REDUCTIVE DEHALOGENATION OF
CHLOROETHENES, DICHLOROPHENOL, AND CHLOROBENZENES

A Dissertation
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by
Jennifer Mon Yee Fung
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The improper disposal of chlorinated compounds has become an environmental concern and its remediation is a priority. *Dehalococcoides ethenogenes* strain 195 is able to reductively dehalogenate the toxic compounds, tetrachloroethene (PCE), trichloroethene (TCE) and 2,3-dichlorophenol (2,3-DCP) to less toxic products, ethene and 3-monochlorophenol. Its genome contains 19 putative reductive dehalogenase (RD) genes including *pceA* (PCE RD), *tceA* (TCE RD), DET0162 and DET1559. Differential transcription was examined when strain 195 was growing on PCE, TCE, or 2,3-DCP. Gene product and function was confirmed using proteomic analysis and resting cell assays. When grown on PCE or TCE transcript levels of *tceA*, *pceA* and DET0162 were several folds higher than housekeeping gene, *rpoB*. DET1559 was only expressed in PCE-grown cells. In 2,3-DCP-grown cells, *pceA* and DET0162 were the only RD genes expressed and *tceA* transcript level was the 300-fold lower than in PCE-grown cells. DET0162 contains a translational stop codon and is presumed nonfunctional and was not detected in proteomic analysis. PceA was the only RD detected in 2,3-DCP-grown cells. PCE- and 2,3-DCP-grown resting cells were able to reductively dehalogenate PCE and 2,3-DCP without lag. PceA has been identified as the 2,3-DCP reductive dehalogenase. In the case of dichlorobenzene (DCB) and monochlorobenzene (MCB), no microorganism
has been identified capable of their reductive dehalogenation. Using historically chlorobenzene-contaminated sediments, microcosms were used to enrich for DCB and MCB dehalogenators. Sediment microcosms were able to reductively dehalogenate all three DCB isomers to MCB and benzene over 5,000 µmoles/liter in some microcosms. If only given MCB, reductive dehalogenation to benzene only occurred in specific sediment samples and was considered unreliable. Inoculating sediment microcosms with sediment slurry from DCB- or MCB-dehalogenating microcosm reduced lag time of MCB reductive dehalogenation to benzene and allowed for reliable growth on MCB in the absence of DCBs. A 1,2-DCB mixed culture was established by transferring sediment slurry from DCB-dehalogenating microcosms into mineral salts medium supplemented with yeast extract. 16S rRNA gene clone library of the mixed culture contained sequences identical >99% to Dehalobacter restrictus, a known dehalogenator. Dehalobacter population, monitored by real time PCR, was linked to reductive dehalogenation of DCBs and its 16S rRNA gene sequences accounted for 53% of the total bacterial sequences in the mixed culture. As the toxicity of the chlorinated compounds can be altered by reductive dehalogenation, characterization of dehalogenating microorganisms is needed to design effective bioremediation strategies.
BIOGRAPHICAL SKETCH

Jennifer Mon Yee Fung was born on July 17, 1979 in San Francisco, California to John and Helen Fung. Influenced by family and school trips to the beach, Lake Tahoe, and Muir Woods, Jennifer pursued an education in the biological sciences. At Skyline Community College, she was introduced to the diverse world of microbiology and took a special interest in the field of bioremediation. In 1999 Jennifer transferred to the University of California, San Diego, Revelle College, to pursue a bachelor degree in microbiology and began working in the laboratory of Dr. Bradley Tebo at Scripps Institution of Oceanography where she studied hexavalent chromium reduction by *Shewanella* spp. Determined to gain a deeper understanding of microbially-mediated bioremediation, Jennifer joined the graduate program in the Department of Microbiology at Cornell University in 2002. At Cornell University, Jennifer worked under the guidance of Dr. Stephen Zinder on reductive dehalogenation of halogenated pollutants. Upon completion of her degree, Jennifer will begin work on bacterial production of biofuels at LanzaTech in Auckland, New Zealand.
This work is dedicated to my parents, John and Helen Fung, as well as my sisters and brother, Christina, Denise and Clifford Fung, for their unconditional support during my entire educational pursuit.
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CHAPTER ONE
REVIEW OF REDUCTIVE DEHALOGENATION

Introduction

The degradation of chlorinated compounds has been the focus of numerous studies due to their extensive use in industrial applications and subsequent disposal into the environment. The Environmental Protection Agency (Clean Water Act) has designated a large number of chlorinated compounds as priority pollutants, including polychlorinated biphenyls (PCBs), chlorinated benzenes, ethenes, methanes, phenols, and dioxins (www.epa.gov/watertrain/cwa/). These compounds are either known or potential causative agents of serious health complications such as liver and kidney cancers (20). Since many chlorinated compounds are dense non-aqueous phase liquids (DNAPLs), they have the ability to migrate into the anoxic region of the sediment and serve as electron acceptors in a process known as respiratory reductive dehalogenation (Figure 1.1).

Figure 1.1. Two mechanisms of reductive dehalogenation A) hydrogenolysis of cis-dichloroethene to vinyl chloride as observed in *Dehalococcoides* and B) dichloroelimination of 1,2-dichloroethane to ethene as observed in *Desulfitobacterium dichloroeliminans* strain DCA1
This process can be found across different phyla such as *Chloroflexi*, *Firmicutes*, and *Proteobacteria* in a variety of environments (Table 1.1) (55). Depending on the microorganism, this physiological process can be the only or one of many respiratory pathways. Understanding the microbial community involved in reductive dehalogenation is critical, as the fate of chlorinated compounds in the environment can be determined by the presence of specific dehalogenators (19).

