 55, 55, 33	220	0	VC & ethene	+
LoTCEB12	38, 4, 4, 4, 2	52	22, 55, 55, 55, 33	220	100	VC & ethene	+
HiTCE	38, 4, 5, 5	52	77, 77, 66	220	0	VC & ethene	-
HiTCEB12	38, 4, 5, 5	52	77, 77, 66	220	100	VC & ethene	-
NoTCE[b]	38, 4, 5, 5	52	0	0	0	None	-
NoTCEB12[c]	38, 4, 5, 5	52	0	0	100	None	-
One-time experimental cultures	Lactate (mmol)		TCE added on 14th day of incubation (μmol)	Dhc strain inoculated with TCE addition	Vitamin B$_{12}$ (μg/L)	Dechlorination products	Methane produced
	Feeding regimen[a]	Total					
ΔHiTCE[d]	38, 4, 5, 5	52	0	-	0	VC & ethene	-
ΔHiTCE+[d]	38, 4, 5, 5	52	7	-	0	VC & ethene	-
NoTCE+$_{HiTCE}$[e]	38, 4, 5, 5	52	4	HiTCE[†]	0	VC & ethene	-
NoTCE+$_{195}$[e]	38, 4, 5, 5	52	7	Strain 195[f]	0	VC & ethene	-

[a] each number represents an individual amendment.
[b] This enrichment is manipulated from HiTCE.
[c] This enrichment is manipulated from HiTCEB12.
[d] This culture is subcultured (5%) from HiTCE.
[e] This culture is subcultured (5%) from NoTCE.
[f] 1% inoculation.

4.2.2 Synthesis and purification of corrinoid species

[Ade]Cba and [2-MeAde]Cba were extracted from 6 L of *Salmonella enterica* serovar Typhimurium strain LT2 grown aerobically for 48 h at 37 °C in NCE medium containing 10 mM glycerol, 80 mM 1,2-propanediol and 1 μM Cbi, as described previously (Gray and Escalante-Semerena, 2009a). [5-OHBza]Cba was extracted from 4 L of *Methanosarcina barkeri* strain Fusaro grown anaerobically for 14 days at 34 °C in defined mineral salt medium (He *et al.*, 2007) containing a N_2/CO_2 headspace, 0.5% (v/v) methanol and a modified Wolin vitamin solution without cobalamin. Cells were harvested by centrifugation (8,000×g, 4 °C, 20min). Corrinoids were extracted from cell pellets as described in the following section.

HPLC purification was performed with an Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector set at 362 and 525 nm. Samples were injected onto an Agilent Eclipse plus C_{18} column (5 μm, 9.4 × 250 mm) at 45 °C, with 1.8 ml min^{-1} flow rate. Samples were separated using acidified water and methanol (0.1% formic acid) with a linear gradient of 10% to 40% methanol over 17 min. Fractions were collected using an Agilent 1200 series fraction collector and dried under vacuum. The concentrations of cobamides were measured spectrophotometrically at 367.5 nm in deionized water. A molar extinction coefficient of 30,800 M^{-1} cm^{-1} was used for quantification (Allen and Stabler, 2008). The identity of each corrinoid was confirmed by mass spectrometry.

[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).

Monocyanocobyric acid standard was prepared as described by Butler and colleagues (Butler *et al.*, 2006), with the following changes: after evaporation to dryness on a rotary evaporator, the reaction mixture residue was dissolved in 100 mL of deionized water and 100 μL of 0.1M potassium cyanide was added. 5 mL aliquots were desalted with a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA), and eluted in 3 mL methanol. The eluates were dried overnight in a vacuum dessicator, resuspended in deionized water, and stored at -80 °C. HPLC purification of monocyanocobyric acid from the eluates was performed with an Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector. Samples were injected onto an Agilent Eclipse plus C_{18} column (5 μm, 9.4 × 250 mm) using a solvent system consisting of deionized water (A) and methanol (B) with the solvent gradient described by Butler *et al.* (2006). Fractions were collected using an Agilent 1200 series fraction collector, dried under vacuum, and resuspended in deionized water.

4.2.3 Corrinoid and lower ligand extraction

Cell pellets in 200-300 mL culture were collected by centrifugation at 15,000 × g for 10 min at 4 °C, and stored in a 50 mL centrifuge tube at -80 °C before extraction. Supernatant was filtered by a 0.2 μm filter and loaded onto a Sep-Pak C_{18} cartridge

(Waters, Milford, MA), which was conditioned by 3 mL 100% methanol and 6 mL milliQ water. The cartridge was then washed with 50 mL milliQ water, and eluted with 3 mL 100% methanol. The eluate was collected in a 50 mL centrifuge tube and stored at -80 °C before extraction. For extraction, cells were resuspended in 20 mL 100% methanol. 20 mg KCN per gram of cell pellets or per equivalent volume of supernatant was added. All samples were then incubated in 60 °C water bath for 1.5 hours with thorough vortex every 20 min throughout the incubation. After the incubation, cell pellet samples were centrifuged at 40,000 g, 4 °C for an hour to eliminate the cell debris. Suspension of cell pellet samples, together with supernatant samples, was dried by a rotary evaporator. The residue from each sample was redissolved in 20 mL milliQ water and then loaded onto a new Sep-Pak C_{18} cartridge, washed with 10 mL milliQ water and eluted with 2 mL 100% methanol. The eluate was collected into two 1.5 mL microcentrifuge tubes and vacuum-dried by an Eppendorf Vacufuge plus vacuum concentrator (Eppendorf, Hauppauge, NY). The residues in the two tubes were redissolved and combined in 200-300 μL milliQ water to make up a concentration factor of 1000 after the entire extraction procedure. All samples were stored at -20 °C prior to LC/MS/MS measurement.

4.2.4 Detection of corrinoids and lower ligands on liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS)

The LC/MS/MS method was performed using Agilent 6410 triple quad LC/MS system (Agilent Technologies, Santa Clara, CA) and modified according to the methods described in Allen and Stabler (Allen and Stabler, 2008). LC column was Agilent Eclipse Plus C_{18}, 1.8 μm, 3.0 × 50 mm (Agilent Technologies, Santa Clara, CA), with column temperature controlled at 40 °C. 0.5 mL/min gradient elution was used with initial solvent conditions of 82% MilliQ water with 0.1% formic acid (solvent A) and 18% methanol with 0.1% formic acid (solvent B) held for 3 min, increased to 21% B immediately and held for 2 min, increased to 100% B over 0.1min and held for 1 min, decreased back to 18% B over 0.1 min and held for 3.8 min. The injection volume was 10 μL. In order to identify the signature ions of each corrinoid and lower ligand species, MS2 scan was used with the fragmentor set at 135 volts, as well as product ion scan with the collision energy of 45 volts for each corrinoid and lower ligand species, except Cbi, for which 65 volts was used. Signature transitions were used for multiple reaction monitoring (MRM) on LC/MS/MS to fulfill quantitative analysis. Serial dilution of corrinoid and lower ligand standards in MilliQ water with concentrations from 1 nM to 100 μM were used to establish calibration curves of each compound. The limit of detection (LOD) and the limit of quantification (LOQ) were also determined.

In order to investigate the recoveries of the extraction procedure, corrinoid or lower ligand standards with a known concentration in MilliQ water (1 nM for each corrinoid and 100 nM for each lower ligand) were added into 200 mL fresh medium and then extracted through the entire procedure as described above. The extracted standards were measured by LC/MS/MS. The concentrations of corrinoid standards before extraction were quantified by extinction coefficient method (Allen and Stabler, 2008), while the concentrations of lower ligand standards before extraction were determined by dissolving calculated amount of solid powder measured on an analytical balance in MilliQ water. Recoveries were calculated using the measured concentration after extraction divided by

the calculated concentration before extraction. We presume that the cell lysis and cyanidation processes are thorough and complete, thus the recoveries could be regarded the same for both cell pellet and supernatant samples. Concentrations of detected compounds in those two kinds of samples were both reported in the unit of nM, which represents nmoles per liter of culture. The sum of cell pellet and supernatant concentrations were reported as total concentration of the targeted compound.

4.2.5 Validation of the LC/MS/MS method

Two parallel sets of samples were prepared for the method validation. In each set cell pellets and supernatant of strain 195 pure culture or one of the enrichment cultures were separated by centrifuging at 15,000 × g for 15 min at 4 °C. Two fresh media controls with different vitamin B_{12} concentrations were prepared in parallel. One set of samples was measured using extraction and detection methods described by Allen and Stabler (Allen and Stabler, 2008) while the other set was extracted and measured by methods described in this study for comparison.

4.2.6 BluB bioassay

Detection of DMB from enrichment samples by *Sinorhizobium meliloti* bioassay was performed as in Crofts *et al*. (publication in progress). Briefly, a culture of *S. meliloti bluB* was grown in M9 minimal media supplemented with 1 mg/mL L-methionine. This indicator strain was diluted to an optical density of 0.04 as measured at 600 nm and grown with samples in a 96-well microtiter plate in 200 µL volumes. Samples included a triplicate series of DMB standards and triplicate enrichment samples serially diluted two fold. The indicator strain was allowed to grow to stationary phase then incubated in the presence of 80 µg/ml calcofluor. The fluorescent phenotype was measured by excitation at 360 nm and emission at 460 nm using a BioTek Synergy 2 plate reader. DMB standards were used to determine the useful range of the fluorescence phenotype and to calculate the concentration of DMB in the enrichment samples.

4.2.7 DNA isolation and quantification by quantitative PCR (qPCR)

Cell pellets in 1.5 mL culture were collected by centrifuging at 15,000 g, 4 °C for 10 min. Genomic DNA (gDNA) was extracted from the pellets using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. To quantify 16S rRNA gene of Dhc, as well as of the other 7 Observed taxa units (OTUs) detected in the four groundwater enrichments, qPCR was applied using primer sets described in chapter 3. Generally, each 20-µL reaction mixture contained 2.5 µL of sample or serially diluted standard, 10 µL 2 × Fast SYBR Green master mix (Applied Biosystems, Foster City, CA), and 0.625 µM of the forward and reverse primers. Purified PCR product of plasmid insertion containing the 16S rRNA gene of each OTU was quantified and used as qPCR standard as described in chapter 3.

4.2.8 Analysis of chlorinated ethenes by gas chromatography (GC)

Chlorinated ethenes, ethene and methane were measured by an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) (Agilent, Santa Clara, CA), as described in 3.2.

4.2.9 The profiles of corrinoid and lower ligand species present in TCE-dechlorinating enrichments

The forms and concentrations of corrinoid and lower ligand species in different TCE-dechlorinating enrichments at the end of cultivation were determined using the method described above. Bottles of autoclaved fresh medium without inoculation were used as abiotic controls, for which corrinoids and lower ligands were measured together with biological samples at the end of incubation. Single measurements were used for each subculture. Measurements on three subcultures were reported as biological triplicates.

Temporal changes of corrinoid and lower ligand species were also investigated in LoTCE, LoTCEB12, HiTCE and HiTCEB12. For each enrichment, multiple bottles were inoculated, 3 of which were sacrificed at each time point for extraction and measurement. According to the end-point results, different sequential subcultures exhibited similar corrinoid and lower ligand profiles, so we only measured corrinoids and lower ligands at different time points over one feeding cycle.

4.2.10 Identification of corrinoid forms produced by supportive microorganisms and utilized by Dhc

In order to investigate the original corrinoid forms produced by supportive microorganisms prior to modification by Dhc, TCE-free enrichments (NoTCE and NoTCEB12) were further constructed as described above. Corrinoid and lower ligand species were determined in NoTCE and NoTCEB12 at the end of incubation (measurements on three subcultures of NoTCE and one subculture of NoTCEB12), as well as a temporal change in NoTCE over one feeding cycle. The same OTUs as detected in HiTCE were quantified by qPCR, in order to correlate corrinoid and lower ligand production with certain Bacterial members. Biological triplicates were used in qPCR measurements.

In order to investigate how native Dhc would salvage corrinoids produced by supportive microorganisms, HiTCE was subcultured into multiple bottles containing the same fresh medium but without TCE (Table 4.1). On the 14th day of incubation, 7 µL of TCE was re-amended into half of the bottles (denoted "ΔHiTCE+"). The other half bottles remained as control (denoted "ΔHiTCE"). In order to test the utilization of corrinoids by non-native Dhc strains, NoTCE was subcultured into 3 sets of bottles (Table 4.1). On the 14th day of incubation, in one set of the bottles, strain 195 isolate was inoculated (1%) with 7 µL of TCE re-amended (denoted "NoTCE+195"). In another set, 1% HiTCE culture was inoculated with a total of 4 µL of TCE. The third set was kept as control. Corrinoid and lower ligand species, as well as the cell growth were determined after TCE dechlorination was complete. Biological triplicates were used in all measurements.

4.3 Results

4.3.1 Establishment and validation of the corrinoid and lower ligand detection methods

9 corrinoid species, excluding Cby, Cbi, [Phe]Cba and [p-Cre]Cba exhibited a signature transition from a doubly charged precursor ion, which appeared to be the corresponding molecular ion of each corrinoid plus 2 H^+ (Table 4.2) to two dominant product ions: a singly charged lower ligand base and an ion with m/s=912. Thus, the lower ligand base plus H^+ could be used as signature product ion for the 9 cobamides. Although the structure of the 912 ion was unclear, since it was commonly observed among these cobamides, it could be used as a qualifier ion (Table 4.2). The unique and predominant product ion of Cbi, Cby and the lower ligand species was used as their signature product ions as listed in Table 4.2. For corrinoids with signature transitions and all lower ligands, multiple reactions monitoring (MRM) on LC/MS/MS was used to do quantification. [Phe]Cba and [p-Cre]Cba were quantified using selected ion monitoring (SIM) as described by Allen *et al*. (2008). The limits of detection (LODs) were determined as the lowest concentration which could be quantitatively detected (Table 4.2). The limits of quantification (LOQs) were determined as the lowest concentration that could be quantitatively detected with the accuracy between 80% and 120% (Table 4.2). For most corrinoid species, the limit of detection was as low as 2 nM (c.a ~ 3 µg/L). The recoveries of each corrinoid species are also listed in Table 4.2. For lower ligands, Bza and 5-OHBza were not able to be recovered by the extraction procedure, which might be due to their higher hydrophilicity and less affinity for the C_{18} cartridge. The other three lower ligands (5-MeBza, 5-OMeBza and DMB), which are associated with the three functional corrinoid forms for strain 195 (Yi *et al*., accepted) were recovered with recoveries listed in Table 4.2.

The parallel comparison between the two detection methods reveals that corrinoid species detected by one method were also detected by the other, and vice versa. Moreover, the concentrations of detectable corrinoid species were comparable, except for Cbi, the detection of which was more sensitive using the method reported in this study.

Therefore, in the following studies, all corrinoid species and 3 recoverable benzimidazolic lower ligands were determined by SPE plus LC/MS/MS method. The reported concentrations were corrected by the concentration factor (1000 ×) and the recoveries.

4.3.2 Corrinoid and lower ligand profiles in different TCE-dechlorinating enrichments

Except cobalamin in B_{12}-amended cultures, above 90% amount of each detected corrinoid was in cell pellets rather than supernatant. Cobalamin, [5-OHBza]Cba, [5-MeBza]Cba, [p-Cre]Cba, [2-MeAde]Cba, [Ade]Cba, and Cbi were the corrionid species detected in SANAS and SANASB12 (Figure 4.2A, Table 4.3). [p-Cre]Cba, cobalamin, [2-MeAde]Cba, [Ade]Cba, and Cbi were the corrinoid species detected in all four groundwater enrichments (Figure 4.2A, Table 4.3), while [5-OHBza]Cba (0.1-0.2 nM)

was only detected in methanogenic enrichments. Similarly, 0.2-0.3 nM of [5-OHBza]Cba was found in SANAS and SANASB12, which also have methanogenic activity (Richardson *et al.*, 2002; Freeborn *et al.*, 2005), suggesting that [5-OHBza]Cba was specifically produced by methanogens in those methanogenic enrichments. Cobalamin was the predominant corrinoid species in SANAS, at concentrations above 0.74 nM (~ 1μg/L), which was reported as the minimum requirement for the growth of strain 195 (He *et al.*, 2007). However, in the B_{12}-unamended groundwater enrichments, [p-Cre]Cba was the most dominant corrinoid species, with cobalamin the next highest. In all tested enrichments, the other detected corrinoids, including Cbi, were below the biologically significant level 0.74 nM (Table 4.3), and no Cby was detected as an intermediate. Interestingly, 66 nM out of 74 nM amended cobalamin remained intact in the supernatant of SANASB12, while in LoTCEB12 and HiTCEB12, with the same amount of amended cobalamin, only less than 1 nM of cobalamin remained intact in the supernatant (Figure 4.2A).

Table 4.2 LC/MS/MS detection and quantification of corrionid and lower ligand species

Corrinoid	Exact mass	Precursor ion (m/z)	Product ion (m/z)	Qualifier ion (m/z)	LOD (nM)	LOQ (nM)	Recovery (%)
Cby	959	960	847.5	N/A[a]	1	1	21 ± 1
Cbi	1041.49[b] 1014[c]	1015	930.6	N/A	10	50	108 ± 0.1
Cobalamin	1354.56	678.3	147.1	912	2	5	70 ± 15
[Bza]Cba	1326.53	664.3	119.1	912	2	5	81 ± 20
[5-MeBza]Cba	1340.53	671.3	133.1	912	2	5	87 ± 19
[5-OHBza]Cba	1342.53	672.3	135.1	912	2	5	74 ± 6
[5-OMeBza]Cba	1356.54	679.3	149.1	912	2	5	82 ± 18
[5-OMe, 6-MeBza]Cba	1370.56	686.4	162.9	912	2	5	64 ± 11
[2-SMeAde]Cba	1389.60	695.9	181.9	912	2	5	46 ± 8
[Ade]Cba	1343.53	672.8	136.1	912	2	5	59 ± 27
[2-MeAde]Cba	1357.55	679.8	150.1	912	2	5	64 ± 23
[Phe]Cba	1302.52	652.3	N/A	N/A	200	1000	85 ± 18
[p-Cre]Cba	1316.54	659.3	N/A	N/A	200	1000	81 ± 18
Lower ligand							
Bza	118.14	119.1	65	N/A	2	2	N/A
5-MeBza	132.16	133.1	77	N/A	1	2	36 ± 5
DMB	146.19	147.1	131	N/A	1	2	127 ± 14
5-OMeBza	148.20	149.2	79	N/A	1	2	27 ± 3
5-OHBza	134.13	135.1	53	N/A	2	2	N/A

[a] not applicable; [b] mass of dicyanocobinamide; [c] mass of monocyanocobinamide

For the lower ligand production, DMB was the most abundant in the supernatant of all enrichments, with a concentration of 2-4 nM in B_{12}-unamnded cultures (Figure 4.2B). 5-MeBza was detected in the SANAS and SNASB12 (Figure 4.2B), consistent with the detection of the corresponding cobamide [5-MeBza]Cba in these two cultures. Notably, over 10 times more DMB was found in LoTCEB12 and HiTCEB12, compared to SANASB12 (Figure 4.2B). This explains the dramatic decrease of cobalamin in LoTCEB12 and HiTCEB12 but not in SANASB12, which is due to biological transformation of cobalamin to other undetectable forms resulting in liberation of the lower ligand DMB.

Temporal changes of corrinoids and lower ligands were further examined in the four groundwater enrichments in order to investigate the transformation of corrinoids among different species. [p-Cre]Cba and cobalamin were the two dominant corrinoid forms throughout the entire incubation, while the concentrations of other detected corrinoids were consistently lower (< 0.74 nM). [p-Cre]Cba dramatically increased to 10-25 nM in all four enrichments after two days. However, in LoTCE and HiTCE, it decreased significantly from 13 nM and 20 nM to 3 nM and 7.5 nM, respectively after 5-6 days at the time point when the first dose of TCE (2 µL for LoTCE and 7 µL for HiTCE) was degraded (Figure 4.3A & C). While in the corresponding B_{12}-amended cultures, it remained constant (Figure 4.3B & D) without dramatic decrease during the rest of the incubation.

Cobalamin in LoTCE and HiTCE promptly increased to 1-2 nM during the first 2 days. Afterwards, it continued to increase to a maximum of 2-3 nM and then decreased. It took 8 and 5 days to reach a maximum level of cobalamin for LoTCE and HiTCE, respectively, when the same total amount of TCE (7 µL) was degraded. The associated lower ligand DMB remained at low levels while cobalamin was being produced and started to increase when the production of cobalamin stopped (Figure 4.3A & C). In the B_{12}-amended enrichments (Figure 4.3B & D), most of the amended cobalamin was detected in the supernatant during the first 2 days, but started to decrease dramatically afterwards. The total cobalamin remained less than 10 nM after 6 days. Meanwhile, DMB in the supernatant increased after 2 days and reached a plateau with a maximum of 60-80 nM after 6 days. However, there is no obvious correlation between the production of [p-Cre]Cba and the decrease of cobalamin.
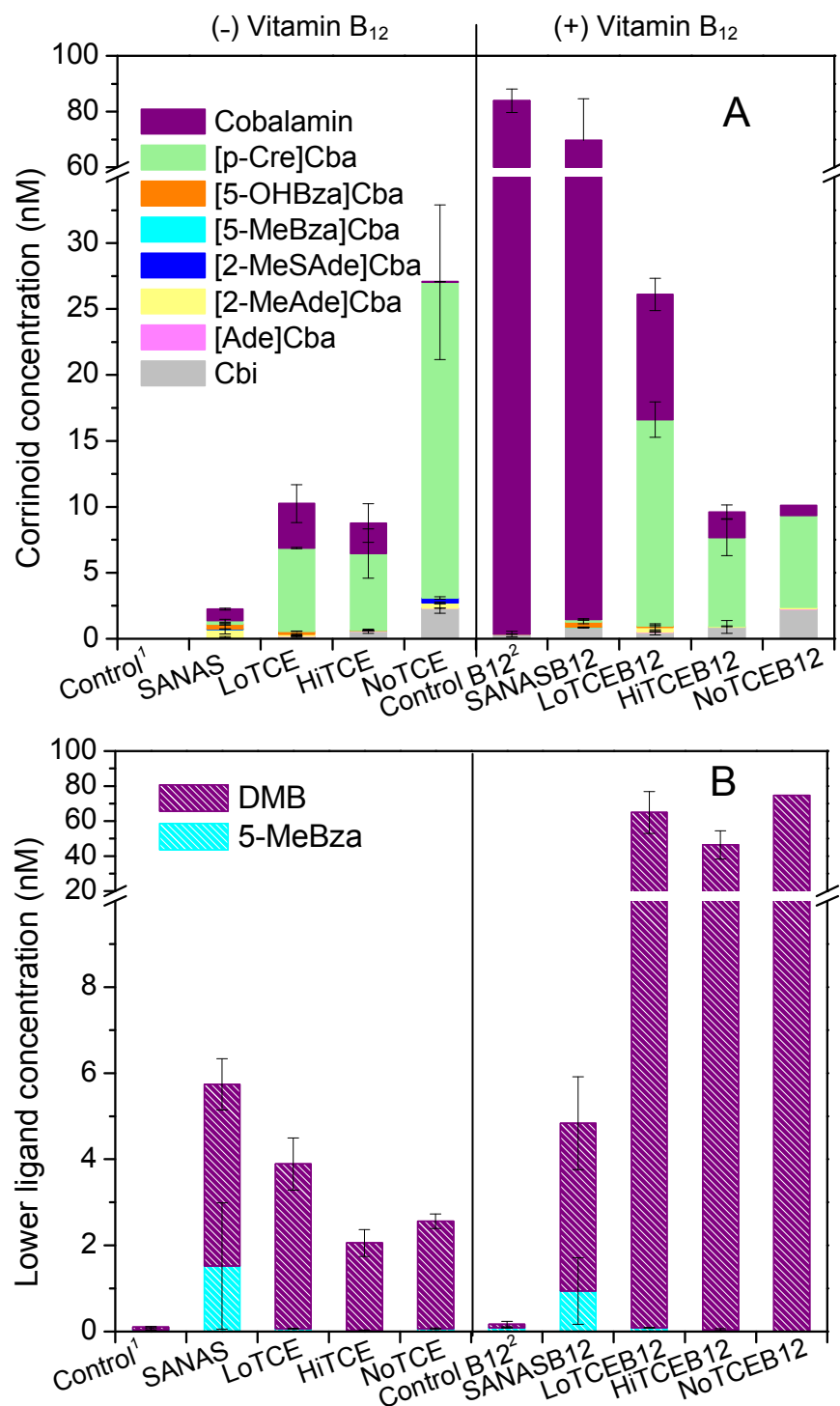
Figure 4.2 Total corrinoid (A) and lower ligand (B) concentrations at the end of the incubation of each enrichment culture ([1] Abiotic control without exogenous vitamin B_{12}; [2] Abiotic control with 100 μg/L vitamin B_{12}; Note: most of the cobalamin in SANASB12 was detected in the supernatant; for other biological samples, corrinoids were mostly detected in the cell pellets. Lower ligands were mostly detected in the supernatant)

Table 4.3 Corrinoid detection in different dechlorinating enrichment cultures

Unit: nM	Cobalamin	[5-MeBza] Cba	[5-OHBza] Cba	[Ade] Cba	[2-MeAde] Cba	[2-SMeAde] Cba	[p-Cre] Cba	Cbi
Abiotic ctrl (without B$_{12}$)	0*	0	0	0	0	0	0	0
Abiotic ctrl (with 74nM B$_{12}$)	**83 ± 4**	0	0	0	0.011 ± 0.005	0	0	0.34 ± 0.20
SANAS sup	0.073 ± 0.063	0	0	0	0.019 ± 0.010	0	0	0
SANAS cell	**0.81 ± 0.02**	0.012 ± 0.007	0.37 ± 0.06	0.062 ± 0.059	0.59 ± 0.36	0.017 ± 0.0001	0.3 ± 0.1	0.039 ± 0.028
SANASB12 sup	**66 ± 15**	0	0.07 ± 0.021	0	0	0	0	**0.78 ± 0.03**
SANASB12 cell	**2.3 ± 0.3**	0.012	0.32 ± 0.14	0.015 ± 0.003	0.015 ± 0.011	0	0.2 ± 0.1	0.068
LoTCE sup	0.17 ± 0.06	0	6.5 ± 3.7×10^{-3}	0	0.012 ± 0.008	0	0.42 ± 0.01	0.040 ± 0.031
LoTCE cell	**3.2 ± 1.4**	0	0.22 ± 0.01	0.023 ± 0.019	0.13 ± 0.04	0	**5.89 ± 0.05**	0.13 ± 0.03
LoTCEB12 sup	0.45 ± 0.17	0	0	0	0.0075 ± 0.0010	0	0.4 ± 0.2	0.18 ± 0.06
LoTCEB12 cell	**9.0 ± 1.2**	0	0.10 ± 0.06	0.014 ± 0.0003	0.36 ± 0.27	0	**15.2 ± 1.3**	0.31 ± 0.18
HiTCE sup	0.10 ± 0.04	0	0	0	0	0	0.38 ± 0.04	0.011 ± 0.001
HiTCE cell	**2.2 ± 1.5**	0	0	0.012 ± 0.006	0.026 ± 0.017	0	**5.5 ± 1.9**	0.56 ± 0.15
HiTCEB12 sup	0.55 ± 0.14	0	0	0	0.0094 ± 0.0059	0	**0.80 ± 0.01**	0.26 ± 0.16
HiTCEB12 cell	**1.4 ± 0.5**	0	0	0	0.032 ± 0.016	0	**6.0 ± 1.4**	0.64 ± 0.46
NoTCE sup	(6± 1)×10^{-3}	0	0	0.028 ± 0.007	0.17 ± 0.02	0.21 ± 0.06	**8.5 ± 0.4**	0.36 ± 0.07
NoTCE cell	0.04 ± 0.01	0	0	0.006 ± 0.001	0.23 ± 0.06	0.16 ± 0.07	**15.5 ± 5.9**	1.9 ± 0.4

*Below detection limit;
Bolded figures indicate concentration above biologically significant level for Dhc 0.74 nM (1 μg/L).
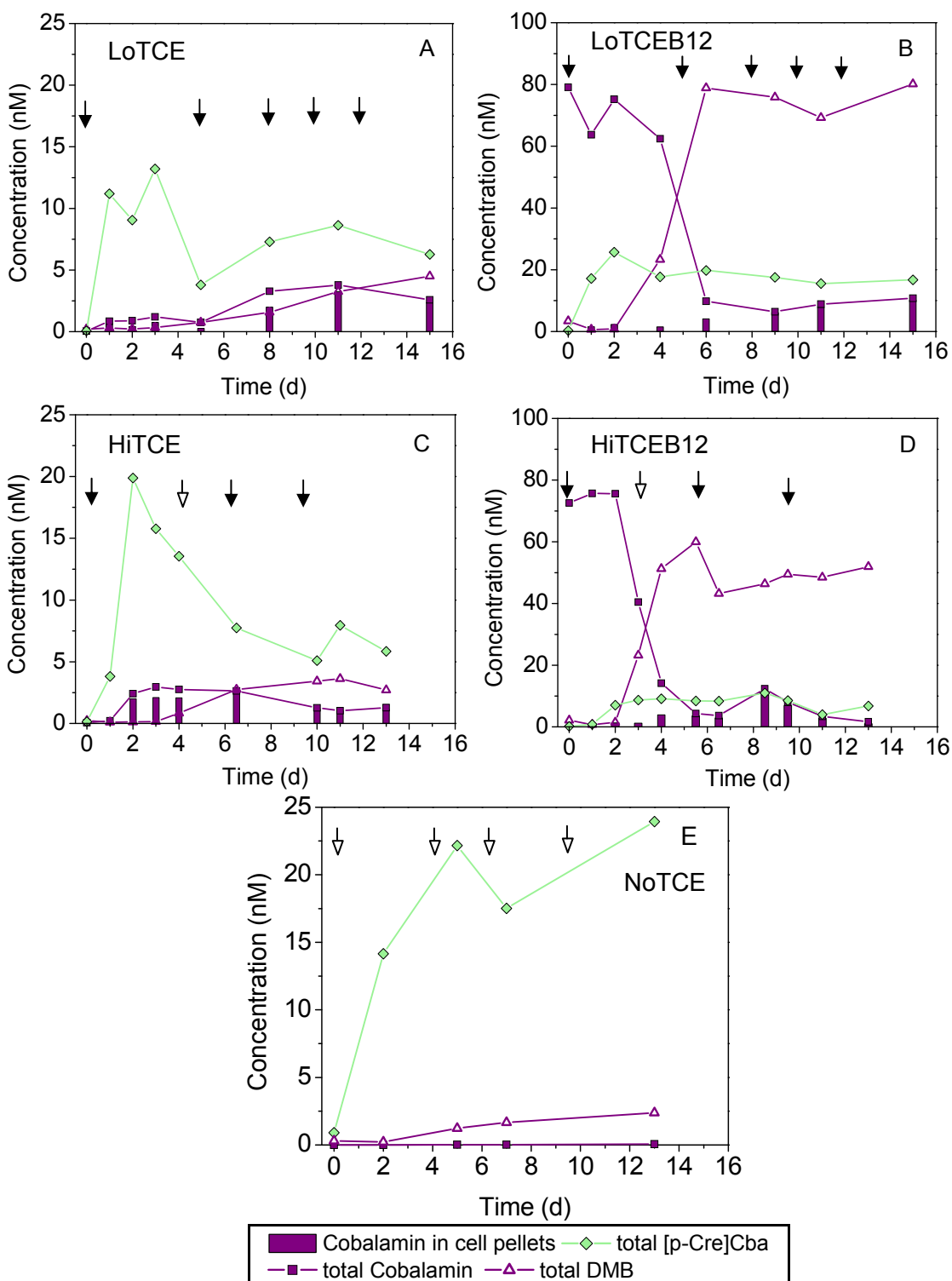
Figure 4.3 Temporal changes of [p-Cre]Cba, Cobalamin, and DMB in groundwater enrichments (A: LoTCE, B: LoTCEB12, C: HiTCE, D: HiTCEB12, E: NoTCE. ↓ indicates amendments of lactate and TCE, ▽ indicates amendment of lactate only, added amounts are according to Table 1. Note: Y-axis Scales in A, C, E are different from those in B & D).

4.3.3 Corrinoid forms produced by supportive microorganisms and the utilization by Dhc

In order to determine the forms of corrinoids originally produced by the supportive microorganisms without the modifying effect of Dhc, corrinoid and lower ligand species, as well as community structures were examined in the NoTCE enrichment. As expected, negligible growth of Dhc ($< 10^4$ 16S rRNA gene copies/mL) in NoTCE was observed by qPCR (Figure 4.4B). However, surprisingly, little cobalamin (< 0.1 nM) was detected in NoTCE (Figure 4.2A). Furthermore, time-course results exhibited little cobalamin production throughout the whole incubation (Figure 4.3E), indicating the production of cobalamin was associated with the growth of Dhc. For [p-Cre]Cba in NoTCE, instead of a dramatic decrease such as that observed in HiTCE, after 5 days it peaked and remained at ~ 22 nM, similar levels as reached in HiTCE after 2 days. Moreover, about 20-50% of total [p-Cre]Cba in NoTCE was detected in supernatant, while in HiTCE, almost all of the [p-Cre]Cba was detected in the cell pellets (Figure 4.3C & E). [2-MeAde]Cba increased in NoTCE compared to HiTCE (Figure 4.2A), and [2-SMeAde]Cba was also detected in NoTCE, while it was below detection limit in HiTCE (Figure 4.2A). Since the concentration of these two corrinoids were below biologically significant levels during the whole incubation, they were considered of less importance than [p-Cre]Cba. In addition, in the B_{12}-amended, TCE-free enrichment NoTCEB12, where the growth of Dhc was also eliminated, the amended 74 nM cobalamin decreased to 0.77 nM after 13 days of incubation (Figure 4.2A), in the meanwhile, about 58 nM DMB was detected in the supernatant (Figure 4.2B), similarly with HiTCEB12, indicating it was other microorganisms that transformed the amended cobalamin rather than Dhc.

Interestingly, although little cobalamin was produced when the growth of Dhc was inhibited, DMB was still produced in NoTCE with a similar concentration as in HiTCE (Figure 4.2B and Figure 4.3E), indicating an anaerobic production of DMB for purposes other than cobalamin biosynthesis. The production of DMB in NoTCE was corroborated by alternative DMB detection methods (BluB bioassays), which resulted in similar DMB concentrations (Figure 4.6).

In terms of the community structure (Figure 4.4B & Figure 4.5B), an increase of growth was observed in the PDS group (~ 10 times) in NoTCE compared with HiTCE, while significant decrease in *Desulfovibrio*_GW by one order of magnitude, indicating its close relationship with Dhc. The growth of *Clostridium*_GW decreased by two fold. No significant effect was observed for *Spirochaetes*_GW and *Bacteroides*_GW.
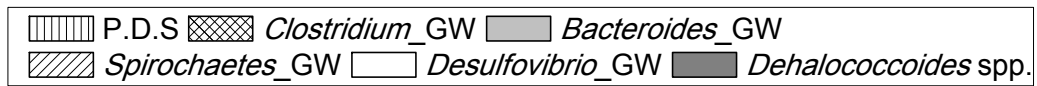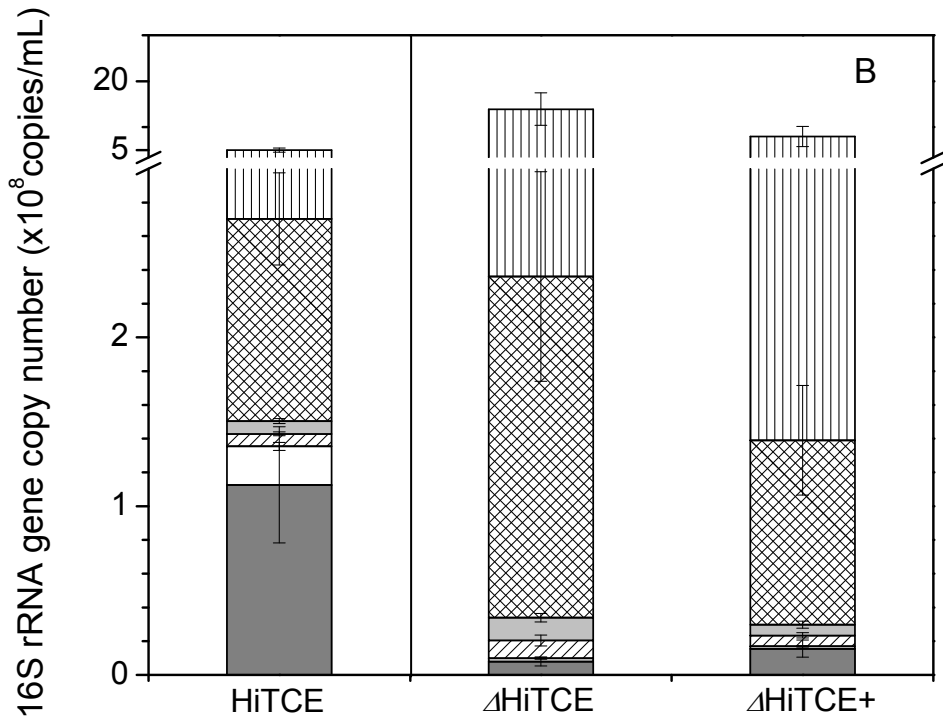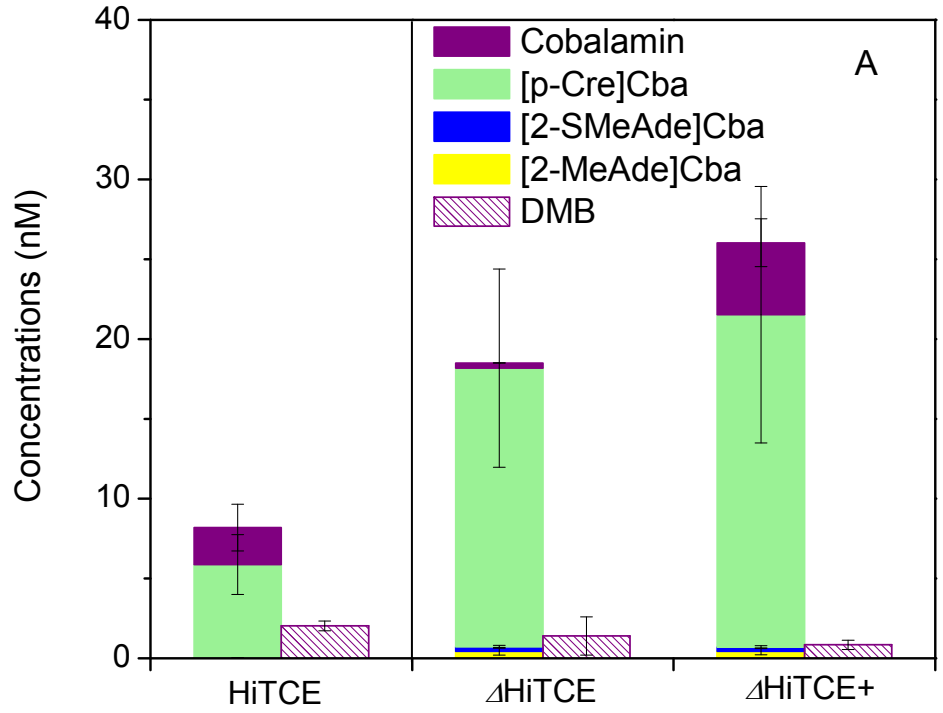
Figure 4.4 Comparison of cobamide and lower ligand production (A) and cell growth (B) between *ΔHiTCE* and *ΔHiTCE+*, HiTCE is shown as reference.
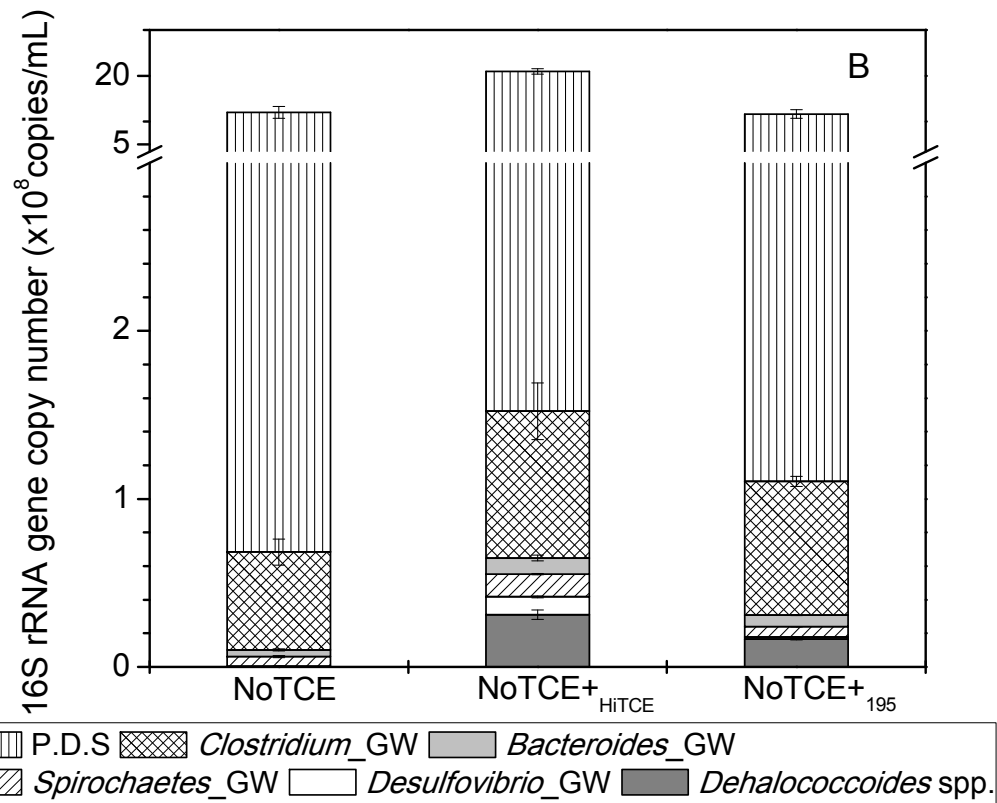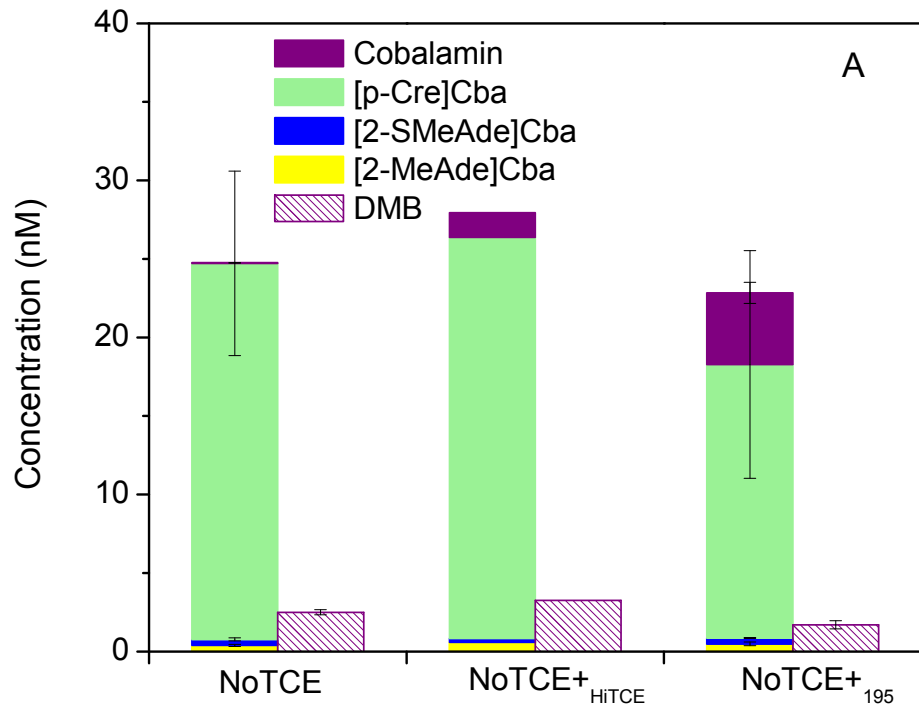
Figure 4.5 Comparison of cobamide and lower ligand production (A) and cell growth (B) among NoTCE, NoTCE+$_{HiTCE}$, and NoTCE+$_{195}$.
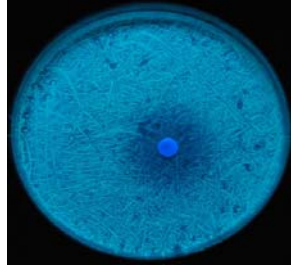
A. Supernatant extract of NoTCE (1000×)
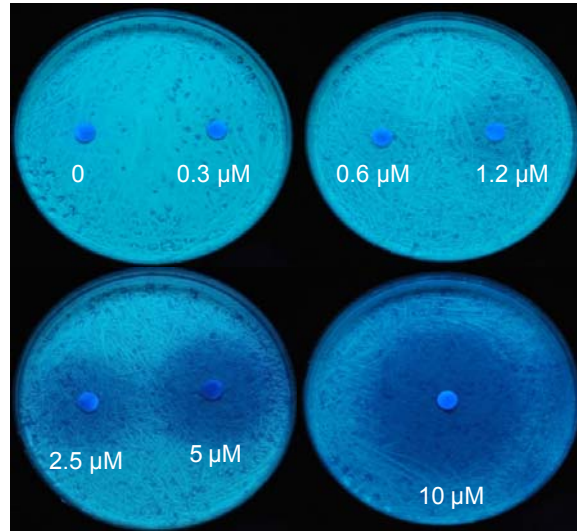
B. DMB standards

C. Results summary from 3 different detection methods

Unit: nM

Sample	LC/MS/MS	BluB bioassay (semi-quantification)	Sm bioassay
NoTCE sup	2.4 ± 0.2	2.5	5.3 ± 0.7

Figure 4.6 BluB assay results

Results from the NoTCE enrichment suggest that cobalamin detected in HiTCE came from the modification of [p-Cre]Cba by Dhc in the presence of DMB. In order to further test this hypothesis, we stopped adding TCE in ΔHiTCE for one feeding cycle, and then re-amended TCE into ΔHiTCE+. As expected, cobalamin production ceased in ΔHiTCE, and was restored in ΔHiTCE+ (Figure 4.4A). No significant difference in terms of other corrinoid forms and DMB was observed between ΔHiTCE and ΔHiTCE+. The cobalamin concentration in ΔHiTCE+ was similar to that of HiTCE. An increase in [p-Cre]Cba was observed in ΔHiTCE compared with HiTCE. The growth of Dhc doubled in ΔHiTCE+ compared with ΔHiTCE, while other OTUs exhibited slight decay (Figure 4.4B).