The calculated redox potential of halogenated substrates is between 200-400 mV, well situated in the range of anaerobic respiration (11). The halogenated substrates can vary from complex aromatics like 1,2,3,4-tetrachloronapthalene to simple two carbon compounds like chloroethenes (55). Some bacteria such as *Geobacter lovleyi* and *Desulfitobacterium* spp. are able to carry out alternative forms of respiration such as sulfate reduction and can use multiple substrates as electron donors (59, 64). Other bacteria such as *Dehalococcoides* spp. and *Dehalobacter* spp. are known only to respire halogenated compounds and utilize hydrogen as the electron donor (55).

Insight into the molecular mechanisms of reductive dehalogenation has been accumulating due to the sequenced genomes of dehalogenators such as *Dehalococcoides* spp. and *Desulfitobacterium* spp. (26, 44, 54). There has been much progress in the understanding of the molecular biology of reductive dehalogenases (RD), the proteins responsible for reductive dehalogenation. In general, RDs are membrane bound and contain a corrinoid cofactor except for the 3-chlorobenzoate RD of *Desulfomonile tiedjei*, which contains a heme group (42). Most RD genes are flanked by putative regulatory genes, and
several show signs of genetic mobility (26, 30). Some bacteria hold large reservoirs of RD genes, like *Dehalococcoides* strain CBDB1 with 32, of which only two have known function (2, 26). While the large number of RD genes suggests a great capacity for reductive dehalogenation, little is known about substrate specificity and range.

The goals of this review are to:

1. Give an overview of the diversity of dehalogenating microorganisms and their suite of chlorinated substrates
2. Survey the molecular biology of reductive dehalogenation

**Microbiology of Reductive Dehalogenation**

*Dehalococcoides and its relatives*

*Dehalococcoides* spp. and its relatives, strain DF-1 and o-17, are members of the *Chloroflexi* phylum and specialize in reductive dehalogenation (9, 55, 67). *Dehalococcoides ethenogenes* strain 195 was the first bacterium isolated able to completely reductively dehalogenate perchloroethylene (PCE) to vinyl chloride (VC) and eventually, ethene with the last step being cometabolic (34). The genome of strain 195 reveals 19 potential RD genes including the characterized *pceA* (DET0318), the gene responsible for PCE to TCE reductive dehalogenation and *tceA*, responsible for TCE to ETH (29, 54). Strain 195 was later shown to dehalogenate chlorinated aromatics as well as polybrominated diphenyl ethers (1, 13, 18). Strains BAV1 and VS have been isolated and reductively dehalogenate VC as an energetically favorable reaction but are unable to dehalogenate PCE or TCE (8, 17). Strain CBDB1
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Halogenated Compound</th>
<th>e-donor</th>
<th>Genome Sequenced</th>
<th>Total No. of RD Genes</th>
<th>Identified RD genes</th>
<th>Phylum</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehalococcoides strain 195</td>
<td>CE, CP, CB, PCB, PCDD/F, CN</td>
<td>H₂</td>
<td>yes</td>
<td>19</td>
<td>2 (pceA, tceA)</td>
<td>Chloroflexi</td>
<td>(1, 13, 29, 34, 54)</td>
</tr>
<tr>
<td>Dehalococcoides strain CBDB1</td>
<td>CB, CP, CD</td>
<td>H₂</td>
<td>yes</td>
<td>32</td>
<td>1 (cbrA)</td>
<td>Chloroflexi</td>
<td>(1-3, 6)</td>
</tr>
<tr>
<td>Dehalococcoides strain BAV1</td>
<td>CE</td>
<td>H₂</td>
<td>yes</td>
<td>10</td>
<td>1 (bvcA)</td>
<td>Chloroflexi</td>
<td>(17, 25)</td>
</tr>
<tr>
<td>Strain DF-1</td>
<td>CB, PCB</td>
<td>H₂</td>
<td>no</td>
<td>unk</td>
<td>0</td>
<td>Chloroflexi</td>
<td>(66, 67)</td>
</tr>
<tr>
<td>Strain TCA1</td>
<td>CA</td>
<td>formate, H₂</td>
<td>no</td>
<td>unk</td>
<td>0</td>
<td>Firmicutes</td>
<td>(58)</td>
</tr>
<tr>
<td>Dehalobacter restrictus</td>
<td>CE</td>
<td>H₂</td>
<td>no</td>
<td>unk</td>
<td>1 (pceA)</td>
<td>Firmicutes</td>
<td>(21, 31)</td>
</tr>
<tr>
<td>Desulfotobacterium hafienae strain Y51</td>
<td>CE, CA</td>
<td>formate, lactate, pyruvate</td>
<td>yes</td>
<td>2</td>
<td>1 (pceA)</td>
<td>Firmicutes</td>
<td>(64)</td>
</tr>
<tr>
<td>Desulfotobacterium hafienae strain DCB-2</td>
<td>CP, CE, 3Cl4OHPA, TCMP, TCHQ</td>
<td>formate, lactate, pyruvate</td>
<td>yes</td>
<td>7</td>
<td>1 (cprA)</td>
<td>Firmicutes</td>
<td>(64)</td>
</tr>
<tr>
<td>Sulfurospirillum multivorans</td>
<td>CE</td>
<td>formate, H₂</td>
<td>no</td>
<td>unk</td>
<td>1 (pceA)</td>
<td>ε-proteobacteria</td>
<td>(40, 51)</td>
</tr>
<tr>
<td>Geobacter lovleyi sp. nov. strain SZ</td>
<td>CE</td>
<td>acetate, H₂</td>
<td>yes</td>
<td>2</td>
<td>1 (pceA)</td>
<td>δ-proteobacteria</td>
<td>(59)</td>
</tr>
</tbody>
</table>