Further, the HiTCE enrichment and strain 195 isolate were inoculated into separate bottles of NoTCE with the re-amendment of TCE to test the effects of re-introduction of Dhc to those enrichments. Complete dechlorination occurred concomitantly with the production of cobalamin (Figure 4.5A) and cell growth (Figure 4.5B) in both NoTCE+$_{HiTCE}$ and NoTCE+$_{195}$. Cobalamin concentration was similar to that in HiTCE and ΔHiTCE+ (Figure 4.4A), and the growth of Dhc reached a similar level with that in ΔHiTCE+ (Figure 4.4B).

4.4 Discussion

The traditional microbiological assay of corrinoids (Chanarin and Muir, 1982; Kelleher and Broin, 1991) or the previously developed analytical methods such as HPLC+UV (Whitman and Wolfe, 1984; Dalbacke and Dahlquist, 1991; Guggisberg et al., 2012), that were commonly used in food, clinical and pharmaceutical fields, suffered from disadvantages associated with inability of differentiating corrinoid forms and of high limits of quantification (> 0.1 mg/L, c.a 74 nM). Unlike previous quantitative or semi-quantitative methods, the LC/MS/MS method established and applied in this study is a modification from a recently reported LC/MS method by Allen and Stabler (Allen and Stabler, 2008), using multiple reaction monitoring (MRM) instead of selected ion monitoring (SIM). The use of signature transitions and a qualifier molecule results in higher accuracy and sensitivity. The LC/MS/MS method enables us not only to distinguish 13 corrinoid and 5 benzimidazolic lower ligand forms, but also to quantify as low as 5 nM (c.a. 7 μg/L) of each, except for [p-Cre]Cba and [Phe]Cba. Studies have shown that various forms of corrinoids are produced or salvaged by corrinoid-dependent organisms living in different natural habitats, such as cyanobacteria and algae in ocean and freshwater (Croft et al., 2005; Tanioka et al., 2009), methanogens and acetogens (Whitman and Wolfe, 1984; Krautler et al., 1987; Stupperich et al., 1988a), as well as dechlorinators from sediment, soil and groundwater (Banerjee and Ragsdale, 2003; Men et al., submitted). So the ability to identify and quantify diverse corrinoid species present in communities would be of great significance to help us improve our understanding of their ecological relationships based on corrinoid utilization.

This is the first study to identify the various forms of corrinoids and lower ligands present in Dhc-containing microbial communities with/without exogenous vitamin B$_{12}$, as well as the utilization of corrinoids by Dhc in a community. [p-Cre]Cba was the most dominant corrinoid species in the four dechlorinating communities enriched from groundwater whether exogenous B$_{12}$ was added or not, whereas much less [p-Cre]Cba was found in SANAS and SANASB12 enrichments, where cobalamin dominated. [p-Cre]Cba was first isolated from acetogenic bacteria *Sporomusa ovata* (Stupperich et al., 1988a), which is currently the only known bacteria to preferably biosynthesize phenolic cobamides (i.e. [Phe]Cba and [p-Cre]Cba) (Chan and Escalante-Semerena, 2011). In *Sporomusa ovata*, it was suggested that [p-Cre]Cba has a function as methyl carrier in acetate-forming reactions (Stupperich et al., 1988a). Moreover, these two phenolic cobamides cannot establish a coordination bond with the cobalt ion of the corrin ring, in other words, they only form the base-off, rather than base-on conformation, limiting the use of [Phe]Cba and [p-Cre]Cba to enzymes that use cobamide in base-off conformation, such as methionine synthase (metH) in *Salmonella enterica* (Chan and Escalante-Semerena,

2011). This distinctive feature might make these two forms of corrinoids undetectable by traditional microbiological assays using other indicative strains, which could result in an underestimation of the total corrinoids. In NoTCE, where no dechlorination occurs and the growth of Dhc was inhibited, little cobalamin was produced, leaving [p-Cre]Cba as the dominant corrinoid form and indicating that the supportive microorganisms preferentially produce the [p-Cre]Cba form rather than cobalamin. However, the production of cobalamin and the growth of Dhc were restored in ΔHiTCE+, NoTCE+$_{HiTCE}$ and NoTCE+$_{195}$ when dechlorination was restored, which indicates that cobalamin is the preferred corrinoid form modified by Dhc from other corrinoid forms. Moreover, in HiTCE, where active dechlorination and growth of Dhc occur, [p-Cre]Cba exhibited a substantial decrease, while in NoTCE, the total [p-Cre]Cba remained at high levels, with no observed decrease. A higher proportion of [p-Cre]Cba was detected in supernatant samples of NoTCE, whereas [p-Cre]Cba was mostly found in cell pellets of HiTCE. In addition, it took a shorter time for HiTCE to reach the maximum level of [p-Cre]Cba than NoTCE, suggesting a stimulated production of [p-Cre]Cba when dechlorination of Dhc is active. No such trend was observed for other corrinoid forms. Therefore, it is likely that Dhc took up [p-Cre]Cba provided by other microorganisms and converted it into cobalamin in the presence of DMB. A study using the strain 195 isolate indicated that although [p-Cre]Cba was not a functional cobamide for strain 195 to use directly, in the presence of DMB, strain 195 was able to replace the original lower ligand base in [p-Cre]Cba with DMB, resulting in the formation of cobalamin (Yi *et al.*, accepted). *CbiZ* is thought to be the gene involved in this salvaging pathway (Gray and Escalante-Semerena, 2009b; Yi *et al.*, accepted). And a pervious genus-wide microarray analysis on HiTCE demonstrated that among the 22 *cbiZ*-like genes in the four Dhc genomes (i.e. CBDB1, 195, BAV1, and VS), duplicated homologous *cbiZ*-like genes in strain 195 (DET0653/0687) were present in the Dhc genomes in HiTCE (see Table 3.5). The restored production of cobalamin in ΔHiTCE+, NoTCE+$_{HiTCE}$ and NoTCE+$_{195}$ also corroborated the hypothesis in the study by Yi *et al*. (accepted).

A previous study (chapter 3) characterized the microbial structures of the four groundwater enrichments, indicating a dominance of three close related Bacterial species (PDS) most similar with *Pelosinus*, *Dendrosporobacter* and *Sporotalea*, which belong to the *Sporomusa-Pectinatus-Selenomomas* group (Moe *et al.*, 2011; Men *et al.*, submitted), as well as the presence of bacterial species (*Clostridium*_GW and *Desulfovibrio*_GW) most similar to *Clostridium* and *Desulfovibrio*, the two commonly known corrinoid-producing genera (Renz, 1999; Men *et al.*, submitted). [5-MeOBza]Cba, [5-MeO, 6-MeBza]Cba, and cobalamin can be produced by *Clostridium* (Stupperich *et al.*, 1988a; Renz, 1999), [5-MeBza]Cba by sulfate-reducing bacteria (Krautler *et al.*, 1988), and two purine cobamides (guanylcobamide and hypoxanthylcobamide) by *Desulfovibrio vulgaris* (Guimaraes *et al.*, 1994). However, the growth of *Desulfovibrio*_GW decreased significantly in NoTCE (Figure 4.4B), which did not correlate with the consistently high level of [p-Cre]Cba (Figure 4.3E), therefore, *Desulfovibrio*_GW is less likely to contribute to the production of [p-Cre]Cba. Given that the growth of *Clostridium*_GW was 10 times lower in LoTCEB12 than in LoTCE (see Figure 3.5), which does not correspond to the high production of [p-Cre]Cba in LoTCEB12, *Clostridium* is unlikely the [p-Cre]Cba producer. Instead, they might produce other forms of corrinoid, resulting

in their advantage for growth in the absence of cobalamin. Given that the [p-Cre]Cba producer *Sporomusa ovata* was assigned to *Sporomusa-Pectinatus-Selenomomas* group (Collins *et al.*, 1994), the same phylogenetic group as *Pelosinus*, *Dendrosporobacter* and *Sporotalea* under the same family of Veillonellaceae, it becomes more likely that the P.D.S contributed to the production of [p-Cre]Cba. However, so far, little is known about the corrinoid productivity of any species belonging to those three genera. Further corrinoid-related investigations on related species are being carried out in our on-going studies.

Not all Dhc-containing communities employ the same corrinoid utilization mechanisms. Different communities may have different structures, thus the corrinoid profiles might vary correspondingly. In this study, different corrinoid profiles were observed among different B_{12}-free enrichments. In SANAS, cobalamin was the dominant corrinoid form (representing ~ 39% of the total corrinoids), whereas [p-Cre]Cba dominated in LoTCE and HiTCE, suggesting a different cobalamin source in SANAS. Recent metagenomic analysis on ANAS, the origin inoculum of SANAS, found that one Dhc strain (ANAS2) contained in this community possesses a nearly complete up-stream cobalamin biosynthesis pathway (Brission *et al.*, 2012), indicating that besides remodeling other forms of corrinoids, cobalamin found in SANAS might also come from the *de novo* biosynthesis of strain ANAS2. No matter what the source of cobalamin in SANAS, LoTCE or HiTCE, the final concentrations of cobalamin in each community were all around 1-3 nM (ca. 1.4-4 µg/L), which was consistent with the reported minimum requirement of 1 µg/L vitamin B_{12} for growing strain 195 in isolation (He *et al.*, 2007).

Another specific corrinoid profile that resulted from different community structures was the production of [5-OHBza]Cba only in the methanogenic communities, indicating that [5-OHBza]Cba (a.k.a factor III) is typically produced by methanogens, which is consistent with previous findings (Pol *et al.*, 1982; Whitman and Wolfe, 1984; Krautler *et al.*, 1987; Stupperich *et al.*, 1987). However, it less likely represents the major contributor to the corrinoid requirement by Dhc due to its absence in HiTCE and other enrichments. Relatively small amounts of [5-MeBza]Cba were found in SANAS and SANASB12, but were undetectable in the groundwater enrichments.

[2-MeAde]Cba and [Ade]Cba (pseudoB12) were the corrinoids generated in all enrichment cultures, albeit in small amounts. [Ade]Cba is reported to be the dominant corrinoid produced anaerobically by *Clostridium cochlearium* and *Lactobacillus reuteri* (Taga and Walker, 2008). Studies show that [2-MeAde]Cba and [Ade]Cba can also be produced under strict anaerobic growth of *Salmonella typhimurium*, which is known to produce cobalamin in the presence of oxygen (Keck and Renz, 2000). It is likely that [2-MeAde]Cba and [Ade]Cba could be alternatively produced by certain corrinoid-producing microorganism when its designated lower ligand bases were not available.

Moreover, Dhc was shown to modify other forms of corrinoids, such as [p-Cre]Cba into cobalamin only if DMB was present (Yi *et al.*, accepted). Since no exogenous DMB was provided, DMB must be generated in those communities, as was shown in each of the enrichments used in this study, even in NoTCE, where the growth of Dhc was limited and

little cobalamin was generated. The presence and quantity of DMB was corroborated by different detecting methods. This is an unusual, but interesting finding since the production of DMB has always previously been found concomitant with the production of cobalamin. The similar amount of DMB detected in NoTCE indicates that DMB is generated anaerobically without being related to cobalamin synthesis. Basically, DMB is biosynthesized aerobically or anaerobically through different pathways. Under aerobic conditions, DMB is known to be synthesized from riboflavin, and BluB is the identified enzyme carrying out this function (Taga *et al.*, 2007), while under anaerobic condition, it is formed in *Eubacterium limosum* from several building blocks, such as glutamine, glycine and formate, which are also involved in purine-nucleotide biosynthesis (Munder *et al.*, 1992). The enzymes involved in the anaerobic pathway, so far, have not been identified. However, *in vitro* studies have demonstrated that DMB could also be formed by nonenzymatic transformation of FMN in the presence of oxygen (Maggio-Hall *et al.*, 2003; Taga and Walker, 2008). It is possible that DMB could also be formed abiotically under anaerobic condition from different biologically produced precursors, although little is known about this potential at this point. Due to the importance of DMB in the corrinoid-remodeling processes of Dhc and other cobalamin-salvaging anaerobes, further investigations are needed in order to understand the anaerobic DMB synthesis mechanisms and pathways. Since Dhc are able to transform most natural forms of cobamides into cobalamin as long as DMB is available (Yi *et al.*, accepted), DMB seems to become a keystone molecule indicative of cobalamin availability for dechlorination processes in the natural environments where various corrinoids could be produced by other microorganisms.

To summarize, by applying a newly established LC/MS/MS method, we identified various corrinoid and lower ligand species present in different Dhc-containing communities with/without exogenous vitamin B_{12}. The mechanisms employed by Dhc in a community to utilize corrinoids provided by other microorganisms were further investigated. Cobalamin is the preferable corrinoid species used by Dhc, which could be formed by Dhc themselves using other available corrinoid forms, such as [p-Cre]Cba in the presence of the preferable lower ligand DMB. Different community structures may result in different corrinoid and lower ligand profiles. The anaerobic production of DMB is of great importance to corrinoid utilization by Dhc. These findings will shed light on the understanding of cobalamin salvaging and modification pathways in Dhc-containing communities, species-function correlations, ecological relationships among community members, as well as the identification of environmental biomarkers for monitoring and enhancement of *in situ* bioremediation of chlorinated solvents. Moreover, the related findings, as well as the methods established in this study could also benefit the understanding of other corrinoid-dependent functional microbial communities in the natural environments.

4.5 Acknowledgements

5 Differential Gene Expression in TCE-dechlorinating enrichments under Different Cobalamin Stress and Growth Phases

5.1 Introduction

Chlorinated ethenes have been common groundwater contaminants in the U.S. for decades (Doherty, 2000a; Doherty, 2000b; Moran *et al.*, 2007). *Dehalococcoides mccartyi* (Dhc) are currently the only known bacterial strains capable of complete dechlorination of perchlorinated ethene (PCE) and trichloroethene (TCE) to the innocuous end product ethene through reductive dechlorination (Maymó-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003b; He *et al.*, 2005; Sung *et al.*, 2006b). Dhc have been found to be widely distributed in many contaminated environments, such as soil, sediments as well as groundwater aquifers (Hendrickson *et al.*, 2002). Therefore, as a cost-effective and environmentally friendly approach to treat aquifers contaminated with chlorinated ethenes, *in situ* bioremediation utilizing Dhc is a promising option.

However, due to the long doubling-times and fastidious nutrient requirements of Dhc, it is challenging to stimulate them to grow to dominance in subsurface communities and their dechlorination activities can be adversely affected by various environmental factors during bioremediation (Aulenta *et al.*, 2006). In order to properly monitor, diagnose, and manipulate the bioremediation processes, to get a better understanding on the growth-limiting factors, biomarkers indicative of robust dechlorination activities are needed.

One of the typical molecular biomarkers is quantification of reductive dehalogenase (RDase) genes. RDases are the enzymes that catalyze reductive dechlorination, using hydrogen as the sole electron donor for Dhc and requiring corrinoids, typically cobalamin (vitamin B_{12}), as an obligate co-factor. Four RDase genes, *pceA*, *tceA*, *vcrA* and *bvcA* have been identified to be associated with different steps in the complete reductive dechlorination of PCE (Magnuson *et al.*, 2000). Studies have shown a correlation between their expression levels and dechlorination activities (Lee *et al.*, 2006; Rahm *et al.*, 2006b; Morris *et al.*, 2007; Behrens *et al.*, 2008; Da Silva and Alvarez, 2008). Besides RDase genes, other oxidoreductase genes involved in dehalorespiration, such as those encoding hydrogenases, formate dehydrogenases, and ATPases were also considered to be useful indicators of dechlorination activities (Morris *et al.*, 2006).

Recently, we integrated the availability of four Dhc genome sequences (strain 195, CBDB1, BAV1, and VS), with high-throughput microarray technology to design microarrays that target Dhc genomes and all known RDases on a Dhc genus-wide microarray (Lee *et al.*, 2011). In addition, another array-based pangenome probe set was designed based on the same four Dhc genomes, as well as the metagenome of a highly enriched mixed culture KB-1 (Hug *et al.*, 2011). These microarray platforms provide us more comprehensive analyses of Dhc genes of interest beyond the RDases genes and respiratory genes commonly studied. In this way, we are able to effectively and simultaneously query genes involved in other important physiological processes, such as corrinoid transport and biosynthesis, amino acid transport and biosynthesis, and stress response. The whole genome microarray has been successfully applied to strain 195 in studies of temporal transcriptomic analysis (Johnson *et al.*, 2008) and in evaluating the effects of corrinoid levels on gene regulations (Johnson *et al.*, 2009). The genus-wide

microarray has also been successfully applied to a well-characterized Dhc-containing enrichment (ANAS) to investigate the presence of Dhc genomes and the global gene expression under conditions of periodic substrate supply (West *et al.*, 2008; West *et al.*, in review). Comparing the global expression of Dhc genes under different growth conditions in a community will allow us to identify potential molecular biomarkers associated with the environmental conditions that may be encountered in bioremediation applications.

In a previous study (Chapter 3), the genomic comparison of four groundwater enrichments (i.e. LoTCEB12, LoTCE, HiTCEB12, and HiTCE) using the genus-wide microarray (See 3.3.2) indicated that the enrichments all shared a common Dhc core genome that most closely resembles strain 195. In order to further analyze the genes associated with the change from corrinoid-unlimited to corrinoid-limited conditions, in this study, transcriptomic analyses were conducted on the two non-methanogenic enrichments HiTCEB12 and HiTCE, during the early exponential phase when TCE was partially dechlorinated and cells starting to grow. Moreover, the temporal expression of Dhc genes in HiTCE were also investigated during different growth phases (early exponential, late exponential, stationary phases). Since corrinoid levels may vary from site to site during bioremediation, it is of great significance to understand the transcription-level response of Dhc species in communities under corrinoid stress, which could guide us towards the identification of potential biomarkers indicative of corrinoid availability during bioremediation applications.

5.2 Materials and methods

5.2.1 Cultures and growth conditions

Groundwater enrichments HiTCEB12 and HiTCE were used in this study, that were constructed and maintained as described in 3.2.2. Basically, HiTCEB12 was grown in 100 mL basal medium amended with a total of 5.3 mM lactate as electron donor and 220 µmol TCE as electron acceptor, 100 µg/L of vitamin B_{12} was added as co-factor; while HiTCE was grown under the same condition as HiTCE except that no exogenous vitamin B_{12} was amended. Both enrichments were able to degrade TCE to VC and ethene within 13 days, and exhibited similar cell growth of Dhc, as shown in Figure 3.1.

5.2.2 Cell collection and RNA extraction

According to the dechlorination performance of HiTCEB12 and HiTCE (Figure 3.1 C&D), in order to compare gene expression between HiTCEB12 and HiTCE, cell pellets of each enrichment were collected on day 2, when TCE dechlorination was beginning, cell growth was occurring (data not shown) and cobalamin in HiTCE started to be produced (Figure 4.3C). In order to investigate the differential gene expression during different growth phases in HiTCE, three time points were selected as day 2 (T1), day 9.5 (T2), and day 13 (T3) (see Figure 3.1 C&D for reference). T1 counts as early exponential phase, T2 as late exponential phase when 3rd dose of TCE was degraded, and T3 as stationary phase. Cells were collected by centrifugation at 15,000 × g for 10 min at

4 ºC. Supernatant was discarded, and cell pellets were stored at -80 ºC prior to RNA extraction.

RNA was extracted and purified using the acidic phenol (pH=4.3) chloroform method. The cell pellets were resuspended 1.0 ml pH 4.3 buffer-equilibrated phenol (Sigma-Aldrich, St. Louis, MO), and transferred in to a 2 mL Nuclease-free microcentrifuge tube amended with 1 g 100-µm-diameter zirconia-silica beads (Biospec Products, Bartlesville, OK). In the tube, 250 µl lysis buffer (50mM sodium acetate, 10mM EDTA; pH 5.1) and 100 µl 10% sodium dodecyl sulfate were added. The cells were lysed by heating them to 65°C for 2 min, bead beating them with a Mini Bead Beater (Biospec Products) for 2min, incubating them for 8 min at 65°C, and bead beating them for an additional 2 min. Cellular debris was collected by centrifugation (14,000 × g for 5 min at 4°C), and the aqueous lysate was transferred to a new, 1.5 mL Nuclease-free microcentrifuge tube. The aqueous lysate was extracted two times with 1 volume of phenol (pH 4.3)-chloroform-isoamylalcohol (25:24:1, v/v) (Sigma-Aldrich, St. Louis, MO), and one time with 1 volume of chloroform-isoamylalcohol (24:1, v/v). RNA was precipitated by adding 0.5 volume of 7.5 M ammonium acetate and 2 volume of 100% ethanol and incubating for at least 2 hours. The precipitate was collected by centrifugation (21,000 × g for 30 min at 4°C), washed once with 80% cold ethanol, collected by centrifugation again. Pellets were vacuum-dried, re-suspended in 30 µL nuclease-free water, quantified by Nanophotometer P300 (Implen, Inc., Westlake Village, CA), stored at -80 ºC prior to transcriptomic microarray analysis.