CE, chloroethenes; CP, chlorophenols; CB, chlorobenzenes; PCB, polychlorinated biphenyls; PCDD/F, polychlorinated dibenzio-p-dioxins/furans; CN, chlorinated naphthalenes; CD, chlorinated dioxins; CA, chlorinated ethanes; 3Cl4OHPA, 3-chloro-4-hydroxy-phenylacetate; TCMP, 2,3,5,6-tetrachloro-4-methoxyphenol; TCHQ, tetrachlorohydroquinone.
was the first isolated bacterium able to reductively dehalogenate chlorinated benzenes and dioxins but it is unable to grow on chlorinated ethenes (3, 6). The genome sequence of *Dehalococcoides* strain CBDB1 revealed 32 potential RD genes including *cbdbA84 (cbrA)*, responsible for 1,2,3-trichlorobenzene reductive dehalogenation (2). Twelve RD genes between strain 195 and CBDB1 share up to 95.4% amino acid sequence similarity, suggesting a partial overlap of substrates (26). Multiple RD genes have been identified in *Dehalococcoides* strains FL-2, KB1 (*Dehalococcoides*-containing mixed culture), and BAV1 (14, 14, and 10, respectively) (22). With their large reservoir of RD genes, *Dehalococcoides* spp. are likely to have a diverse substrate range that will be useful for site remediation (Figure 1.2).

*Dehalococcoides* has also been shown to be important in bioremediation field studies and dehalogenating mixed cultures. Field studies done in a variety of contaminated sites throughout North America and Europe indicate that *Dehalococcoides* is the key player in the complete reduction of chlorinated ethenes (19). In sediment-free mixed culture derived from the Housatonic River, Massachusetts, *Dehalococcoides* population growth has been linked to the reductive dehalogenation of Aroclor 1260 (polychlorinated biphenyl mixture) (4). Similar results were shown with trichlorinated dibenzo-*p*-dioxins with mixed cultures originating from the Spittelwasser, Germany (12). With sediment microcosms derived from three different sites (New York, California, and Maryland), Yan *et al.* (68) demonstrated 2,3,4,5-tetrachlorobiphenyl dehalogenation and presence of *Dehalococcoides*-like populations. Commercially available mixed culture, KB-1, where the bacterial population.
Figure 1.2. Phylogenetic tree of reductive dehalogenases of *Dehalococccoides* strain 195 (DET), strain CBDB1 (cbdb), strain BAV1 (bav), *Desulfitobacterium hafniense* strain DCB-2 (Dhaf), *Geobacter lovelyi* strain SZ (Glov) and *Photobacterium profundum* strain 3TCK (3TCK). The length of the bar represents 10% sequence diversity. Protein distances were calculated with Prodist using Jones-Taylor-Thornton matrix and tree drawn with Neighbor-joining algorithm.
contains *Dehalococcoides* spp., has been successfully used to bioremediate sites contaminated with chlorinated ethenes (32).

Strains DF-1 and o-17 are related to *Dehalococcoides* with 89% 16S rRNA gene identity to strain 195 and they reductively dehalogenate PCB from the *para* position and the *ortho* position respectively (9, 67). Strain DF-1 is the only isolated microorganism shown to be capable of growth by reductively dechlorinating PCBs. It can only use hydrogen as an electron donor and requires an unknown growth factor from a *Desulfovibrio* (33). Strain o-17 is grown as a mixed culture and uses acetate to drive reductive dehalogenation.

*Dehalobacter*

*Dehalobacter* spp. are members of the *Firmicutes* and are only known to respire halogenated compounds. *Dehalobacter restrictus* can reductively dehalogenate PCE and TCE to *cis*-dichloroethene (cDCE) and only uses hydrogen as an electron donor (21). Strain TCA is the first bacterium shown to dehalorespire chloroethanes and can use formate or hydrogen as an electron donor (58). Grostern *et al.* (16) used a combination of culture independent method to identify *Dehalobacter* sequences in a chloroethane dehalogenating mixed consortium. A *Dehalobacter* sp. has been identified in a coculture with *Sedimentibacter* sp. that is able to reductively dehalogenate β-hexachlorocyclohexane to MCB and benzene (62).

*Desulfitobacterium*

*Desulfitobacterium* spp. are also members of *Firmicutes* but show a marked difference in physiological characteristics compared to *Dehalobacter* and...
Dehalococcoides. Desulfitobacterium spp. can utilize a diverse range of electron acceptors such as nitrate, sulfite, and fumarate along with halogenated substrates. As a genus, it is capable of dehalogenating chloroethenes, -ethanes, -phenols, -butanes and more. When presented with multiple electron acceptors: nitrate, sulfite, 3-chloro-4-hydroxyphenylacetic acid (3Cl4OHPA), and fumarate, Desulfitobacterium dehalogenans reductively dehalogenates 3Cl4OHPA and nitrate simultaneously followed by sulfite and fumarate. Certain strains such as D. dichloroeliminans strain DCA1 reductively dehalogenate via dichloroelimination instead of hydrogenolysis (10). The genome of Desulfitobacterium hafniense strain Y51, a bacterium capable of reductively dehalogenating chloroethanes and PCE, contains two reductive dehalogenase genes including pceA (44, 60). The genome of Desulfitobacterium hafniense strain DCB-2, a bacterium capable of reductively dehalogenating chlorophenols and 3Cl4OHPA, is being annotated and tentatively contains seven RD genes. Strain DCB-2 has a larger substrate range than strain Y51 so a larger library of RD genes was expected. A genome comparison between this strain and Y51 will reveal evolutionary insight into the development of reductive dehalogenation.