5.2.3 Microarray protocol

The genus-wide microarray targeting four Dhc genomes (i.e. strain 195, CBDB1, BAV1 and VS) was designed and applied as previously described in Lee *et al*. (Lee *et al.*, 2011). The microarray was processed according to the instructions given in section 3 of the Affymetrix GeneChip expression analysis technical manual (www.affymetrix.com) (Affymetrix, Santa Clara, CA). Basically, cDNA was synthesized from ~ 10 µg total RNA, then fragmented, labeled, and hybridized to each array. The hybridized arrays were stained, washed and then scanned with an Affymetrix Scan 3000 scanner. Biological triplicate analyses were performed for each culture under each condition.

5.2.4 Microarray data analysis

In this study, transcription of Dhc genes to RNA were analyzed, and we use the term "gene expression" to refer to the hybridizating of cDNA generated by reverse transcription of transcripts indicated by the corresponding signal intensity.

The microarray data were 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 arrays.

For comparison between HiTCEB12 and HiTCE on day 2, the normalized signal intensities were analyzed using t-test with *p* value below 0.05. For comparison among

multiple time points of HiTCE enrichment, the normalized microarray data was performed using the R statistical program (www.r-project.org) (R 2005) with packages available from Bioconductor version 1.9 (www.bioconductor.org) (Gentleman *et al.*, 2004) as described by Johnson *et al*. (Johnson *et al.*, 2008; Johnson *et al.*, 2009). The Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995) was applied to control the false discovery rate (FDR) below 0.05.   In addition, only genes with absolute hybridization signal intensities greater than 550 (this is higher than usual to eliminate the false positive signals) for at least one condition and with more than two-fold changes between two conditions could be considered to be differentially expressed. Differentially expressed genes at multiple time points in HiTCE were sorted by hierarchical clustering using the "heatplot" function from the "made4" package, generating the dendrogram of those genes.

5.3 Results

5.3.1 Differential gene expression between enrichments with and without exogenous vitamin B_{12}

The difference in gene expression between HiTCEB12 and HiTCE during early exponential phase (day 2) was compared using t-test with *p* value below 0.05. A total of 104 Dhc genes were differentially expressed, 102 of which were significantly up-regulated in HiTCE compared with HiTCEB12. The up-regulated genes in HiTCE were dominated by those encoding proteins involved in metabolism-related functions such as energy production and conversion, amino acid transport and metabolism, as well as inorganic ion transport and metabolism (Figure 5.1 A).

The $\log_2$ ratios of signal intensities of key functional genes in the two cultures are shown in Figure 5.2. Out of the five present RDase genes in both HiTCEB12 and HiTCE (see 3.3.2), only *tceA* (DET0079) was expressed in both enrichments, and no significant regulation was observed (Figure 5.2A). Genes encoding four out of five hydrogenases (Hym, Ech, Hup, Vhu, except Hyc), and three other oxidoreductases (Mod, Fdh, and Nuo) were actively expressed. Nuo oxidoreductase genes (DET0925-926, DET0933) and Hym hydrogenase genes (DET0145, DET0147-148) were significantly up-regulated in HiTCE compared with HiTCEB12. Three Genes (DET0860, DET0862 and DET0865) in the Ech operon also exhibited > 2-fold up-regulation in HiTCE, whereas the corresponding transcripts in HiTCEB12 were not detected (Figure 5.2B).

Two genes (DET0650 and DET1176) encoding corrinoid transporters were significantly up-regulated in HiTCE compared to HiTCEB12. The homologous genes (DET0657/0691, *cobT*) involved in lower ligand modification and attachment were also significantly up-regulated in HiTCE (Figure 5.2C). However, transcripts of two genes encoding putative cobalamin riboswitches (DET0125-126) (Johnson *et al.*, 2009) were not detected in neither of the two enrichments.

(A)

102 genes total

(B)

606 genes total
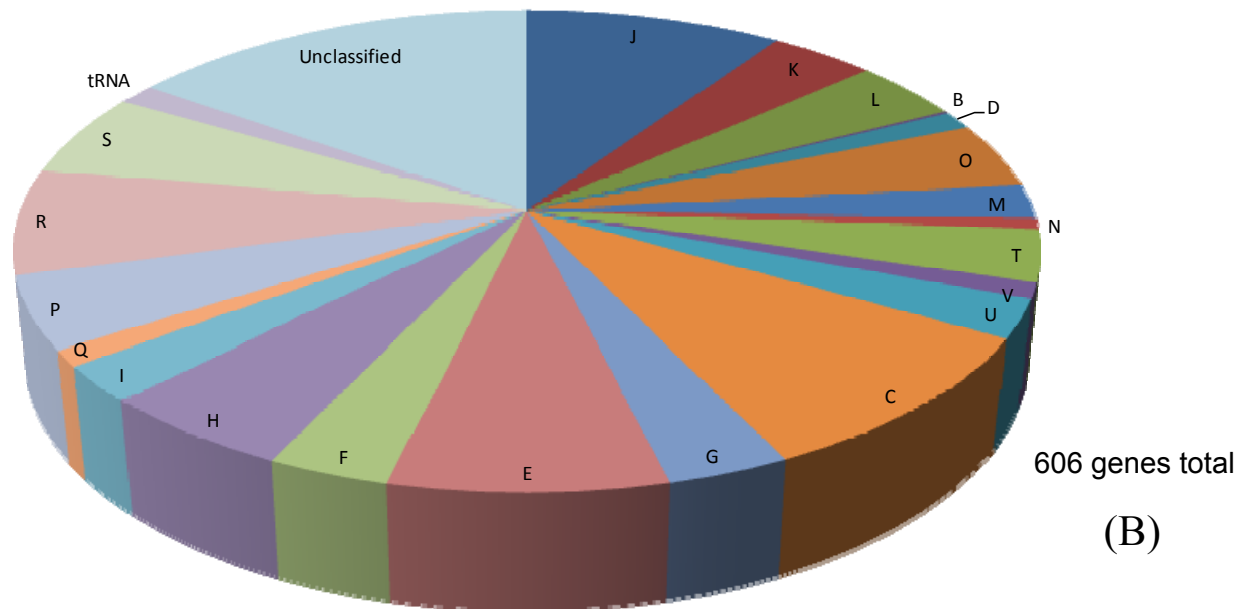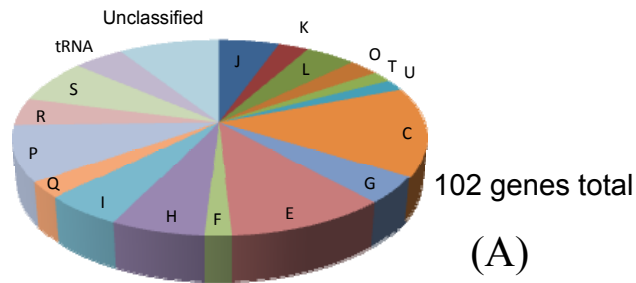
Legend:

Information storage and processing		Cellular processes and signaling	
J	Translation, ribosomal structure and biogenesis	D	Cell cycle control, cell division, chromosome partitioning
A	RNA processing and modification	Y	Nuclear structure
K	Transcription	V	Defense mechanisms
L	Replication, recombination and repair	T	Signal transduction mechanisms
B	Chromatin structure and dynamics	M	Cell wall/membrane/envelope biogenesis
Metabolism		N	Cell motility
C	Energy production and conversion	Z	Cytoskeleton
G	Carbohydrate transport and metabolism	W	Extracellular structures
E	Amino acid transport and metabolism	U	Intracellular trafficking, secretion, and Vesicular transport
F	Nucleotide transport and metabolism	O	Posttranslational modification, protein turnover, chaperones
H	Coenzyme transport and metabolism	**Poorly characterized**	
I	Lipid transport and metabolism	R	General function prediction only
P	Inorganic ion transport and metabolism	S	Function unknown
Q	Secondary metabolites biosynthesis, transport and catabolism		

Figure 5.1 Clusters of orthologous groups (COGs) distributions of up-regulated genes in HiTCE compared with HiTCEB12 (A) and differentially expressed genes under different growth phases in HiTCE (B).
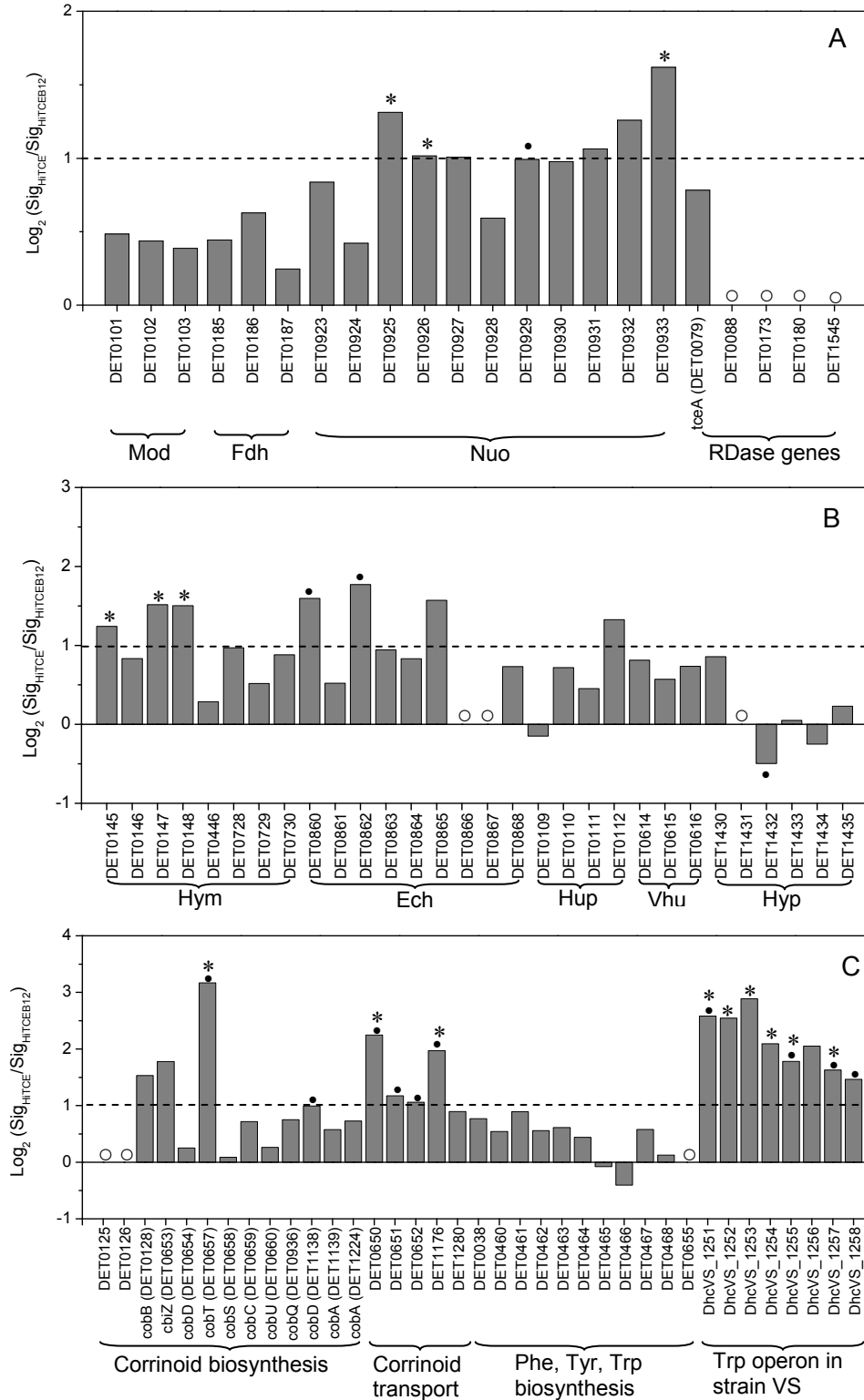
Figure 5.2 Log₂ signal intensity ratio between transcripts of Dhc in HiTCE and HiTCEB12. All measurements are averages from three biological replicates. Dashed lines indicate the 2-fold difference in signal intensities. "○" indicates no transcripts detected in both cultures (signal intensity < 550). "•" indicates transcripts detected in one of the two cultures. "*" indicates significant difference based on t-test $p<0.05$.

Interestingly, almost the entire tryptophan operon, which is more similar to that of strain VS (DhcVS_1251-1258) than strain 195, was significantly up-regulated in HiTCE compared with HiTCEB12 (Figure 5.2C). However, other genes involved in aromatic amino acids biosynthesis were not significantly regulated.

Other significantly up-regulated genes in HiTCE include genes encoding ATP synthase (DET0557-565), and degV family protein (DET1264-1267), the function of which has not yet been characterized.

5.3.2 Differential gene expression during different growth phases in enrichment without exogenous vitamin B_{12}

Besides the investigation of differential gene expression between Dhc genomes in enrichments with and without exogenous cobalamin (i.e. HiTCEB12 and HiTCE), we also investigated the differentially expressed Dhc genes in HiTCE, the enrichment without exogenous cobalamin, over a time-course. According to physiological measurements of HiTCE (see Figure 3.1D and Figure 4.3C), three time points of interest were selected: T1 (day 2) is considered early exponential phase, when dechlorination and cell growth have recently started and hydrogen and corrinoids have been highly produced; T2 (day 9) represents the late exponential phase, when the dechlorination of the 3rd dose of TCE was finishing and all related processes have been well-established; and T3 (day 13) as stationary phase when TCE is depleted and VC dechlorinated to ethene.

A total of 606 out of 1302 Dhc genes in HiTCE were differentially expressed. Among those assigned to cluster of orthologous groups (COGs), the top three differentially expressed genes were those with predicted functions of energy production, translation, ribosomal structure and biogenesis, as well as amino acid transport and metabolism (Figure 5.1B). Other regulated genes include those with predicted functions of coenzyme transport and metabolism, transcription, replication, recombination and repair, as well as inorganic ion transport and metabolism.

The hierarchical clustering and dendrogram of those differentially expressed genes exhibit five clusters (Figure 5.3). Cluster I and II were closely related. Cluster I includes genes with the highest expression at T2 and low expression at both T1 and T3, while Cluster II includes genes more highly expressed at both T1 and T2 than T3. Similarly, cluster IV and V were closely related with Cluster IV containing genes more highly expressed at both T2 and T3 than T1, while Cluster V contains genes with the highest expression at T3. Genes in cluster III were most highly expressed at T1, and then were down-regulated.

The COG distributions of genes within each cluster are shown in Figure 5.4. About 3/4 differentially expressed genes belong to cluster I and II. Moreover, genes with well-characterized functions in cluster I and cluster II exhibited similar and relatively even distributions, except that cluster I contains more translation and biogenesis related genes. The overall functional gene up-regulation could be explained by the higher dechlorination and cell growth activities at T2.

75
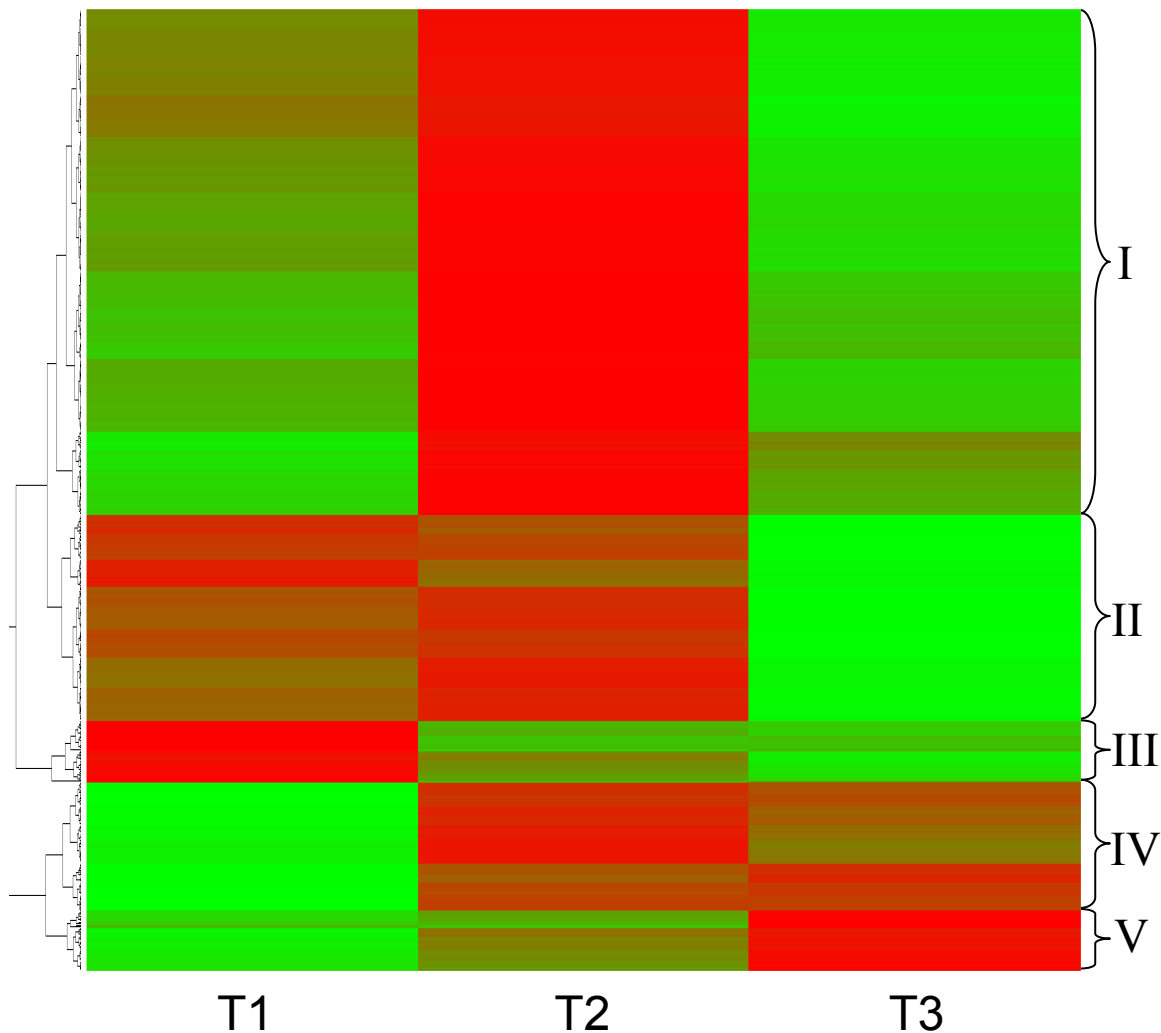
Figure 5.3 Hierarchical clustering and dendrogram of differentially expressed genes (false discovery rate, <0.05; signal intensity > 550, > 2-fold difference). The color gradient from green to red represents increasing microarray hybridization intensity.

Cluster IV contains higher proportions of genes involved in posttranslational modification, protein turnover, chaperones, coenzyme transport and metabolism. Onc corrinoid transpter gene (DET0650), one corrinoid biosynthesis gene *cobD* (DET0654), two genes encoding hydrogenase-related proteins (*hypCD*, DET1433-1434), as well as two genes encoding radical SAM domain proteins (DET1314 in strain 195 and DhcVS_1417 in strain VS).

Genes encoding transcriptional regulators and inorganic ion transporters were more abundant in Cluster V, including one transcriptional regulator gene (Dhc_VS_1415) and two Fe^{2+} transporter genes (DhcVS_1277-78) in strain VS, as well as *hypA* (DET1430) and a heat shock gene (DET1401) in strain 195. These genes were up-regulated in T2 to T3, indicating that they were playing important roles during the transition from exponential to stationary phases.

Most notable are the genes more highly expressed at T1 compared with T2 and T3 indicated by cluster III. Most genes in cluster III have predicted functions in energy production, including those encoding Hup, Hym hydrogenases (DET0110-112, DET0145-148), three other oxidoreductase, Mod (DET0101-102), Fdh (DET0186-187), and Nuo (DET0925-927, 930-933), as well as ATP synthase genes (DET0559-565). One gene with distinct expression dynamics in this cluster is another oxidoreductase encoding gene (DET0736), which was down-regulated from T1 to T2 and up-regulated again from T2 to T3. This gene is predicted to encode a dehydrogenase with specificity to short-chain alcohol.
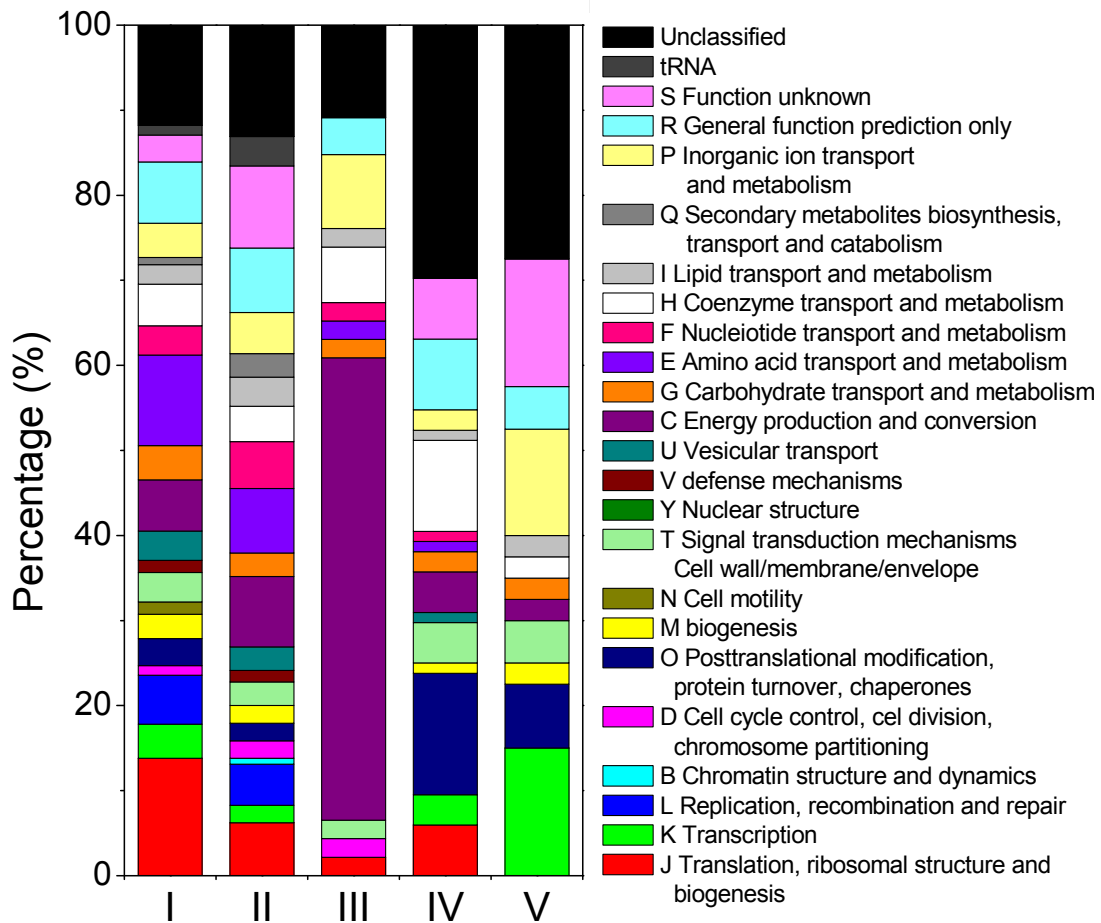
Figure 5.4 COG distributions of the differentially expressed genes in each hierarchical cluster as indicated in Figure 5.3.

The time-course expression levels of four hydrogenase operons (Hup, Hym, Vhu, and Ech) in the strain 195 genome, as well as Mod, Fdh and Nuo operons are shown in Figure 5.5A & B. Transcripts of another hydrogenase operon (Hyc) were not detected. The Hup operon was most highly expressed. Besides those differentially expressed hydrogenase and oxidoreductase genes, other genes within the same operon also exhibited the same

expression dynamics as shown in cluster III. Differently, the expression levels of Vhu operon (DET0614-616) and another Hym operon (DET0728-730) remained similar from T1 to T2, and then down-regulated from T2 to T3. Genes in the hydrogenase maturation related Hyp operon (DET1430-1435), except DET1431, were more highly expressed at T2 and T3 (Figure 5.5B).

According to previous gDNA microarray results (see 3.3.2), the HiTCE enrichment contains five RDase genes (DET0079, DET0088, DET0173, DET0180, and DET1545) in its Dhc genome. Only transcripts of *tceA* (DET0079) were detected at very high levels, while those of the other four genes were not detected (signal intensity < 550) (Figure 5.5A). In addition, no RDases of non-Dhc bacteria were detected in either the gDNA or expression analysis. Therefore, *tceA* was the only identified functioning RDase gene throughout the entire feeding cycle. Although the signal intensities of the transcript of *tceA* increased from T1 to T2 and decreased from T2 to T3, no statistically differential expression was found among the three growth phases.

Among the corrinoid transport and biosynthesis genes, DET0650 was most highly expressed, which encodes a corrinoid transporter. While other corrinoid-related genes were stably expressed at relatively low levels. Transcripts of DET0125 and DET0126, which are predicted to encode putative cobalamin riboswitches, were not positively detected in any of the three time points, indicating they were not functioning in HiTCE. Genes involved in aromatic amino acid biosynthesis in the strain 195 genome exhibited up-regulation from T1 to T2 and down-regulation from T2 to T3, whereas the VS tryptophan operon was stably expressed at relative low levels during the entire time-course (Figure 5.5C).

5.4 Discussion

Differential gene expression between HiTCEB12 and HiTCE

The two non-methanogenic groundwater enrichments, HiTCEB12 and HiTCE exhibited similar physiological characteristics, in terms of dechlorination and cell growth, although 100 µg/L exogenous cobalamin was amended to HiTCEB12, while not to HiTCE (See 3.3.1). Global gene expression of the Dhc genome in the two enrichments was compared at early exponential phase. At this time point, dechlorination and cobalamin utilization had just started. Thus, it should reflect the different responses of Dhc in HiTCEB12 with cobalamin ready to use with those in HiTCE where it has to be scavenged from other sources. Results indicate that almost all differentially expressed genes when grown without exogenous cobalamin were up-regulated genes with functions in metabolism, such as energy production, nutrient transport and amino acid biosynthesis, which indicates an increased need of energy and nutrients when cobalamin stress was imposed.
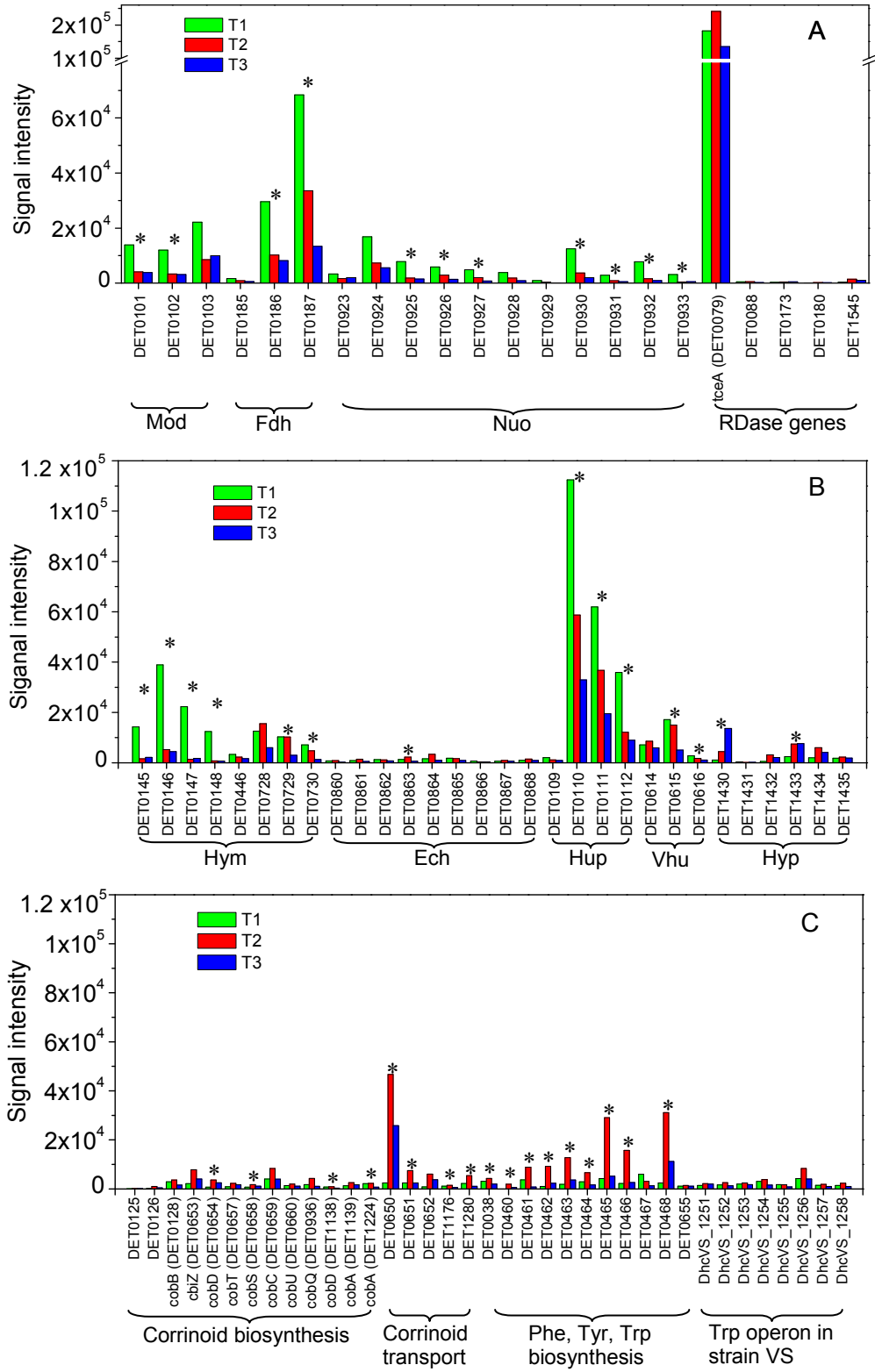
Figure 5.5 Signal intensities of selected genes in key functional groups at time points T1, T2 and T3. "*" indicates genes that were differentially expressed according to statistical criteria. Note: Scale of y axis in (A) is different from those in (B) and (C).

In terms of the energy-related genes, the functional RDase gene *tceA* was actively expressed in the two enrichments without significant difference, which is consistent with the observed similarity in dechlorination activities. However, some other genes encoding oxidoreductases along the respiration chain were up-regulated, including genes in the Nuo operon (DET0925-926, DET0933) and Hym operon (DET0145-148), as well as ATPase genes (DET0561-565). Nuo is a membrane-bound NADH-ubiquinone oxidoreductase and Hym is a membrane-bound hydrogenase, both of which play roles in the electron transport network of Dhc species (Seshadri *et al.*, 2005). The up-regulation of these genes together with ATPase genes suggests additional stress on energy conservation under the cobalamin-limited condition during the early dechlorination period. More energy was probably required for corrinoid salvaging activities.

Another noteworthy response under cobalamin-limited condition is the up-regulation of genes encoding a lower ligand activation protein (CobT), and two corrinoid transporters. CobT catalyzes the formation of alpha-5,6-dimethylbenzimidazole adenine dinucleotide (α-DAD) using NAD+ and the lower ligand 5, 6-dimethylbenzimidazole (DMB) as substrates in cobalamin biosynthesis (Maggio-Hall and Escalante-Semerena, 2003). It is required in exogenous DMB utilization, and a *cobT* mutant, corrinoid-dependent strain of *Salmonella enterica* serova Typhimurium LT2 was not able to synthesize cobalamin even when the precursor cobinamide (cbi) and DMB were provided (Anderson *et al.*, 2008). The up-regulation of *cobT* gene in HiTCE suggests an activity of DMB attachment by Dhc, while the result that *cobT* was not actively expressed in HiTCEB12 indicates it is not essential when exogenous cobalamin was provided. In addition, transcripts of *cbiZ* (DET0653), which encodes the enzyme catalyzing the removal of lower ligand and nucleotide loop from adeninylcobamide by *Rhodobacter sphaeroides* (Gray and Escalante-Semerena, 2009a), exhibited > 2-fold increase in HiTCE compared with HiTCEB12, indicating increased corrinoid remodeling and modification activities. The concomitant up-regulation of corrinoid transporter genes also indicates the increasing need of other corrinoids from other community members to satisfy the requirement by RDases when exogenous cobalamin was not provided. Further, the transport efficiency of other corrinoids might be lower than that of cobalamin. The two putative cobalamin riboswitches (DET0125-126) up-regulated in strain 195 grown in isolation under cobalamin-limited conditions were not actively expressed in neither of the two enrichments at early exponential phase in this study, which indicates that although exogenous cobalamin was not provided in HiTCE, corrinoids were still made available sufficiently to Dhc species by other community members.

Interestingly, the entire tryptophan operon, which closely resembles the tryptophan operon in strain VS, was up-regulated in HiTCE. Tryptophan (Trp) is a very hydrophobic amino acid, which shows significantly higher content in membrane-associated proteins than soluble proteins (Vonheijne, 1986; Schiffer *et al.*, 1992). Trp residues play important roles in the translocation of the protein through the membrane, and serve as anchors on the periplasmic side of the membrane (Schiffer *et al.*, 1992). The increased requirement for tryptophan under cobalamin-limited conditions is likely related to the up-regulation of genes encoding membrane-associated proteins, such as respiratory proteins and ABC transporters, in which tryptophan might be structurally essential.

Differential gene expression at different growth phases in HiTCE

Besides comparative transcriptomic analyses between HiTCEB12 and HiTCE, the differential gene expression over a time-course in HiTCE could also give us information associated with dechlorination activities under cobalamin-limited growth condition. First of all, *tceA* was the only known RDase gene highly expressed over the entire cultivation, while the other four RDase genes (DET0088, DET0173, DET0180 and DET1545) detected in genomic analysis were not actively expressed (Figure 5.5A), nor were they detected in HiTCEB12 at early exponential phase (Figure 5.2A). This is different than previous studies, in which DET1545 was highly expressed in strain 195 grown in isolation (Johnson *et al.*, 2008) and other Dhc-containing mixed cultures (Waller *et al.*, 2005; Rahm *et al.*, 2006b; West *et al.*, in review). The substrates and specific functions of DET1545 still remain elusive. Therefore, the two enrichments in this study have a simple RDase network employing a single functional cobalamin-dependent RDase (TceA) to carry out the TCE dechlorination.

Similar to other enrichment studies (Rahm *et al.*, 2006b; West *et al.*, in review), the actively expressed oxidoreductase genes in HiTCE include those encoding Hup, Hym, Vhu hydrogenases, formate dehydrogenase (Fdh) and molybdopterin oxidoreductase (Mod), while genes encoding Ech exhibited rather low transcript levels and the Hyc operon was not actively expressed at all. The Hup operon was most highly expressed among the four expressed hydrogenase operons, which is consistent with the pure culture studies (Morris *et al.*, 2006; Johnson *et al.*, 2008) and the enrichment study by Rahm *et al.*(Rahm *et al.*, 2006b), in which *tceA* and *pceA* were the major functional RDase genes. However, the study of dechlorinating enrichment ANAS exhibited higher expression in Hym and Vhu operons than the Hup operon. ANAS has *tceA* and *vcrA* as the major functional RDase genes, and *vcrA* was more highly expressed than *tceA*. These results indicate that the expression of the Hup operon may be more likely related to the expression of *tceA* and *pceA*, which are specific to PCE/TCE dechlorination to VC. In addition, Hup, Hym, Mod, Fdh and Nuo operons were mostly expressed during the early exponential phase, which is consistent with results from other studies (Rahm *et al.*, 2006b; Johnson *et al.*, 2008; West *et al.*, in review). The higher expression of these oxidoreductase genes correlates with the increasing hydrogen concentration during early exponential phase. Hydrogen serves as the electron donor for Dhc in reductive dechlorination, thus hydrogenases play an essential role in withdrawing electrons from hydrogen and donating it to other electron carriers along the electron transport chain. Therefore, it is reasonable that the hydrogenase genes were up-regulated to initiate the dechlorination at an early stage.

Last and notably, one corrinoid transporter encoding gene (DET0650) was most highly expressed among corrinoid-related genes. Similarly, it is also found to be more highly expressed than other cytoplasmic corrinoid biosynthesis genes in ANAS, which is maintained with low amounts of exogenous cobalamin (West *et al.*, in review), while not in the strain 195 isolate where sufficient exogenous cobalamin was amended (Johnson *et al.*, 2008). Moreover, DET0650 was up-regulated more than 2-fold in HiTCE compared

with HiTCEB12. DET0650 has recently been re-annotated as *btuF*, which encodes the periplasmic corrinoid-binding protein in an ATP-binding cassette (ABC) transport system (*btuFCD*) (Yi *et al.*, accepted) (http://img.jgi.doe.gov). BtuF was identified as an essential periplasmic cobalamin-binding protein with high affinity for cobalamin in other corrinoid-dependent Bacterial and Archaeal species (Cadieux *et al.*, 2002; Woodson *et al.*, 2005; Boiani *et al.*, 2009). However, the affinity of BtuF for other corrinoid forms remains unknown. The up-regulation and higher expression level of DET0650 in HiTCE suggests lower affinity of BtuF encoded by DET0650 for p-cresolylcobamide, which was confirmed to be produced by supportive microorganisms in the community rather than cobalamin (see 4.3).

In summary, this study investigated the transcription level responses of Dhc strains in mixed cultures enriched with and without exogenous cobalamin. The *cobT* gene (DET0657), corrinoid transporter genes, such as DET0650, tryptophan operon in strain VS, as well as Hym and Nuo operon were up-regulated under cobalamin-limited, corrinoid-salvaging conditions, thus may serve as potential biomarkers indicative of cobalamin stress and indigenous corrinoid utilization within a community. Further temporal gene expression analysis of the HiTCE enrichment emphasizes the important role played by the corrinoid transporter gene (DET0650) in corrinoid import. Expression levels of RDase genes reveal a distinct dechlorination system carried out by a single identified RDase (TceA), which was closely related to the expression of Hup hydrogenase gene and is associated with the dechlorination of TCE to VC and ethene. These findings could provide us useful guidance in monitoring and diagnosing dechlorination performance in chlorinated ethene bioremediation applications.

5.5 Acknowledgements

6 [*] Syntrophic Growth of *Dehalococcoides mccartyi* strain 195 with previously identified supportive microorganisms in defined consortia: Global Transcriptomic and Proteomic Analyses

6.1 Introduction

Dehalococcoides mccartyi (Dhc) are, thus far, the only known bacteria capable of completely dechlorinating tetrachloroethene (PCE) and trichloroethene (TCE) to the benign product ethene (Freedman and Gossett, 1989; Maymó-Gatell *et al.*, 1997; Holliger *et al.*, 1998b; Cupples *et al.*, 2003; He *et al.*, 2003b; Smidt and de Vos, 2004). They exhibit low growth rates, specific obligate nutrient requirements (hydrogen as electron donor, acetate as carbon source, cobalamin as co-factor), and non-robust physiology when grown in isolation, resulting in limited dechlorination activity and sub-culturing reproducibility (Distefano *et al.*, 1992; Maymó-Gatell *et al.*, 1997; He *et al.*, 2003a; He *et al.*, 2003b). Consequently, developing methods to improve the robust growth and dechlorination activity of Dhc would be useful for developing improved bioremediation strategies.

Dhc are commonly found in microbial communities that contain other anaerobes, such as *Desulfovibrio*, *Eubacterium*, *Acetobacterium, Citrobacter, Spirochetes*, and *Clostridium* (Richardson *et al.*, 2002; Ritalahti and Löffler, 2004; Duhamel and Edwards, 2006; Lee *et al.*, 2006), which are able to ferment organic substrates into hydrogen and acetate. Studies of a previously described dechlorinating microbial community ANAS, showed that it was dominated by three bacterial groups: Dhc, *Desulfovibrio*, and *Clostridium*, with the hydrogenotrophic methanogen *Methanobacterium congolense* (MC) as the dominant archaeal species (Richardson *et al.*, 2002). Studies in Chapter 3 also observed the presence of *Desulfovibrio* spp. in dechlorinating enrichments. Previous studies have shown that *Desulfovibrio vulgaris* Hildenborough (DVH) can transform lactate to acetate and H_2 when grown syntrophically with a H_2-utilizing methanogen (McInerney and Bryant, 1981; Schink, 1997). It is possible that DVH might also play a role in syntrophic interactions with other hydrogenotrophic microorganisms like Dhc.

Corrinoids serve as an essential co-factor of reductive dehalogenases (RDases), which are not able to biosynthesized *de novo* by sequenced Dhc strains (Yi *et al.*, accepted). However, pure culture studies have shown that strain 195 were able to remodel other corrinoid forms into cobalamin in the presence of the lower ligand 5, 6-dimethylbenzimidazole (DMB) (Yi *et al.*, accepted). Similarly, studies in previous chapters of this dissertation have demonstrated that the cobalamin detected in the enrichment without exogenous cobalamin was formed by Dhc using *p*-cresolylcobamide ([p-Cre]Cba), which might be produced by *Pelosinus* spp., and DMB produced by other unknown organisms within the community. This makes *Pelosinus* spp., which were also dominantly detected in lactate amendment metal-reducing enrichments (Moe *et al.*, 2011; Mosher *et al.*, 2012), important in corrinoid transfer in dechlorinating communities.

Methanogens are commonly found to be growing concomitantly with Dhc within chloroethene degrading communities, as shown in Chapter 3. It has been hypothesized that methanogens play an important role in the anaerobic dechlorination by cometabolic processes (Vogel and McCarty, 1985; Fathepure and Boyd, 1988; Gantzer and Wackett, 1991). In addition, methanogens were found to compete for hydrogen with dehalogenators (Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Yang and McCarty, 1998).

Although there have been a number of studies evaluating the link between methanogens and Dhc (Fathepure and Boyd, 1988; Freedman and Gossett, 1989; Smatlak *et al.*, 1996; Fennell *et al.*, 1997; Löffler *et al.*, 1997; Yang and McCarty, 1998; Booker and Pavlostathis, 2000; Heimann *et al.*, 2006), the role of methanogens in dechlorinating communities remains unclear.

We have previously generated defined consortia containing *D. mccartyi* strain 195 (DE195) and *Desulfovibrio desulfuricans*, using lactate as the electron donor (He *et al.*, 2007). In that single-transfer experimental co-culture, PCE was dechlorinated to vinyl chloride (VC) and ethene with concomitant growth of DE195, whose density was 1.5 times greater than when grown in isolation (He *et al.*, 2007).

In this study we constructed two defined DE195-containing consortia capable of sustained robust growth (> 15 subcultures) with lactate as electron donor and carbon source. These two consortia, a co-culture containing DE195 and DVH, and a tri-culture containing DE195, DVH and MC, dechlorinate TCE to VC and ethene consistently and reproducibly. Physiological characteristics were quantified and transcriptomic and proteomic analyses were applied to evaluate the effects of long-term syntrophic growth on this Dhc strain. The dechlorination activities of DE195/DVH co-culture, DE195/DVH/MC tri-culture, and the tri-culture containing DE195/DVH and *Pelosinus fermentans* strain R7 in the absence of exogenous cobalamin were also examined.

6.2 Materials and methods

6.2.1 Bacterial cultures and growth conditions

DE195 was grown in 160-mL serum bottles containing 100 mL basal medium as previously described (He *et al.*, 2007), with $H_2:CO_2$ (20:80 v/v) headspace, 5 mM acetate, 78 μmol TCE, and 100 μg/L cobalamin (vitamin B_{12}). DVH was grown in the same basal medium as DE195 with $N_2:CO_2$ (90:10 v/v) headspace and 5 mM Na_2SO_4. *Pelosinus fermentans* strain R7 (PF) was obtained from ATCC (ATCC No. BAA-1133) and reactivated using assigned liquid medium without the addition of cobalamin. After four sub-culturing events, PF was maintained in the same basal medium as the DE195/DVH co-culture amended with 38 mM lactate, but without cobalamin.

Co-culture containing DE195 and DVH (DE195/DVH) were grown in the same medium with 5 mM lactate and $N_2:CO_2$ (90:10 v/v) headspace. The ratio of DE195 to DVH cells in the initial syntrophic co-culture was 10:1. Three parallel bottles were established as biological triplicates for each subculture. Subcultures were established with transfers of the co-culture (10% v/v) from one of the three bottles into three newly prepared bottles containing 90 mL fresh medium every 10 days for 20 subcultures (~ 66 generations) prior to the described experiment. Another co-culture containing DVH and MC (DVH/MC) was prepared by inoculating 10 mL of each into 80 mL lactate medium. After 3 subcultures (10% v/v), the defined tri-culture (DE195/DVH/MC) was constructed by adding 10 mL of DVH/MC and 10 mL of DE195/DVH to 80 mL lactate medium in triplicate bottles. The tri-culture was maintained in the same medium as the co-culture, with the exception that TCE was added in ~40 μmol doses in order to avoid MC

inhibition. A DE195/DVH co-culture with ~40 µmol TCE was also constructed as a control. Transfers of the co-culture (10% v/v) with low TCE dosage and the tri-culture (10% v/v) from one of the three bottles into three newly prepared bottles containing fresh medium were made every 10 days for 16 subcultures (~ 53 generations) prior to the described experiment.

In the experiments testing the availabilities of corrinoid supply by DVH and PF to DE195, cobalamin-free co-culture was constructed by inoculating 1% pre-grown normal co-culture into cobalamin-free basal medium with other ingredients the same. Cobalamin-free tri-culture was constructed by inoculating 1% pre-grown normal co-culture and 2% pre-grown PF into cobalamin-free basal medium the same as used for cobalamin-free co-culture. Dechlorination activities were examined in cobalamin-free co- and tri-cultures with and without the addition of 36 nM DMB.