Other Dehalogenating Microorganisms and Sediment Microcosms

Other dehalogenating bacteria include Sulfurospirillum multivorans (ε-Proteobacteria) and Geobacter lovleyi strain SZ (δ-Proteobacteria) (51, 59). S. multivorans can reductively dehalogenate PCE using either hydrogen or formate as an electron donor. It is also capable of using other electron acceptors such as fumarate and selenate. Its PCE reductive dehalogenase
(PCE RD) is the best-characterized RD (see below). It is constitutively expressed, and when grown on PCE is membrane-associated and when grown on fumarate is located in the cytoplasm. *G. lovleyi* strain SZ is unique in its ability to simultaneously dehalogenate PCE and reduce radionuclides like hexavalent uranium. It may play a major role in bioremediation of sites contaminated with toxic solvents and metals. *Photobacterium profundum* strain 3TCK (δ-Proteobacteria) contains a putative reductive dehalogenase gene but is not known to perform reductive dehalogenation (63).

Reductive dehalogenation has also been described in different environments though the activity has not been linked to any specific bacteria. In the case of dichlorobenzene, it has only been demonstrated in sediment microcosms. Bosma *et al* (5) showed that flow-through columns from Rhine River, Netherlands, sediments were able to reductively dehalogenate all three DCB isomers to MCB, with 1,2-DCB consumed first after 7 days. A microbial consortium derived from Rhine River sediments was able to reductively dehalogenate 1,2,4-TCB to MCB with 1,4-DCB being the major intermediate, and 1,3- and 1,2-DCB produced in trace amounts (35).

**Cometabolic Reductive Dehalogenation**

A large number of halogenated compounds are reductively dehalogenated as cometabolic reactions (18, 34, 49). As mentioned above, the final dechlorination of vinyl chloride to ethene in strain 195 is cometabolic. The vinyl chloride is unable to induce *tceA* and depends on the presence of higher-chlorinated ethenes. The removal of the final chlorine determines the toxicity of the end product and is a critical step in remediation. The reductive
dehalogenation of complex aromatic structures like polybrominated diphenyl ethers (PBDE) has been shown to occur in a variety of bacteria such as strain 195 and *S. multivorans* but only occurs when grown with PCE. It is unknown if the PCE RD is responsible for the reductive dehalogenation or if PCE stimulates a RD of unknown function. Sediment microcosms derived from the Saale River, Germany show the reductive dehalogenation of MCB to benzene to only occur during dehalogenation of higher chlorinated benzenes (45). Benzene is easier to degrade than MCB under anaerobic conditions to nontoxic endproducts such as carbon dioxide or methane (7).

**Biochemistry and Genetics of Reductive Dehalogenation**

*Common Features*

The reductive dehalogenation of halogenated compounds is carried about by reductive dehalogenases (RDs). Though phylogenetic relationship between dehalogenating bacteria is distant, features of their RDs are remarkably similar. Common features shared by these proteins include the presence of a twin arginine transport signal sequence (ssTAT), often used for transport of folded proteins containing cofactors into the periplasm, iron-sulfur cluster-binding motifs, corrinoid, and the presence of a second open reading frame (ORF) usually named the “B” protein i.e. *tceB* (Figure 1.3) (55). The B ORFs are predicted to encode hydrophobic polypeptides believed to serve as membrane anchors. It is hypothesized that the iron-sulfur cluster and corrinoid are involved in electron transfer. Chemical studies have shown that free cobalamin catalyzes reductive chlorination of chloroethenes and -benzenes while iron-sulfur clusters do not (14). The RD enzymatic cofactor vitamin B$_{12}$ apparently catalyzes reductive dehalogenation of chlorinated ethenes and
benzenes (14). Schmitz et al. (50) found the reductive dehalogenation of chloropropene by PceA in *S. multivorans* to proceed via radical formation from an donated electron from the B$_{12}$ cofactor. Isolation of the enzymes from strain 195 involved in reductive dechlorination of PCE to ETH had shown a single protein of 51 kDa, PCE RD, and a single protein of 61 kDa, TCE RD (29). The gene encoding TCE RD was subsequently cloned and sequenced using degenerate primers and found to share similar features to a previously isolated RD; PCE RD from *S. multivorans* and *ortho-*chlorophenol RD (CprA) from *Desulfitobacterium dehalogenans* (40, 61).

Figure 1.3. Schematic of RD gene structure and key protein features.

Substrate specificity in RDs shows a restricted range. Studies on TCE RD of strain 195 show that while it is able to dehalogenate both branched and brominated compounds, there is a clear preference for linear chlorinated compounds of two to three carbons (28). The substrate range of PCE RD of strain 195 has not been determined. The PCE RD of *Dehalobacter restrictus*
and *Desulfitobacterium* sp. strain Y51 is able to dehalogenate a wide range of compounds from TCE to hexachloroethane but not DCE (31, 60). The underlying explanation for the differences in substrate range is currently unknown. Because *Dehalococcoides* spp. do not grow to very high density it has been difficult to perform extensive biochemical analysis on their RDs, and sufficient material for enzyme assay can only be obtained from highly mixed-cultures containing *Dehalococcoides* (29, 39). Attempts to functionally express RD genes in *E. coli* thus far have not been successful (41, 48, 60). Other methods such as proteomics have been employed to monitor the expression and presence of RDs under various physiological conditions to study the molecular basis of *Dehalococcoides* spp. unique physiological abilities. Morris et al. (37) identified peptides of RDs from strain 195, strain CBDB1, the characterized mixed-culture KB1 and an uncharacterized TCE-dehalogenating mixed-culture from Savannah River National Laboratory site that could be used as markers of reductive dehalogenation activity.