6.2.2 DNA extraction and cell growth quantification

Cells from 1.5 mL culture sampled from each biological replicate were collected by centrifugation ($21,000 \times g$ for 10 min at 4°C), and genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Quantitative PCR (qPCR) using SYBR® Green-based detection reagent (Applied Biosystems, Foster City, CA) was applied to quantify 16S rRNA genes. Briefly, each 20-µL reaction mix contained 2.5 µL of gDNA sample or 10-fold serially diluted standard, $1 \times$ fast SYBR Green master mix, and 0.625 µM of forward and reverse primers (Table 6.1). gDNA of DE195, DVH and MC isolates were quantified using Nanodrop 3300 fluorometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's instructions and used as standards for qPCR. Cell density was determined using the equation given by Ritalahti *et al*. (Ritalahti *et al.*, 2006). According to the USDOE IMG website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), the genome sizes for DE195 and DVH are 1.5M bp and 3.8 M bp, respectively. Due to the lack of genomic information for MC, we assumed the genome size for MC to be 2M bp according to the average genome size of sequenced hydrogenotrophic methanogens.

6.2.3 Cell collection for transcriptomic and proteomic analysis

Cells were all collected during exponential phase when approximately 90% of TCE was dechlorinated (i.e. DE195: day 7; DE195/DVH fed by 78 µmol TCE: day 5; DE195/DVH fed by 40 µmol TCE and DE195/DVH/MC: day 3). To collect sufficient material for transcriptomic microarray analysis, 15 subcultures were inoculated and grown from the triplicate bottles of each culture (i.e. pure DE195, DE195/DVH, DE195/DVH/MC). Then, for each biological triplicate, cells from 5 bottles were collected by vacuum filtration (100 mL culture per filter). Each filter was placed in a 2 mL microcentrifuge tube, frozen with liquid nitrogen, and stored at −80˚C until processing. For proteomic analysis, a total of 12 bottles of DE195 and DE195/DVH were inoculated and grown, and cells were collected by centrifugation ($12,000 \times g$ for 5 min at 4° C) in a nitrogen-flushed 250-mL centrifuge bottle. Supernatant was discarded, and cell pellets were frozen at −80˚C and shipped overnight on dry ice to Oak Ridge National Laboratory, where they were pooled prior to dividing into three analytical replicates for further analysis.

6.2.4 RNA extraction

RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with the addition of a bead-beating step using 1 g 100-µm-diameter zirconia-silica beads (Biospec Products, Bartlesville, OK) after addition of the lysis buffer. Contaminating DNA in the RNA samples was removed by DNase I treatment using a DNA-free kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNA was quantified on the Nanodrop 3300 fluorometer using the Quant-iT RiboGreen RNA Assay kit (Invitrogen Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions.

6.2.5 Transcriptomic microarray

Microarrays targeting the genome of DE195 were designed and applied as previously described by West *et al*. (West *et al.*, 2008) and Johnson *et al*. (Johnson *et al.*, 2008). The microarray platform (GPL6336) was deposited to the NCBI Gene Expression Omnibus database (Johnson *et al.*, 2008). cDNA was synthesized from 10 µg RNA, then fragmented, labeled, and hybridized to each array. The hybridized arrays were stained, washed and then scanned with an Affymetrix Scan 3000 scanner. All procedures were performed according to the protocol outlined in Section 3 of the Affymetrix GeneChip Expression Analysis Technical Manual (www.affymetrix.com). Three replicate arrays were analyzed for each condition.

6.2.6 Microarray data analysis

The microarray analyses were performed using the R statistical program (www.r-project.org) (R 2005) with packages available from Bioconductor version 1.9 (www.bioconductor.org) (Gentleman *et al.*, 2004) as described by Johnson *et al*. (Johnson *et al.*, 2008; Johnson *et al.*, 2009). The Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995) was applied to control the false discovery rate (FDR) below 0.05. In addition, only genes with absolute hybridization signal intensities greater than 200 for at least one condition and with more than two-fold changes between two conditions could be considered to be significantly regulated and used for further analyses.

6.2.7 Validation of microarray data

Various transcripts that were observed to be significantly differentially transcribed from microarray results were quantified by RT-qPCR using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The same RNA samples were processed as used in the microarray analysis. Targeted genes and the appropriate primer sets are listed in Table 6.1.

6.2.8 Proteomic analysis

DE195 and DE195/DVH cell samples were analyzed via 2-dimensional nanoES 2d LC-MS/MS as described in (Verberkmoes *et al.*, 2009). Briefly proteomes were digested in peptides with sequencing grade trypsin (Promega, Madison WI) then separated via 2-

dimensional HPLC (SCX/RP) into a nanoelectrospray source connected to a Linear ion Trap Orbitrap with full scans at 30K in Orbitrap and five subsequent data dependent MS/MS scans in the linear ion trap. MS/MS spectra were searched with SEQUEST (Eng *et al.*, 1994) against a database created with both DE195 and DVH predicted protein sequences, common contaminants as well as common lab protein standards. Peptide identifications were filtered and sorted into proteins with DTASelect (Tabb *et al.*, 2002) as previously described (Verberkmoes *et al.*, 2009). Contrast (Tabb *et al.*, 2002) was used to display all proteins across runs, differentially expressed proteins were identified based on the following criteria (Chourey *et al.*, 2006): at least under one condition, > 40% sequence coverage, >5 unique peptides, and ≥ 2-fold difference in spectral counts identified between DE195/DVH and DE195 isolate. Data identification as well as the actual MS/MS spectra from every peptide and accessory scores are available at http://compbio.ornl.gov/Dehalo195_CoCulture/.

6.2.9 Cobalamin measurements

The same pre-treatment procedure and detection method as described in 4.3.3 and 4.3.4 were used to determine the forms and concentrations of corrinoids produced by PF and DVH pure cultures.

6.2.10 Analytical methods

Chloroethenes and ethene were analyzed using gas chromatograph with a flame ionization detector (GC-FID) as described by (Lee *et al.*, 2006). Hydrogen was analyzed by gas chromatography with a reductive gas detector (Trace Analytical, Menlo Park, CA). Organic acids were analyzed by high-performance liquid chromatography (HPLC) equipped with a UVD 170S UV detector as described previously (He *et al.*, 2007).

6.2.11 Accession number
The microarray data in this study (GSE26815) was deposited in the National Center of Biotechnology Information Gene Expression Omnibus database.

Table 6.1 Forward and reverse primers used for qPCR and RT-qPCR assays

Gene (Locus tag)	Predicted function	Primer Sequences (5'-3', forward and reverse)
DET0079	reductive dehalogenase tceA	GTGAACTGGGCTATGGCGAC TGGCGGCATATATTAGGGCA
DET0126	anthranilate phosphoribosyltransferase	TTACGCTATGAGTGTAATCGTTTCG CCGCCAGAAAGAATACGGAG
DET0173	reductive dehalogenase	CACTCACCCCATAGATGCCG TTGTGGCAGGTATGGCAGAA
DET0198	glutaredoxin family protein	CGGGTCTGAGAGGGATAAAGC GACGGGTTAAGGGCGCTAAG
DET0461	chorismate mutase prephenate dehydratase	AGTGGAAAAAGGAATGGCCC AGTGAGTTTTCCACCGGCAC
DET0462	chorismate synthase	TCCGCCAGAATATCAGCCAG TGGCTGTAAGGTCAGGGCAG
DET0651	ABC-type cobalamin Fe3+-siderophores transport systems	CTGGCGGCAATCTTTGCTAT AAAAAACGGCACTTCAACCG
DET0657	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	GGGAATGCTAAGCCCCAAAC GCCAGCCATGGTGATAATGG
DET0659	alpha-ribazole-5-phosphate phosphatase	CTGCGGTCAGGATAGATGCC CCATGCAGCGTTTAAGAGGG
DET0660	cobinamide kinase cobinamide phosphate guanylyltransferase	CTCACGGGAAAAATGCCG CCAGCAGGCTAATGCAGTCC
DET0876	reductive dehalogenase	TGGCCTTCTCAATGGATTCC CCATAGCCGAAAGCCTTGTC
DET0927	proton-translocating NADH-quinone oxidoreductase, H	CCAACAGAGCCGGTCCTTT TGATAGCATCTGCAATGGGC
DET0928	proton-translocating NADH-quinone oxidoreductase, I	AGGAGACCTGTATTGCCTGCC CCTTTACCGGACAGGCTCTG
DET0930	proton-translocating NADH-quinone oxidoreductase, K	TGCTGAACGCAGTTACCATTG CGGCAGAAAAACGGTTAAAGG
DET0931	proton-translocating NADH-quinone oxidoreductase, L	TTAGGTTCGGGCAGCGTAAA TTCGCGGATATCAAATGTGC
DET0955	bacterioferritin domain protein	TGGATATGACCGGCCAAACT GCCCTATATTGGCCTGCATC
DET0956	superoxide dismutase	GGTAAATCCGCCCCCTTATC TGAGAGAGCCTGGTAAAGTTTGC
DET1257	acetylglutamate kinase	GCTCTCTTCAGGCACAGCCT CATGAGCGGAGCTTGGGTAT
DET1488	tryptophan synthase, alpha subunit	AACTTGCCGGCTGATTTGAG GGCGGTTTTCCTTACCCTGT
DET1535	reductive dehalogenase	TCATTCCACACTCTCACGGC CCGACTACCCCAAGGCTTTT
DET1545	reductive dehalogenase	GCCATTCCTGCCAGAAGTGT TGGATATAACCTGAGGCGGG
DET16S	16S ribosomal RNA	CTTCGATCGGTAGCTGGTCTG TCTCAGTCCCAGTGTGGCTG
Dv16SC	16S ribosomal RNA of *Desulfovibrio vulgaris* Hildenborough	AATCGGAATCACTGGGCGTA CCCTGACTTACCAAGCAGCC
Methanobacterium[a]	16S ribosomal RNA	GGGTTGTGAGAGCAAGAGCC GCCTGGAACCTTGTCTCAGG
Lactobacillus[b]	16S ribosomal RNA	GCCACATTGGGACGTAGACA CTACTGCTGCCTCCCGTAGG
E. coli K4379	16S universal RNA	GTAGCGGTGAAATGCGTAGA CTACCAGGGTATCTAAT

[a] *Methanobacterium congolense* NCBI Accession No. AF233586
[b] *Lactobacillus delbrueckii* NCBI Accession No. EU886725

6.3 Results

6.3.1 Syntrophic growth of DE195 in co- and tri-cultures

TCE degradation was substantially faster in the sytrophic cultures versus the isolate. That is, while it took 20 days for DE195 to dechlorinate 78 μmol TCE to VC and ethene when grown alone (ca. 3.8 ± 0.1 μmol per day), it took only 7 days (ca. 11.0 ± 0.01 μmol per day) in the co-culture, the DE195/DVH/MC tri-culture took 4 days to dechlorinate 40 μmol TCE (ca. 10.1 ± 0.3 μmol per day), compared to 6 days (ca. 7.9 ± 0.5 μmol per day) in the co-culture control (Figure 6.1). The amount of ethene produced from VC in the co-culture was higher compared with the isolate, and similar compared with the tri-culture (Figure 6.1). By the 20th subculture of the co-culture, the syntrophic growth of DE195 was substantially more consistent and robust than growth in isolation. For example, while subculturing 10 vials of DE195 isolate resulted in only 6 successful cultures, all of the 10 vials inoculated with DE195/DVH subcultures grew successfully. Similar results were observed over multiple subsequent subcultures. The density of DE195 grown in the 20th subculture of the co-culture was approximately 2 times higher than when DE195 was grown alone (Figure 6.2A) (two-tailed student's t-test p=0.002). The density of DE195 grown in the 16th subculture of the tri-culture was lower than the co-culture control (Figure 6.2A) (two-tailed student's t-test p=0.04). The growth of DVH in the tri-culture was 5 fold greater than that in the co-culture control. The ratio of DE195 to DVH cells in co-culture remained approximately 5:1 (Figure 6.2B) over multiple subsequent subcultures, while the ratio of DE195 to DVH cells in the tri-culture remained around 1:1.5 (Figure 6.2B). Further, the ratio of DE195 to MC and DVH to MC remained at approximately 4:1 and 6:1, respectively (Figure 6.2B). The cell yield of DE195 in the co-culture was higher ($9.0 \pm 0.5 \times 10^7$ cells per μmol Cl⁻ released) than that of DE195 in pure culture ($6.8 \pm 0.9 \times 10^7$ cells per μmol Cl⁻ released) (two tailed student's t-test p=0.02), while the cell yield of DE195 in the tri-culture ($7.3 \pm 1.8 \times 10^7$ cells per μmol Cl⁻ released) had no significant difference from that of the co-culture control ($10 \pm 1.6 \times 10^7$ cells per μmol Cl⁻ released) (two tailed student's t-test p=0.12).

When DE195 was grown in isolation, 500 μmoles acetate was added to the basal medium, far exceeding the stoichiometric requirement of 0.03 μmoles for growing DE195 to 10^8 cells mL⁻¹ and assuming a cell composition of $C_5H_7O_2N$ (Rittmann and McCarty, 2001; Cupples *et al.*, 2003). In the co- and tri-cultures, lactate was provided as the electron donor and carbon source, with the expectation that DVH would ferment it into acetate and hydrogen with the stoichiometry given in Table 1, thus supporting the hydrogen and acetate requirements of DE195 and MC. However, the growth of DVH on lactate is only thermodynamically favorable when sufficient hydrogen is consumed by another strain (DE195 for dechlorination and/or MC for hydrogenotrophic methanogenesis (Table 6.1)), such that sustained survival of the constructed consortia requires syntrophic association among the species. The 500 μmoles lactate provided per bottle was partially consumed in the co-culture but was completely consumed in the tri-culture (Figure 6.3), with near stoichiometric acetate production in both. The co-culture also generated 140 μmoles (Figure 6.3A) hydrogen indicating that approximately 40% of the lactate consumed provided electrons for dechlorination. In the tri-culture, hydrogen was completely

consumed by day 6 and 250 μmoles methane were generated (Figure 6.3B), indicating that approximately 90% of the lactate electrons were consumed by methanogenesis while only 10% were used for dechlorination. Although MC consumed most of the generated hydrogen in the tri-culture, the aqueous hydrogen concentration never dropped below 5 nM, remaining above the hydrogen threshold (2 nM) for dechlorination by *Dehalococcoides* (Yang and McCarty, 1998). Consequently, competition for hydrogen between MC and DE195 was not observed to affect dechlorination in this study.
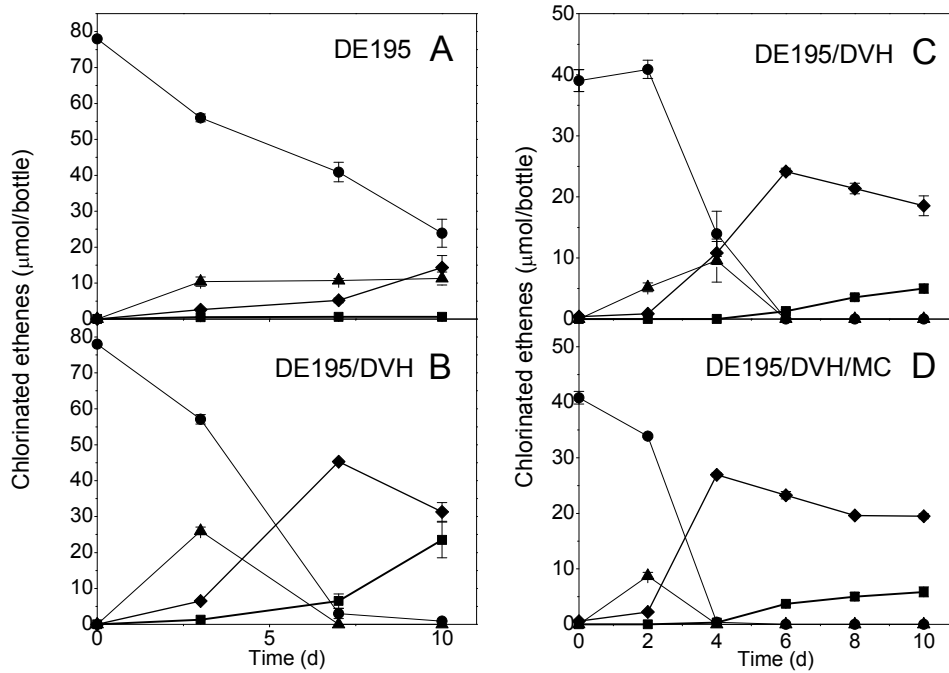
Figure 6.1 Temporal changes in the quantities of solvents for (A) DE195 fed ~78 μmol TCE, (B) DE195/DVH fed ~78 μmol TCE, (C) DE195/DVH fed ~40 μmol TCE, and (D) DE195/DVH/MC fed ~40 μmol TCE. All measurements are averages from three biological replicates and error bars are the standard deviation; (●) TCE, (▲) c-DCE, (♦) VC, (■) ethene.

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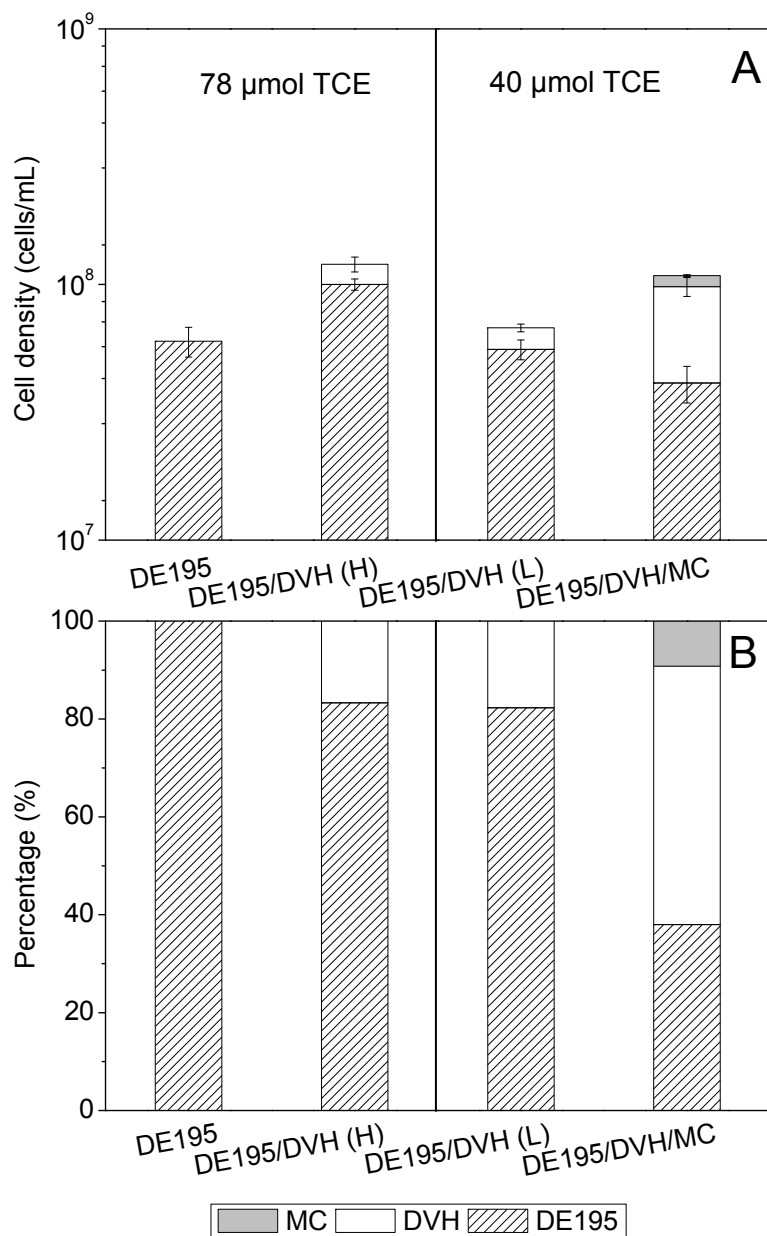
Figure 6.2 Cell density (A) and percent of each species in syntrophic cultures (B) for DE195 fed ~78 µmol TCE; DE195/DVH(H) fed ~78 µmol TCE; DE195/DVH(L) fed ~40 µmol TCE).
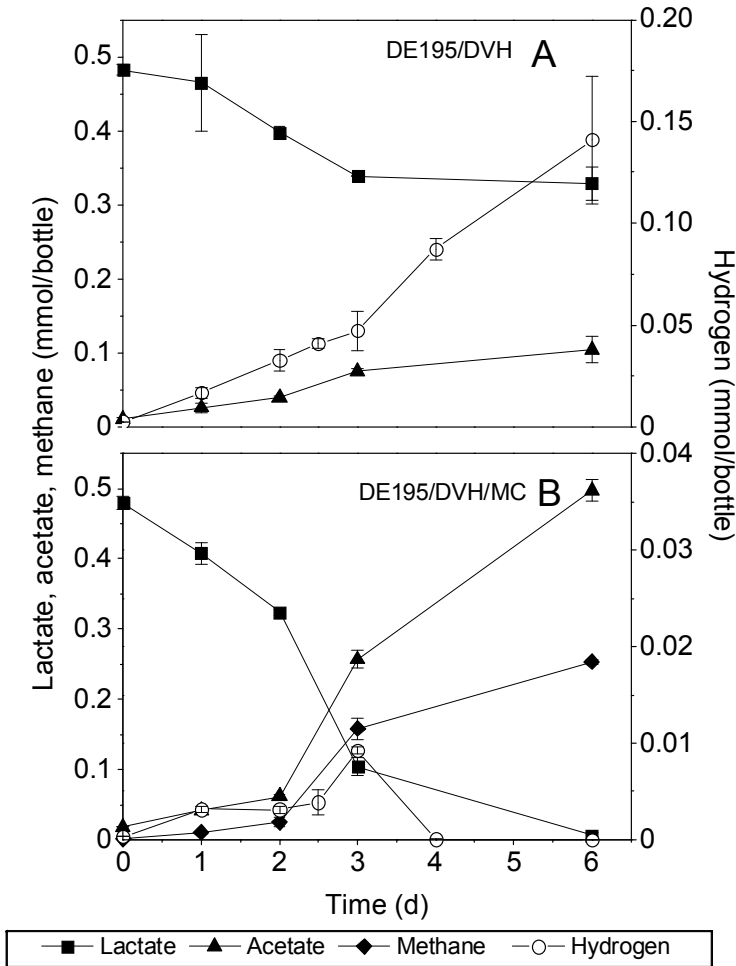
Figure 6.3 Consumption of lactate and production of acetate, hydrogen, and methane in (A) DE195/DVH and (B) DE195/DVH/MC (both fed by ~40 μmol TCE). Note: different H_2 scales in (A) and (B).

6.3.2 Transcriptomic microarray validation and analysis

Copy numbers of mRNA from 22 genes targeted by the microarray were quantified by RT-qPCR and compared to signal intensities measured by the microarray. All genes showed the same direction of transcription regulation, validating the microarray results

Transcriptomic microarray analysis of DE195 grown in the co-culture and in isolation identified 102 genes that were differentially transcribed (Figure 6.4). However, no DE195 genes were differentially transcribed between the co- and tri-culture conditions, indicating that the presence of MC in the constructed consortium did not significantly affect gene expression of DE195 (data not shown).
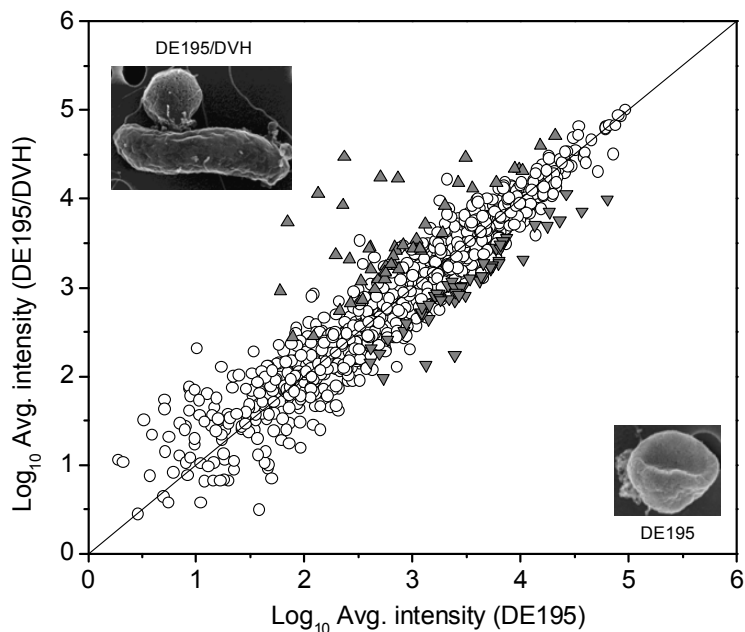
Figure 6.4 Plot of signal intensities of transcripts from DE195 grown alone vs. signal intensities of transcripts from DE195/DVH (colored data points represent statistically significant differential transcription, avg. intensity > 200, p < 0.05, > 2-fold difference; genes significantly up-regulated ($\triangle$) or down-regulated ($\triangledown$) in DE195/DVH compared to DE195. All measurements are averages from three biological replicates; left-upper corner: SEM photo of DE195/DVH; right-lower corner: SEM photo of DE195).

Cobalamin-associated genes

Cobalamins, including vitamin B_{12}, are corrinoid-based essential co-factors of reductive dehalogenases (RDases) (Smidt and de Vos, 2004). They cannot be synthesized *de novo* by DE195 (Seshadri *et al.*, 2005) nor by other sequenced *Dehalococcoides* strains. The cobalamin co-factor salvage and transport genes (DET0650-0652/DET0684-0686), as well as the genes annotated to construct and attach the lower ligand base to cobyric acid (DET0657-0660/DET0691-0694) and its associated riboswitch were significantly down-regulated in the co-culture compared with in isolation (Figure 6.5C). Interestingly, DET0125 and DET0126, two putative cobalamin riboswitches that were previously shown to be down-regulated in DE195 by excess cyanocobalamin (Johnson *et al.*, 2009), were also down-regulated in the co-culture.

Radical SAM domain superfamily

Proteins within this family have many functions in the biosynthesis of DNA precursors, vitamins, and co-factors (Sofia *et al.*, 2001) in biodegradation pathways. Among the 15 genes that are predicted to encode SAM proteins within the genome of DE195, 8 genes were actively transcribed and 4 genes (DET0622, DET1280, DET1368, and DET1629) were down-regulated in the co-culture (Figure 6.5C) while one gene (DET1314) was significantly up-regulated in the co-culture as compared to the DE195 isolate.

Genes associated with membrane-bound oxidoreductase complexes

Predicted oxidoreductase complexes identified in the genome of DE195 include: molybdopterin oxidoreductase (Mod), putative formate dehydrogenase (Fdh), NADH-ubiquinone oxidoreductase (complex I, Nuo), as well as six hydrogenase complexes (i.e. Hup, Hym, Hyc, Vhu, Ech and Hyp) (Seshadri *et al.*, 2005). In this study, the entire operon for the Nuo (DET0923-933) was significantly down-regulated in the co-culture compared with the isolate (Figure 6.5A). Within the six hydrogenase complexes, the Hym operon (DET0145-148) was observed to be significantly down-regulated, whereas a gene predicted to encode a HymA subunit but not associated with other hydrogenase genes (DET0446) was significantly up-regulated in the co-culture. Other genes within the hydrogenase complexes did not show significant regulation (Figure 6.5B), or were not actively transcribed in either culture condition (i.e. Hyc operon).

Reductive dehalogenases
The genome sequence of DE195 revealed 19 potential RDase genes (Seshadri *et al.*, 2005), 5 of which (DET0079, DET0162, DET0180, DET0318, and DET1559), were actively transcribed in the isolate with an additional 4 up-regulated in the co-culture (DET0311, DET1171, DET1522, and DET1545). Although the transcript levels of *tceA* (DET0079) were at the same level between the isolate and co-culture, all the other actively transcribed RDase genes were up-regulated in the co-culture (Figure 6.5A).

Twin arginine transport system
These proteins are important for secretion outside the cytoplasmic membrane of folded proteins such as reductive dehalogenases (RDases), hydrogenase complexes (e.g. Hup), and the formate dehydrogenases (e.g. Fdh). The gene predicted to encode one of these proteins, TatC, a secretion-independent translocase (DET1599) was significantly down-regulated in the co-culture.

Genes involved in amino acid biosynthesis
Several genes involved in amino acids biosynthesis were significantly differentially transcribed. Genes predicted to encode glutamate synthase and chorismate mutase/prephenate dehydratase (DET0038 and DET0461) were significantly down-regulated in the co-culture (Figure 6.5C). DET0461 is a gene involved in the biosynthesis of chorismate, a precursor of all aromatic amino acids (i.e. tryptophan, tyrosine and phenylalanine). The other genes within the chorismate operon (DET0462-DET0468), except DET0464 and DET0468 were also down-regulated, but less than two-fold difference (Figure 6.5C). The only amino-acid synthesis gene significantly up-regulated in the co-culture was DET1484, predicted to encode indole-3-glycerol phosphate synthase which is associated with tryptophan synthesis.

Stress-related genes
Genes significantly up-regulated in the co-culture included those predicted to encode glutaredoxin family protein reductase (DET0198, 4-fold), ferredoxin-thioredoxin reductase (DET0199, 3-fold), superoxide dismutase (DET0956, 4-fold), antioxidant alkyl hydroperoxide reductase (AhpC) (DET1581, 3-fold), and the alpha crystallin heat-shock family protein (DET0954, 9-fold). Alpha-crystallin related proteins have chaperone-like properties including the ability to prevent the precipitation of denatured proteins and to

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increase cellular tolerance to stress. DET1178 and DET1580, predicted to encode MarR and TetR transcriptional regulators, respectively, were significantly up-regulated in the co-culture, while another MarR family protein-encoding gene DET1536 was significantly down-regulated. MarR mediates response to multiple environmental stresses (Tropel and van der Meer, 2004) and the TetR family has been linked with cell density-sensing regulatory cascades (Ramos *et al.*, 2005). Interestingly, the observed stress response was not accompanied by a reduction in apparent growth rate of DE195 grown in the co-culture.

Genes with unknown functions
The most up-regulated genes in the co-culture, but poorly transcribed in the pure culture were DET0765 (153-fold) and DET1322 (117-fold), whose associated functions have not been well annotated. Both genes are small but present in all *Dehalococcoides* species. The gene adjacent to DET0765 (DET0766) is predicted to encode a V-type H (+) translocating pyrophosphatase suggesting a putative function in the regulation of proton transport. The gene adjacent to DET1322 (DET1323) is predicted to encode a dephospho-CoA kinase, involving in the synthesis of Coenzyme A, which is part of a pathway for acetate assimilation (Seshadri *et al.*, 2005).

6.3.3 Proteomic analysis

A total of 610 and 530 proteins were positively detected among the 1249 and 1251 transcripts of open reading frames (ORFs) detected in the transcriptomes of DE195 and DE195/DVH, respectively. According to the proteome of DE195, genes in COG categories of translation, as well as nucleotide metabolism and transport were mostly expressed (> 80%). Among the most abundant proteins are chaperonin GroEL (encoded by DET1428), formate dehydrogenase alpha subunit (encoded by DET0187), TceA, as well as a cobalamin uptake and salvaging protein CobT (encoded by DET0657/0691). In the co-culture, 86 proteins were determined to be significantly up-regulated (Table 6.2), while 34 proteins were significantly down-regulated (Table 6.3) compared with the DE195 isolate. 24 up-regulated proteins were ribosomal proteins, indicating the robust growth of DE195 in the co-culture. 4 hydrogenases, VhuA, HymB, HymC, and Ech encoded by DET0615, DET0729, DET0730, and DET0866, respectively, were also found to be significantly up-regulated in the co-culture (Table 6.2), while no significant differential transcription of these genes was found. Nevertheless, while transcripts for genes (DET0145-0148) predicted to encode HymA, HymB, HymC and HymD hydrogenases were found to be significantly down-regulated in the co-culture, no significant difference was found in the expression of corresponding proteins. Interestingly, cobalamin uptake and salvaging proteins CobT and CobU (encoded by DET0657/0691 and DET660/0694) were found to be less abundant in the co-culture than in the isolate (Table 6.3), consistent with the transcriptomic data. Among the 19 RDases, only TceA and the RDase encoded by DET1559 were detected in both cultures, neither of which exhibited significant regulation. In contrast, transcriptomic data showed no significant regulation of *tceA*, but a more than 2-fold up-regulation of DET1559 and another highly transcribed RDase gene (DET1545)
in the co-culture (Figure 6.5A).
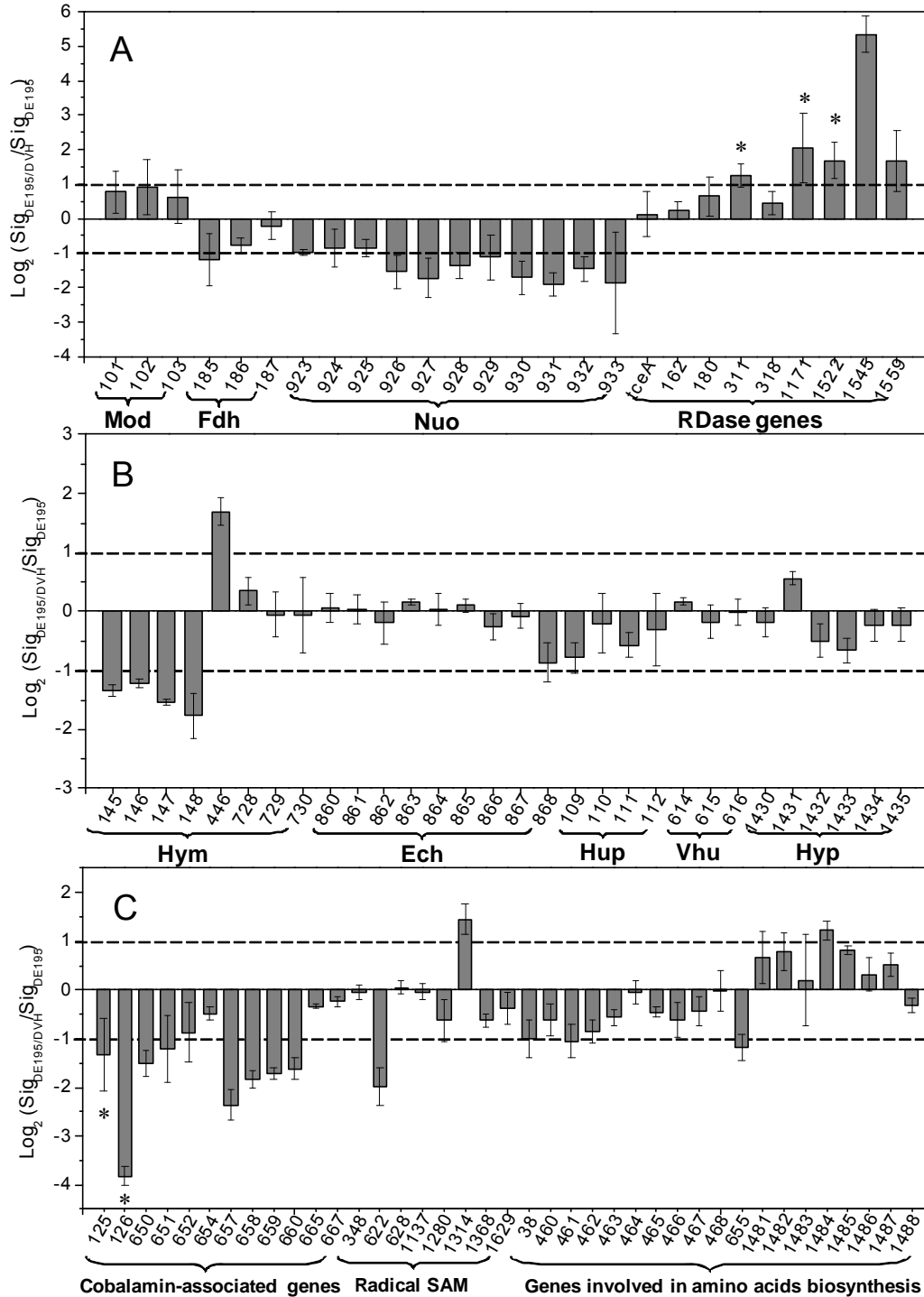
Figure 6.5 Log$_2$ signal intensity ratio between transcripts of DE195/DVH and DE195. All measurements are averages from three biological replicates and error bars are standard deviations. All X axis labels are designated for DE195 gene loci (e.g. DET0101). Dashed lines indicate the 2-fold difference in signal intensities. * indicates genes not actively transcribed (signal intensity < 200) in one of the two cultures.

Table 6.2 Up-regulated proteins in proteome of DE195 grown in pure and co-cultures

Protein-coding Gene locus	Expressed in DE195	Ratio of DE195/DVH to DE195	Description
DET0028	Y	2.1	universal stress protein family
DET0040	Y	2.5	hypothetical protein
DET0118	N	5.5	DJ-1 family protein
DET0297	N	6.1	hypothetical protein
DET0336	Y	2.5	DNA-binding protein, putative
DET0367	Y	3.8	inorganic pyrophosphatase
DET0376	Y	2.3	elongation factor Ts
DET0377	Y	2.7	30S ribosomal protein S2
DET0378	Y	2.3	phosphoribosylformylglycinamidine synthase I
DET0380	Y	2.8	pyridoxine biosynthesis protein
DET0387	N	10.3	hypothetical protein
DET0394	Y	2.1	nucleoside diphosphate kinase
DET0406	Y	2.2	rubrerythrin/rubredoxin
DET0452	Y	2.9	hypothetical protein
DET0453	Y	3.0	fumarate hydratase
DET0455	Y	2.1	HIT domain protein
DET0470	Y	4.5	30S ribosomal protein S12
DET0472	Y	2.1	translation elongation factor G
DET0474	Y	2.6	ribosomal protein L3
DET0477	Y	3.1	50S ribosomal protein L2
DET0479	Y	2.1	ribosomal protein L22
DET0482	N	5.7	ribosomal protein L29
DET0483	Y	3.3	ribosomal protein S17
DET0484	Y	2.4	ribosomal protein L14
DET0485	Y	2.7	ribosomal protein L24
DET0489	Y	2.8	50S ribosomal protein L6
DET0490	Y	2.3	ribosomal protein L18
DET0493	Y	3.3	ribosomal protein L15
DET0495	Y	2.9	adenylate kinase
DET0497	N	2.1	translation initiation factor IF-1
DET0499	Y	4.3	30S ribosomal protein S13
DET0501	Y	2.5	30S ribosomal protein S4
DET0505	Y	7.0	50S ribosomal protein L13
DET0506	Y	4.0	ribosomal protein S9
DET0541	Y	4.0	ribosomal protein L9
DET0560	Y	2.2	ATP synthase F0, B subunit
DET0567	Y	2.7	hypothetical protein
DET0568	Y	3.5	ribosomal protein S16
DET0583	Y	2.2	recombination protein RecR
DET0584	Y	3.7	hypothetical protein
DET0615	Y	2.2	hydrogenase, group 3, VhuA subunit, putative
DET0636	Y	3.4	cell division protein FtsZ

Table 6.2 Up-regulated proteins in proteome of DE195 grown in pure and co-cultures (cont.)

Protein-coding Gene locus	Expressed in DE195	Ratio of DE195/DVH to DE195	Description
DET0709	Y	2.2	trigger factor
DET0729	Y	2.3	[Fe] hydrogenase, HymB subunit, putative
DET0730	Y	2.5	[Fe] hydrogenase, HymC subunit, putative
DET0734	Y	2.8	hypothetical protein
DET0752	N	4.3	translation initiation factor IF-3
DET0760	Y	3.6	peptide deformylase
DET0770	Y	2.9	transcription elongation factor GreA
DET0821	N	5.5	hypothetical protein
DET0827	Y	2.2	3-isopropylmalate dehydratase, small subunit
DET0854	N	4.9	hypothetical protein
DET0866	Y	2.8	hydrogenase subunit, putative
DET0970	Y	2.2	polyribonucleotide nucleotidyltransferase
DET0971	N	3.8	dihydrodipicolinate reductase
DET0985	Y	2.1	transcription elongation factor NusA
DET0987	Y	2.6	acetyltransferase, GNAT family
DET0990	Y	2.3	ribosomal protein L7/L12
DET0991	Y	2.1	ribosomal protein L10
DET0993	Y	2.1	ribosomal protein L11
DET1008	Y	3.3	hypothetical protein
DET1011	Y	3.5	hypothetical protein
DET1026	Y	2.4	hypothetical protein
DET1044	Y	4.4	ribosomal protein S18
DET1045	Y	2.9	single-strand binding protein
DET1053	Y	2.0	hypothetical protein
DET1058	Y	2.4	DNA-binding response regulator
DET1129	Y	2.1	imidazoleglycerol phosphate synthase, cyclase subunit
DET1133	N	6.0	iron-sulfur cluster-binding protein
DET1188	Y	2.3	3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II
DET1274	N	2.3	hypothetical protein
DET1296	Y	3.0	hypothetical protein
DET1318	Y	3.0	hypothetical protein
DET1326	N	4.9	50S ribosomal protein L27
DET1329	Y	2.8	phosphoribosyl-AMP cyclohydrolase
DET1369	Y	4.7	hypothetical protein
DET1382	Y	2.7	nicotinate-nucleotide pyrophosphorylase
DET1400	Y	2.1	co-chaperone protein GrpE
DET1448	Y	6.5	hypothetical protein
DET1585	N	2.9	hypothetical protein
DET1591	Y	2.3	methyltransferase, UbiE/COQ5 family
DET1601	N	6.4	twin-arginine translocation protein, TatA/E family, putative
DET1608	Y	2.2	KH domain/HDIG domain protein
DET1613	N	2.3	conserved hypothetical protein TIGR00104
DET1631	Y	2.4	TPR domain protein
DET1639	Y	3.0	ribosomal protein S20

Table 6.3 Down-regulated proteins in proteome of DE195 grown in pure and co-cultures

Protein-coding Gene locus	Expressed in DE195/DVH	Ratio of DE195/DVH to DE195	Description
DET0396	N	0.1	conserved hypothetical protein TIGR00150
DET0525	N	0.3	hypothetical protein
DET0533	N	0.5	HAD-superfamily hydrolase, subfamily IA
DET0542	Y	0.5	thioredoxin-disulfide reductase
DET0588	N	0.2	hypothetical protein
DET0609	N	0.0	hypothetical protein
DET0657	Y	0.4	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase
DET0660	N	0.2	cobinamide kinase/cobinamide phosphate guanylyltransferase
DET0661	Y	0.3	thioredoxin
DET0664	Y	0.4	hypothetical protein
DET0670	Y	0.2	iron-sulfur cluster binding protein
DET0691	Y	0.4	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase
DET0694	N	0.2	cobinamide kinase/cobinamide phosphate guanylyltransferase
DET0695	Y	0.3	thioredoxin
DET0698	Y	0.4	hypothetical protein
DET0704	Y	0.2	iron-sulfur cluster binding protein
DET0750	Y	0.3	50S ribosomal protein L20
DET0839	N	0.3	phosphoribosylaminoimidazole carboxylase, catalytic subunit
DET0841	Y	0.4	phosphoribosylaminoimidazole-succinocarboxamide synthase
DET0846	Y	0.4	histidyl-tRNA synthetase, putative
DET0851	Y	0.5	sodium extrusion protein NatA, putative
DET0935	Y	0.5	endonuclease III
DET0954	N	0.2	Hsp20/alpha crystallin family protein
DET0956	Y	0.3	superoxide dismutase
DET0963	Y	0.5	3-oxoacyl-[acyl-carrier-protein] synthase II
DET1054	Y	0.5	GTPase domain, tubulin/FtsZ family protein
DET1123	Y	0.5	glutamine synthetase, type I
DET1198	Y	0.3	pyrimidine regulatory protein PyrR
DET1199	Y	0.3	aspartate carbamoyltransferase
DET1278	N	0.5	N utilization substance protein B
DET1357	N	0.2	hypothetical protein
DET1500	N	0.4	hypothetical protein
DET1543	Y	0.4	hypothetical protein
DET1595	Y	0.2	ApbE/NosX family protein

6.3.4 Dechlorination of DE195/DVH and DE195/DVH/PF without exogenous cobalamin but with DMB

DE195/DVH/PF grown without exogenous cobalamin but with 36 nM DMB exhibited similar dechlorination performance to that of DE195/DVH grown with 74 nM cobalamin, whereas the tri-culture without the addition of DMB had little dechlorination activity (Figure 6.6). However, the cobalamin-free co-culture with DMB exhibited similar dechlorination activity to the co-culture without DMB (Figure 6.6), which was much slower than the normal co-culture with added cobalamin. In addition, no dechlorination activity was observed in subsequent subcultures of the co-cultures with and without DMB (data not shown), indicating that the slow dechlorination observed in the first subculture was due to the carry-over of cobalamin from the inoculation stock (theoretically 1 μg/L carry-over from 100 μg/L inoculation stock). The dechlorination activities of different consortia under different cobalamin and DMB conditions are summarized in Table 6.4.
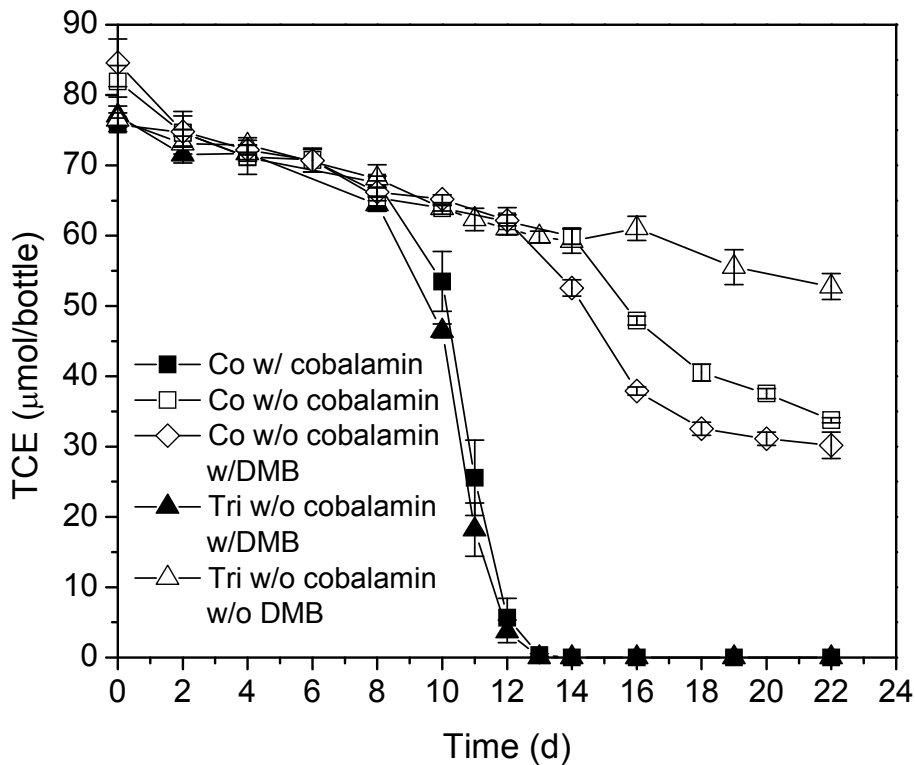
Figure 6.6 TCE dechlorination curves of DE195 grown in the co-culture DE195/DVH (designated as "Co") and the tri-culture DE195/DVH/PF (designated as "Tri"). The final concentration of added cobalamin and DMB was 74 nM (ca. 100 μg/L) and 36 nM, respectively.