**Horizontal Gene Transfer**

Horizontal gene transfer seems to play an important role in reductive dehalogenation. The *pceA* of *Desulfitobacterium hafniense* strain TCE-1 shares 100% nucleotide identity with the *pceA* of *Desulfitobacterium* strain Y51 and PCE-S and *Dehalobacter restrictus* (15, 36). Maillard et al (30) discovered the *pceA* of *D. hafniense* strain TCE-1 is located on a catabolic transposon, Tn-Dha1. Sequence comparison with the *pceA* region of strain Y51 and PCE-S revealed the presence of a similar transposon but not in *D. restrictus*. GC content, nucleotide usage and recombination gene analysis
reveal that 15 of the 19 RD genes, including \textit{tceA}, in \textit{D. ethenogenes} strain 195 are located in atypical foreign regions of the genome (47). Genomic analysis of strain CBDB1 also reveals markers of RD gene recombination events (26).

\textit{Regulation}

Most RD genes are flanked by predicted regulatory genes, indicating regulation on the transcript level. The RD genes of \textit{Dehalococcoides} spp. are located in close proximity to global regulator genes who are members of families such as Crp/Fnr, MarR, and two-component signaling regulators (25, 26, 39). These families regulate a diverse range of functions from antibiotic production to osmotic sensing (24, 52, 65). Through physiological and preliminary transcriptional studies, several RD genes of 195 have shown indications of being under transcriptional regulation. Resting cell activity assays with cells grown on TCE were able to reductively dehalogenate PCE, suggesting that \textit{pceA} was being co-expressed with \textit{tceA} or that both are constitutive (28). The \textit{tceA} of strain 195 does not have adjacent regulatory genes. RD gene expression in strain 195 appears to be temporally regulated. During TCE dehalogenation at the transition point from exponential to stationary phase, four RD genes of unknown function are up regulated (DET0173, DET0180, DET1535, and DET1545) (23).

The best-studied RD regulon is the ortho-chlorophenol gene cluster (\textit{cprTKZEBACD}) of \textit{Desulfitobacterium dehalogens} (46, 56, 57, 61). \textit{cprA} encodes for the chlorophenol reductive dehalogenase and the regulator of chlorophenol reductive dehalogenation, CprK, belongs to the fumarate nitrate
regulator family (FNR). \textit{cprK} is constitutively expressed at low levels but it only binds upstream of \textit{cprB} in the presence of chlorophenol and therefore, regulates chlorophenol reductase though metabolite activation. Activity studies done in the presence of conventional electron acceptors fumarate, sulfite, sulfate or nitrate, did not repress \textit{cprA} expression.

\textit{Electron Transfer}

The mechanism and electron flow of reductive dehalogenation is not very well understood. In studies of PceA and TceA using whole cells and crude extracts of strain 195 and reduced methyl viologen, which is considered unable to cross the cytoplasmic membrane, as an electron donor, reductive dehalogenation occurs, indicating that the reaction occurs in the periplasm (43). This is in contrast to the PceA of \textit{D. restrictus}, which was found to face the cytoplasm and required a menaquinone shuttle (53). In \textit{Desulfomonile tiedjei} strain DCB-1, a novel cytochrome \textit{c} was co-induced during reductive dehalogenation and is thought to be part of the electron shuttling process (27). In strains 195 and CBDB1 no upstream components in the electron transport chain have been identified though they each contain five hydrogenases (Hyc, Ech, Hup, Hym, and Vhu) (26, 54). They also contain an annotated formate dehydrogenase, though neither strain is known to use formate as a growth substrate. In strain 195, Hup and formate dehydrogenase show the highest levels of transcripts and were both detected with high peptide coverage in proteomic studies (38).
Objectives

There has been a large influx of information into the field of reductive dehalogenation with increasing numbers of dehalogenating microorganisms, putative RD gene sequences, and halogenated substrates. While demonstrating that *Dehalococcoides* 16S rRNA genes are present at a contaminated site is useful (19), it is now clear that 16S rRNA genes do not predict function, since strains FL-2, CBDB1, and BAV1 have essentially identical 16S rRNA gene sequences, yet show markedly different dehalogenation spectra. Identifying which genes are expressed during dehalogenation of different substrates may eventually allow clearer diagnoses of microbial activity at contaminated sites. My first objective is to define RD gene function in *Dehalococcoides ethenogenes* strain 195 (chapter two). My approach is to examine differential transcript levels and peptide sequences during growth on different chlorinated compounds.

Little is known about the microorganisms that are involved in the reductive dehalogenation of dichlorobenzenes (DCBs) and monochlorobenzene (MCB). Reductive dehalogenation of DCBs has only been observed in sediment microcosms and no microorganism has been identified to be involved in this process. Though thermodynamically favorable, MCB reductive dehalogenation to benzene has rarely been observed. My second objective is to enrich for dichlorobenzene reductive dehalogenation activity from a historically chlorobenzene-contaminated site using sediment microcosms (chapter three) and to characterize the microorganism(s) responsible (chapter four).
REFERENCES


42. **Ni, S., J. K. Fredrickson, and L. Xun.** 1995. Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase


CHAPTER TWO

EXPRESSION OF REDUCTIVE DEHALOGENASE GENES IN

DEHALOCOCCOIDES ETHENOGENES STRAIN 195 GROWING ON
TETRACHLOROETHENE, TRICHLOROETHENE OR 2,3-
DICHLOROPHENOL

Abstract

Reductive dehalogenase (RD) gene transcript levels in Dehalococcoides ethenogenes strain 195 were investigated using reverse transcriptase quantitative PCR during growth and reductive dechlorination of tetrachloroethene (PCE), trichloroethene (TCE), or 2,3-dichlorophenol (2,3-DCP). Cells grown with PCE or TCE had high transcript levels (greater than that for rpoB) for tceA, which encodes the TCE RD, pceA, which encodes the PCE RD, and DET0162, which contains a predicted stop codon and is considered nonfunctional. In cells grown with 2,3-DCP, tceA mRNA was less than 1% of that for rpoB, indicating that its transcription was regulated. pceA and DET0162 were the only RD genes with high transcript levels in cells grown with 2,3-DCP. Proteomic analysis of PCE-grown cells detected both PceA and TceA with high peptide coverage but not DET0162, and analysis of 2,3-DCP-grown cells detected PceA with high coverage but not TceA, DET0162, or any other potential RD. Cells grown with PCE or 2,3-DCP were tested for the ability to dechlorinate PCE, TCE or 2,3-DCP with H₂ as the electron donor. 2,3-DCP-grown cells were unable to dechlorinate TCE but

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1 Originally published with copyright 2007 from "Expression of reductive dehalogenase genes from Dehalococcoides ethenogenes strain 195 growing on tetrachloroethene, trichloroethene, or 2,3-dichlorophenol" by Fung, J.M., Morris, R.M., Adrian, L., and Zinder, S.H. Appl Environ Microbiol 73 (14) 4439-4445. Reprinted with permission from American Society of Microbiology.
dechlorinated PCE to TCE without a lag, and PCE-grown cells dechlorinated 2,3-DCP without a lag. These results show that 2,3-DCP-grown cells do not produce TceA and that DET0162 is transcribed but its translation product is not detectable in cells. They also demonstrate that PceA is bifunctional, also serving as the 2,3-DCP RD. Chlorophenols naturally occur in soils, and are good candidates for the original substrates for PceA.

Introduction

*Dehalococcoides ethenogenes* strain 195 reductively dechlorinates tetrachloroethene (PCE) and trichloroethene (TCE) to vinyl chloride (VC) and ethene (ETH) (21, 31). In addition to chlorinated ethenes, strain 195 has been found to reductively dechlorinate chlorobenzenes and other chloroaromatics (7), and more recently 2,3-dichlorophenol (2,3-DCP) and 2,3,4 trichlorophenol in the *ortho* position to 3-monochlorophenol (3-MCP) or 3,4-dichlorophenol respectively (1). The reduction of halogenated compounds by *Dehalococcoides* is carried out by membrane bound respiratory reductive dehalogenases (RDs) (12, 19, 26, 27), and although more than 90 RD-homologous genes have been identified in this genus (11, 15, 26, 30), little is known about their specific functions. PCE-RD (PceA) and TCE-RD (TceA) were first characterized in mixed dechlorinating enrichment-cultures containing strain 195, and were found to reductively dehalogenate PCE to TCE and TCE to VC and ETH, respectively (19). The gene encoding TCE-RD was subsequently cloned, sequenced and designated *tceA* (18).

The genome sequence of strain 195 (30) revealed 17 RD-homologous genes in addition to *tceA* (designated DET0079) and *pceA* (designated DET0318, J.
Magnuson, personal communication). Common features of RDs include the presence of a putative twin arginine transport signal sequence used for transport into the periplasm of folded proteins that can contain prosthetic groups, iron-sulfur cluster-binding motifs, and an adjacent "B" RD gene predicted to encode a small hydrophobic protein proposed to serve as a membrane anchor. Two of the RD-homologous genes may not be functional. One (DET0162) contains a verified TGA stop codon that would truncate the predicted gene product from 488 to 59 amino acids, and a shorter corresponding "B" gene (DET0163). DET0088 encodes a protein predicted to be 153 amino acids long corresponding to the C-terminal of other RDs, and lacks a corresponding "B" gene. Sixteen of the 19 RD genes in *Dehalococcoides* have transcriptional regulator genes in close proximity, including *pceA*, suggesting they are transcriptionally regulated. The genome sequence of *Dehalococcoides* strains CBDB1 revealed 32 potential RD genes (15), twelve of which share up to 95.4% amino acid sequence identity with RDs from strain 195, suggesting a partial overlap of substrates. Multiple RD genes have been identified in *Dehalococcoides* strains FL-2, KB1 (mixed culture), and BAV1 (14, 14, and 10, respectively) (10, 34). Although sequenced *Dehalococcoides* genomes share high sequence similarity and synteny among "housekeeping" genes, isolates harbor different suites of RD genes and exhibit different dehalogenation spectra. Identifying which RD genes are expressed during dehalogenation of different substrates can provide insights into reductive dehalogenase function and their potential activity at contaminated sites.
Here we report *D. ethenogenes* RD genes expressed during growth and the reductive dehalogenation of PCE, TCE and 2,3-DCP by comparing RD gene expression and corresponding dehalogenation activities in strain 195. Expression and activity results indicated that *tceA* was under transcriptional control, and LC/MS/MS proteomic approaches identified TceA protein fragments only in the presence of PCE and TCE, but not 2,3-DCP. *pceA* was the only intact RD gene expressed in cells grown with 2,3-DCP and is most likely the 2,3-DCP reductive dehalogenase, which suggests that PceA has broad substrate specificity.

**Materials and Methods**

**Chemicals.** Most chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) at the highest purity available and gases were purchased from Airgas East (Elmira, NY).