Table 6.4 The dechlorination activities of different defined consortia

	DE195/DVH	DE195/DVH/MC	DE195/DVH/PF
w/ cobalamin	√	√	√
w/o cobalamin, w/o DMB	×	×	×
w/o cobalamin, w/ DMB	×	Not tested	√

In terms of corrinoid production of DVH and PF, as expected, 15 nM of [p-Cre]Cba was produced in PF pure culture, whereas none of the 10 tested corrinoid forms was at a detectable level in DVH pure culture. This well explains the above results and demonstrates that *Pelosinus* spp. are responsible for the [p-Cre]cba production and corrinoid transfer to Dhc in the enrichment without exogenous cobalamin described in chapter 3 & 4. However, neither DVH nor PF produced DMB, leaving the DMB producers from the enrichment cultures still unknown.

Hydrogen measurements showed that little hydrogen was generated from lactate fermentation by PF when grown in co-culture with DE195, whereas DVH consistently generated hydrogen (Figure 6.3), suggesting that under cobalamin-limitation, successful dechlorination by Dhc may require cooperation of multiple organisms supportive in different ways.

6.4 Discussion

In this study, DE195 was sustainably grown with DVH in a syntrophic association as a co-culture, as well as with DVH and the hydrogenotrophic methanogen MC as a tri-culture using lactate as sole energy and carbon source with TCE as electron acceptor. In these syntrophic associations, DVH ferments lactate to acetate and hydrogen that becomes available for use by DE195 and MC as carbon source and electron donor, respectively. This syntrophy occurs at trace sulfate concentrations. This approach of defined syntrophic growth enabled direct experimental measurement of the specific effects of associated bacteria on the growth, activity and gene expression of Dhc, similar to the approach taken for the study of *Syntrophomonas wolfei* with *Methanospirillum hungatei* (Beaty and Mcinerney, 1989), for the study of *Desulfovibrio vulgaris* with methanogens (Bryant *et al.*, 1977; Stolyar *et al.*, 2007; Walker *et al.*, 2009) and for the study of *Desulfovibrio sp*. strain SULF1 and *Desulfovibrio fructosivorans* with a dehalorespiring bacterium *Desulfitobacterium frappieri* TCE1 (Drzyzga *et al.*, 2001; Drzyzga and Gottschal, 2002).

In DE195/DVH and DE195/DVH/MC, the dechlorination rates of DE195 were enhanced 2-3 fold over the isolate and cell yields were enhanced in the co-culture by approximately 1.5 times. In addition, the ratio of ethene to VC was higher in the co– and tri–cultures and the subculturing was more reproducibly successful, four phenomena that are commonly observed when Dhc strains are grown in microbial communities (Maymó-Gatell *et al.*, 1997; He *et al.*, 2007). The complete dechlorination but lower cell yield in the tri-culture compared to the co-culture indicated that DE195 uncoupled TCE dechlorination from net cell growth. Johnson *et al*. (Johnson *et al.*, 2008) demonstrated that DE195 uncouples dechlorination from net cell growth associated with stress and

transition into stationary phase. The uncoupling in this study may be associated with the competition for H_2 in the tri-culture caused by MC, although competition for hydrogen between MC and DE195 was not observed to adversely affect the dechlorination rate in this study. Therefore, the presence of the methanogen did not provide benefits beyond those of DVH. Although the presence of methanogens has been shown to promote reductive dechlorination in some communities (Vogel and McCarty, 1985; Heimann *et al.*, 2006), such an enhancement was not observed for Dhc in this study. Further, competition for hydrogen between MC and DE195 was not observed to adversely affect dechlorination in this study. The increase in cell density of DE195 grown in co-culture is similar to the increase previously reported for the single-transfer co-culture containing *Desulfovibrio desulfuricans* strain Essex 6 and 195 (He *et al.*, 2007). A previous study with a co-culture containing *Desulfovibrio* sp. strain SULF1 and a dehalorespiring bacterium *Desulfitobacterium frappieri* TCE1 showed that at low sulfate concentrations and relatively high PCE concentrations, SULF1 was outnumbered by TCE1 with a protein level ratio about 1:10 (Drzyzga *et al.*, 2001). Since SULF1 and TCE1 are similar in biovolume, the cell number ratio of SULF1 to TCE1 should also be about 1:10 which is similar to the results in this study. Interactions among organisms in anaerobic environments where trophic hierarchies occur with functionally different members of the community providing substrates and essential co-factors for each other and removing inhibitory metabolic products are well known in both the environment and in engineered systems such as anaerobic digesters (Schink, 1997; Rittmann and McCarty, 2001).

One possible reason for more robust growth and faster dechlorination of DE195 grown in the co- and tri-cultures is facilitated hydrogen transfer between DVH and DE195 due to physical proximity under the unshaken conditions. Membrane-bound oxidoreductase operons, such as Fdh, Nuo, as well as Hym and Hup operon were down-regulated in DE195 grown in the co-culture compared with the DE195 isolate. These operons are predicted to be components of the electron transport chain, and are important in hydrogen transfer for DE195 (Seshadri *et al.*, 2005). Moreover, the down-regulation of TatC gene, whose product functions in the secretion of folded proteins such as the putative hydrogenase complexes and Fdh, could be a corresponding effect of a down-regulation of those proteins. The down-regulation of these genes suggests that the syntrophic growth of DE195 and DVH might have a different hydrogen transfer system than the DE195 isolate. Transcriptomic analysis of DVH syntrophically grown with a hydrogenotrophic methanogen compared with DVH grown in sulfate-limited monocultures showed that genes encoding hydrogenases Coo, Hyd and Hyn were among the most highly expressed and up-regulated genes (Walker *et al.*, 2009). Therefore, differential regulation of genes encoding hydrogenases of DVH grown in DE195/DVH compared with the DVH isolate should be further investigated, in order to elucidate the interspecies hydrogen transfer during syntrophic growth.

In addition, aromatic amino acids (i.e. tryptophan, tyrosine, and phenylalanine) might play important roles in the robust syntrophic growth of DE195 with DVH. In the co-culture, the down-regulation of genes involved in the biosynthesis of chorismate, a precursor for aromatic amino acid biosynthesis, suggests that DVH might support the

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growth of DE195 by decreasing the need for *de novo* aromatic amino acid biosynthesis, consistent with our recent detailed amino acid study (Zhuang *et al.*, 2011).

One interesting observation is that both transcriptomic and proteomic analyses showed that cobalamin-associated genes, including genes predicted to encode a corrinoid ABC-type transport system (DET0650-0655/DET0684-0689) and a corrinoid salvage system (DET0657-0660/DET0691-0694), were significantly down-regulated in the co-culture, which suggests that DE195 may exert less energy salvaging corrinoids when DVH is present. DET0650-0654 were also found to be significantly down-regulated when DE195 is grown with excess cobalamin, as well as with spent medium of a Dhc-containing enrichment culture (ANAS) compared with DE195 grown with limited cobalamin (Johnson *et al.*, 2009). Interestingly, another significantly down-regulated gene in both this study and the study by Johnson *et al*. (Johnson *et al.*, 2009) was DET0126, which is annotated as an anthranilate phosphoribosyltransferase (TrpD) and reported to have an upstream putative cobalamin-binding riboswitch (Johnson *et al.*, 2009). Although the function of DET0126 remains unclear, its differential regulation within different growth conditions suggests that it is of biological importance to DE195.

DVH possesses a nearly full set of genes required for the biosynthesis of adenosylcobalamin, another form of corrinoids (Rodionov *et al.*, 2004). *Desulfovibrio vulgaris* was also reported to produce two other corrinoids: guanylcobamide and hypoxanthylcobamide (Guimaraes *et al.*, 1994). However, it is revealed by the follow-up studies that DVH in pure culture were not able to produce sufficient amounts of corrinoids or support sustainable growth of DE195 in co-culture. This indicates that although DVH was reported to produce corrinoids, the amount of corrinoid it produces might be sufficient to only cause the response of Dhc on a transcriptomic level, but not on a phenotypic level.

Instead, the production of sufficient amounts of [p-Cre]cba by the PF isolate, combined with the observed complete dechlorination in cobalamin-free DE195/DVH/PF tri-culture with DMB, demonstrated the role of *Pelosinus* spp. on corrinoid transfer to Dhc. However, the fermentation of lactate by PF cannot produce hydrogen in sufficient amounts to support Dhc and PF cannot produce DMB, which indicates cooperation must be needed among different organisms within dechlorinating communities to support Dhc in different ways under cobalamin-limited conditions.

In summary, DE195 exhibits faster dechlorination and more robust growth when grown syntrophically with DVH using lactate as carbon and energy source than when grown in isolation on the gaseous substrate hydrogen with the addition of cobalamin. The difference in gene transcription and protein expression levels between DE195 grown in isolation and with DVH suggests that effective transfer of hydrogen may mainly contribute to the enhanced dechlorination and robust growth of DE195 in the co-culture. These results provide an improved method for rapid and robust growth of *Dehalococcoides* strains using lactate as an inexpensive, widely available substrate. This study also provides strong evidence to demonstrate that *Pelosinus* spp. are playing

important roles in corrinoid supply, and multiple supportive organisms may be needed if cobalamin-limited conditions were encountered in Dhc-containing communities.

6.5 Acknowledgements

7 Conclusions and Future Work

7.1 Summary and conclusions

The primary objective of this research is to understand the ecological relationship between *Dehalococcoides* spp. (Dhc) and supportive microorganisms in a community and identify novel biomarkers for monitoring TCE dechlorination activities in bioremediation. This overall goal was accomplished via fundamental scientific discovery in dechlorinating cultures from complex enrichments originated from contaminated groundwater to simple defined consortia. Together, this body of work serves to advance the science of dehalorespiring communities at both the fundamental and applied levels with the ultimate goal of enhancing the effectiveness of bioremediation.

Hydrogen, acetate, and corrinoid are three major factors required by the growth of Dhc. Hydrogen and acetate could be generated from fermentation of organic compounds such as lactate. Fermentation involves corrinoid-dependent processes (Banerjee and Ragsdale, 2003), thus may produce corrinoid in the course of hydrogen and acetate production. However, no specific studies have focused on corrinoid-providing organisms in a dechlorinating community. In Chapter 3, four enrichments were generated using contaminated groundwater under four different enrichment conditions exploring two parameters: high and low TCE amendments (resulting in inhibited and uninhibited methanogenic activity, respectively); and with and without vitamin B_{12} 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 in dechlorination increased when methanogenesis was inhibited. Biologically significant concentrations (3-4 µg/L) of cobalamin were detected in the enrichments without exogenous vitamin B_{12}, indicating biologically active cobalamin biosynthesis in the cultures. Results from microarrays targeting four sequenced Dhc genomes indicated that the Dhc strains in all enrichments are similar to that of strain 195, lack the major upstream cobalamin biosynthesis pathway rendering them incapable of *de novo* cobalamin synthesis. Seven other Bacterial OTUs were detected by clone libraries. OTUs closest to *Pelosinus*, *Dendrosporobacter*, and *Sporotalea* (PDS) are most dominant. The *Clostridium*-like OTU was most affected by B_{12} amendment and active methanogenesis. Principal component analysis revealed a greater effect of active methanogenesis on the community structures than vitamin B_{12} limitation, although methanogenesis did not exert significant effects on Dhc cell growth or biological corrinoid generation. The phyletic and genomic information of PDS and *Clostridium* species suggests that they have potential in corrinoid supply. Overall, the successful dechlorination in enrichments without exogenous vitamin B_{12} suggests the presence of organisms support the growth of Dhc with corrinoids. The physiological, phyletic and genomic investigation on these communities gives us hints on corrinoid-providing organisms.

The little progress made in corrinoid-related studies in the past might due to the limitation of detection methods. In Chapter 4, a more specific and sensitive corrinoid detection method has been established using LC/MS/MS, which is able to quantitatively differentiate 12 corrinoid species, as well as 5 lower ligands. With this powerful tool, we determined the forms of corrinoids and lower ligands, as well as their concentrations in

eight different Dhc-containing microbial communities enriched with or without exogenous vitamin B_{12}, including the four enrichments described in Chapter 3, two subcultures (SANASB12 and SANAS) of previously reported ANAS enrichment. Different corrinoid and lower ligand profiles were observed. In the absence of exogenous B_{12}, cobalamin was the dominant form in methanogenic, B_{12}-unamended SANAS enrichment, while p-cresolylcobamide ([p-Cre]Cba) was overwhelming than cobalamin in two B_{12}-unamended groundwater enrichments (LoTCE and HiTCE). [p-Cre]Cba in these two enrichments increased to maximum of 13-20 nM and decreased substantially afterwards. Cobalamin concentrations in the three B_{12}-unamended enrichments were 1-3 nM. In the enrichment manipulated from HiTCE (denoted "NoTCE"), where the dechlorination and growth of Dhc were exclusively limited, little cobalamin was detected. [p-Cre]Cba reached and stayed at the similar maximum level as in HiTCE with no substantial decrease. Notably, Anaerobic DMB production was detected in both NoTCE and other B_{12}-unamended enrichments. When the growth of Dhc was limited, a significant decrease of *Desulfovibrio*-like OTU in the community structure was observed, which indicates a close relationship between these two species. The production of cobalamin ceased without the addition of TCE to HiTCE subculture, and restored after TCE was re-amended. When 1% HiTCE culture or strain 195 pure culture was inoculated with TCE into pre-grown NoTCE culture, complete dechlorination occurred concomitantly with cobalamin production and cell growth. A production of 15 nM [p-Cre]Cba was observed in *Pelosinus fermentans* strain R7 pure culture grown on fermentation of 38 mM lactate. These results demonstrate that cobalamin in HiTCE was formed by *Dehalococcoides*, via modification of [p-Cre]Cba, which might be generated by *Pelosinus* sp., in the presence of DMB.

In addition to the study of physiological and phylogenetic characteristics of cobalamin-limited enrichments, the differential gene expression of Dhc grown with and without exogenous cobalamin, together with a time-course transcriptome in enrichment without exogenous cobalamin was investigated in Chapter 5. According to physiological characterization and corrinoid production determined in Chapter 3 and 4, the differential gene expression of Dhc under conditions with and without exogenous cobalamin was studied on day 2, when dechlorination and cell growth started, cobalamin concentration dramatically increased. Results revealed almost all differentially expreesed genes were those up-regulated under cobalamin-limited condition. The up-regulation of the lower ligand cleaving (*cbiZ*, DET0653) and attachment (*cobT*, DET0657) genes and two corrinoid transporter genes (DET0650, DET1176) in the enrichment without exogenous cobalamin indicates more active corrinoid transport and remodeling processes under cobalamin-limited condition. Concomitantly, Hym operon (DET0145-0148), genes in Nuo operon (DET0925-26, DET0933), as well as tryptophan operon in strain VS (DhcVS_1251-58) were also up-regulated, indicating a close correlation with cobalamin stress. Temporal global gene expression of Dhc in the enrichment without exogenous cobalamin exhibited higher expression of DET0650 than other corrinoid-related genes during late exponential phase, suggesting it might be related to the cobalamin stress. Different from study on pure strain 195 grown under cobalamin-limited condition, the two genes encoding putative cobalamin riboswitches (DET0125-26) were not expressed in the enrichment without exogenous cobalamin during the entire cultivation period,

indicating an alternative cobalamin source for Dhc grown in a community. Besides corrinoid-related genes, microarray results also exhibited that *tceA* is the only functioning RDase genes in the enrichment, which is closely correlated to Hup operon. Hydrogenase operons, Hup, Hym and three other oxidoreductase operons, Mod, Fdh and Nuo were all up-regulated during the early exponential phase, indicating an initiation of energy production in dechlorination. Overall, this work provides us possible biomarkers, such as genes involved in corrinoid transport and lower ligand attachment (DET0650 and DET0657), the upregulation of which, combined with the "off" status of cobalamin riboswitches (DET0125-26), could indicate the salvaging of alternative corrinoid source by Dhc under cobalamin-limited conditions.

Given the knowledge of supportive organisms in the aspects of hydrogen and corrinoid supply to Dhc, we successfully simplified the complex enrichment cultures into defined consortia by growing strain 195 (DE195), the most similar pure strain to the Dhc strains in the enrichments with *Desulfovibrio vulgaris* Hildenborough (DVH), *Methanobacterium congolense* (MC) or *Pelosinus fermentans* strain R7 (PF). Physiological, transcriptomic and proteomic analysis were carried out in Chapter 6. Sustainable syntrophic co-culture (DE195/DVH) and tri-culture (DE195/DVH/MC) were successfully constructed using lactate as the electron donor with the addition of 100 μg/L cobalamin. In DE195/DVH and DE195/DVH/MC, maximum dechlorination rates of DE195 were enhanced by approximately 3 times compared with DE195 grown alone. Cell yield of DE195 was also enhanced in the co-culture, while no further enhancement was observed in the tri-culture. The transcriptome of DE195 grown in DE195/DVH was analyzed using a whole-genome microarray targeting DE195, which detected 102 significantly up- or down-regulated genes compared to DE195 grown in isolation, while no significant transcriptomic difference was observed between the co- and tri- cultures. Proteomic analysis showed that 120 proteins were differentially expressed in the co-culture compared to DE195 grown in isolation. Physiological, transcriptomic and proteomic results indicate that the robust growth of DE195 in co- and tri-cultures is due to the advantages associated with the capabilities of DVH to ferment lactate to provide H_2 and acetate for growth, while MC in the tri-culture provided no significant additional benefits beyond those of DVH. Neither DE195/DVH nor DE195/DVH/MC were able to dechlorinate TCE without the addition of cobalamin, while the DE195/DVH/PF triculture successfully degraded TCE with the addition of 36 nM DMB instead of cobalamin, at a similar dechlorination rate to the DE195/DVH co-culture with the addition of 74 nM cobalamin. These results corroborate the important roles played by supportive microorganisms such as *Desulfovibrio* spp., *Pelosinus* spp. for the hydrogen and corrinoid supply to Dhc within dechlorinating microbial communities.

Together, the major contributions of this research were the identification of important supportive microorganisms, the understanding of their relationships to Dhc in the terms of the hydrogen and corrinoid supply, as well as the identification of potential molecular and chemical biomarkers for effective monitoring in field operations. These contributions are within the framework of trying to optimize the bioremediation processes through eliminating potential bottlenecks in cell metabolism and obtaining accurate feedback information from cells that are indicative of their physiology.

7.2 Suggestions for future research

Until a novel group of organisms that are effective in dechlorinating chlorinated ethenes is discovered, Dhc will remain as the key player in bioremediation processes. Strong evidence has been shown that the DMB lower ligand of cobalamin is of more practical importance than cobalamin itself in microbial communities, where the production of diverse forms of corrinoids was demonstrated and the possible corrinoid producers have been identified. However, the addition of either cobalamin or DMB to a field site would be an extra cost and effort, moreover, have the risk of introducing unexpected growth of irrelevant organisms. Therefore, the ideal scenario is to further identify DMB producers that are closely associated with Dhc in Dhc-containing enrichments. The interactive relationship between DMB and corrinoid producers and Dhc could be further investigated, thus promote the extended understanding of dechlorinating microbial communities and the optimization of bioremediation processes, as well.

Moreover, taking advantages of more and more available sequenced genomes and the high throughput microarray and proteomic techniques, future research will seek a systems level understanding of the biology of not only Dhc, but also the supportive organisms. A thorough understanding of the ecological relationship among members within the constructed dechlorinating consortia could provide more accurate guidance to support the growth of Dhc during the bioremediation operations.

Recent advances in the next generation sequencing (NGS) technology lead studies to the reconstruction of all genomes and transcriptomes within a community. The metagenomic and metatranscriptomic analysis of dechlorinating enrichments will re-evaluate previously described community structures and properties and provide systems-level knowledge of biological functions in the community. Furthermore, NGS will expand our research from culture-dependent to culture-independent studies. Obtaining metagenomic and metatrascriptomic information directly from environmental samples will eliminate the laboratorial cultivation bias, thus help us to develop more accurate biomarkers to guide *in situ* bioremediation.

Eventually, field-based research should be conducted to test the biomarkers identified from laboratory-based studies. Correlation of the expression levels of biomarker genes and concentrations of such chemical biomarkers as corrinoids and lower ligands to the dechlorination activities should be investigated, which could optimize the application of these biomarkers to monitor the bioremedial treatment systems.

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