**Growth conditions.** *D. ethenogenes* strain 195 was cultured with PCE, TCE or 2,3-dichlorophenol as previously described (1, 20). Briefly, culture inoculum sizes were 2% (vol/vol) in either 27 ml culture tubes, 120 ml serum vials, or 1000 ml incubation containers containing 10, 50, or 500 ml of growth medium, respectively. Basal salts medium was amended with 2 mM acetate, a vitamin solution containing 0.05 mg of vitamin B$_{12}$ per liter, 10% (vol/vol) filter-sterilized anaerobic digestor sludge supernatant, and 1% (vol/vol) mixed butyrate-PCE culture extract (20). Doses of PCE, TCE and H$_2$ were added as previously described (20), and filter-sterilized stock solution of 2,3-DCP was added with a syringe at increasing doses of 30, 50, 75 µM. Culture tubes were sealed with Teflon-coated butyl rubber stoppers and incubated at 35 °C.
Reductive dechlorination of ethenes was monitored using a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector (8). Chlorophenols were analyzed using high-pressure liquid chromatograph (Beckman Coulter, Fullerton, CA) equipped with an Alltima C8 3µM-bead diameter column (length 53 mM, ID 7mm, Alltech, Deerfield, IL) at ambient temperature. The solvent for isocratic elution was acetonitrile:water:glacial acetic acid (50:50:0.1) at 1.5ml/min and chlorophenols were detected by their absorbance at 220nm. To monitor growth, cells were fixed with 25% formaldehyde, filtered onto 0.2µm GTBP Isopore membrane filters (Millipore, Billerica, MA), and stained with 5µg/ml 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI).

All resting cell assays and RNA and protein extractions were performed with cells harvested near maximum growth and dechlorination rates.

**Nucleic acid extraction.** Prior to extraction, 40ml of PCE-fed or 200ml of 2,3-DCP-fed cultures were placed on ice for 30 minutes, centrifuged at 12,000x g for 10 minutes at 4°C and resuspended in 500 µl of sterile ultrapure RNase-free water. DNA extractions were performed according to Fennell *et al.* (6) except for the elimination of a glass bead homogenization step. In short, cell pellets for RNA extractions were processed using the RNeasy mini kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). RNA was eluted with 50 µl RNase-free water and quantified by measuring absorbance at 260 and 280 nm on a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). RNA samples were treated twice with RNase-free DNase 1 (Fisher Scientific, Rockville, MD) to eliminate contaminating DNA.
**Reverse transcription and quantitative PCR.** First-strand cDNA synthesis reactions were performed with random hexamer primers using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to manufacturer’s instructions. Reactions were performed in 20 µl solutions containing 10 ng of RNA incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min in a DNA Engine PTC-200 thermocycler (MJ Research, Hercules, CA). Quantitative PCR amplifications were performed on triplicate samples using an ABI 7000 Real Time PCR machine (Applied Biosystems, Foster City, CA). Individual reactions contained iQ SYBR Green Super Mix (BioRad, Hercules, CA) with 1 ng of cDNA template and 200nM of primer targeting one of the 19 RD genes or the gene encoding the RNA polymerase beta subunit (rpoB) (Table 2.1). Primers were designed to target RD genes and not to amplify any other sequences in the *D. ethenogenes* genome. Specificity of each primer set was tested by PCR amplification and sequencing of amplified DNA. All primers were tested to amplify at minimum of 97% reaction efficiency for each experiment. PCR amplifications were carried out with the following parameters: 95°C for 10 min., 30 cycles of 95°C for 15s and 60°C for 1 min. Melting curve analysis and amplicon sequencing were used to screen for primer dimers, and RNA samples incubated without reverse transcriptase did not lead to a PCR product showing that no DNA was present. cDNA target amplifications were compared to DNA standards obtained by serial dilution of genomic DNA. RD expression levels were calculated from DNA standard curves generated during each run and with each primer pair and related to corresponding rpoB expression levels. In preliminary experiments, we found that using rpoB expression as standard provided the most uniform and reproducible results compared to other potential standards, including atpA and
the 16S rRNA gene. Rahm et al. (29) found that rpoB was highly expressed in mixed cultures containing *D. ethenogenes* that were actively reductively dechlorinating chloroethenes. Values presented and their standard deviations were taken from triplicate samples from at least two different cultures.

**Mapping transcriptional start sites.** The transcription start sites (TSS) of genes *tceA, pceA, infA* (DET0497, initiation factor IF-1), and DET1407 were identified using the 5’-rapid amplification of cDNA ends (5’-RACE) system according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). A series of two or three nested primers was designed starting 20 bp downstream from the translational start codon (Table 2.1). PCR product was sequenced and aligned with the upstream region of corresponding gene.

**Peptide and identification by NanoLC/MS/MS.** Membrane-enriched proteins were extracted from cells as described in Morris et al. (24) and in-gel-peptide samples were sent to the Cornell University Life Sciences Core Laboratory Center for analysis. Samples were reconstituted in 15 µL of 0.1% formic acid with 2% acetonitrile prior to mass spectrometry (MS) analysis. The nanoLC was carried out by an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). The gel-extracted peptides were injected using a Famous auto sampler onto a C18 µ-precolumn cartridge for on-line desalting and then separated on a PepMap C-18 RP nano column, eluted in a 60-minute gradient of 5% to 45% acetonitrile in 0.1% formic acid at 250 nL/min. The nanoLC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q
Table 2.1 Gene targets and corresponding primer designations and sequences

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a Primer name includes the position number of the first nucleotide the primer binds
b Primers were designed to target the predicted translational stop codon in DET0162
Trap from ABI/MDS Sciex (Framingham, MA) equipped with Micro Ion Spray Head ion source.

The data acquisition on the MS was performed using Analyst 1.4.1 software (Applied Biosystems) in the positive ion mode for information dependent acquisition (IDA) analysis. In IDA analysis, after each survey scan for \( m/z \) 400 to \( m/z \) 1550 and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied for detected ions based on different charge states and \( m/z \) values.

MS/MS data generated from nanoLC/ESI-based IDA analysis were interrogated using the ProID 1.4 search engine (Applied Biosystems) for database searching against the *Dehalococcoides* strain 195 database. One trypsin miscleavage, the carboxyamidomethyl modification of cysteine, and a methionine oxidation were used for all searches. Initial protein identification was limited to peptide hits with > 95% confidence. Subsequent identification was limited to at least one peptide with a Pro Group confidence score >95 and at least one additional peptide with a Pro Group confidence scores >20. Proteins identified by a single peptide (Figure 2.3) had confidence scores >95.

**Resting cell assay of reductive dehalogenase activity.** Reductive dechlorination resting cell assays were performed as described by Magnuson *et al.* and Nijenhuis *et al.* (18, 27) with the following modifications. Cells grown on PCE and DCP were concentrated 10- and 100-fold relative to their original volumes, respectively and were prepared with Ti (III) citrate as a reducing
agent. Assays were set up in 8ml crimp-top vials with either 1ml of cells for PCE and TCE reductive dechlorination assays, or 2ml of cells for the 2,3-DCP reductive dechlorination assay, along with 8.3mM Ti (III) citrate, 57% hydrogen in the headspace and 0.5-1mM chlorinated ethene or 20-50µM 2,3-DCP. Dechlorination was monitored as described above for chlorinated ethenes and phenols.

**Results**

**RD gene transcript levels.** Transcript levels of each of the 19 potential RD genes normalized to *rpoB* (29) in *D. ethenogenes* 195 was examined during exponential-phase growth of cultures on either PCE, TCE or 2,3-DCP (Figure 2.1). In cells growing on PCE or TCE, *tceA* and *pceA* transcript levels were several-fold higher than those for *rpoB*, as were those for DET0162, which is presumably nonfunctional because of a translational stop codon. We confirmed the presence of this stop codon in cultures we were studying by sequencing the PCR product from DET0162 using primers listed in Table 2.1. In PCE-grown cultures transcript levels of DET1559 approached those of *rpoB* but not in TCE-grown cells, a finding we confirmed several times. Transcripts from DET0180 in TCE-grown cells and DET1545 in PCE grown cells were near 10% of the levels of *rpoB* transcripts.

In cells grown with 2,3-DCP, the relative transcript levels of *pceA* were 10-fold higher than those in PCE or TCE-grown cells, as were those for DET0162. In contrast, those for *tceA* transcripts fell three orders of magnitude compared to cells grown on PCE or TCE, suggesting transcriptional control. The only other RD gene with transcripts approaching 10% of that for *rpoB* was DET0180.
Figure 2.1. Transcript levels for genes in the genome of *D. ethenogenes* strain 195 annotated as potential reductive dehalogenases, including *tceA* (DET0079) and *pceA* (DET0318) in cells grown on PCE, TCE or 2,3-DCP. All transcript levels were normalized to that of *rpoB* (R') and error bars represent standard deviations.

**Transcription start sites for tceA and pceA.** We used 5'-RACE to determine TSSs for *tceA*, *pceA*, and two "housekeeping" genes not involved in reductive dehalogenation that are likely to be highly expressed. DET0497 is annotated as encoding translation initiation factor 1 (*infA*) and is highly expressed in growing cells of *Escherichia coli* (3), whereas DET1407 was found to be one of the most abundant membrane-associated proteins in a proteomic survey of strain 195, and was hypothesized to encode part of the S-layer cell wall (25). The TSSs of these genes were determined to be 86-156
bp upstream of the predicted translational start codons (Figure 2.2A). Attempts to determine TSSs for DET0162 and rpoB were not successful.

Potential promoter regions upstream of these TSSs were examined. The canonical sigma\textsuperscript{70} promoter site is TTGACa-(16-19 bp)-TAtAaT-(5-9 bp)-TSS in which capitalized bases are present more than 50% of the time, and these two conserved hexamers are called –35 and –10 regions respectively (16). All four upstream regions had acceptable –10 hexamers, all beginning with TA and ending with T. Moreover, the infA and DET1407 upstream regions had TGTG motifs one base upstream of the predicted –10 region, known to obviate the need for a strong –35 region in \textit{Bacillus subtilis} (33), and tceA had a TG in the same position which can play a similar role in \textit{E. coli} (23).

The only gene we examined with a suitable -35 region was infA, which had the important TTG as the first three bases. DET1407 had essentially no match near -35, but curiously had a TTGACA located only 10 bp upstream of the –10 region. The potential –35 region for tceA had a 4/6 bp match, but lacked a beginning T, and only a 2/6 match could be found for the pceA –35 region. Matches to other potential sigma factor binding sites were not apparent.

We also examined the leader regions between the TSS and the translation start sites for homology with other potential leader regions. BLAST analyses revealed that the upstream region of tceA from strain 195 to be nearly 100% identical with upstream regions of close tceA homologues sequenced from diverse \textit{Dehalococcoides} enrichments and strain FL2 (14) (data not presented). Unfortunately, these sequences began downstream of the
predicted –10 and –35 regions so that these regions could not be compared.

Comparison of the upstream regions of tceA and the vcrA vinyl chloride RD of
Dehalococcoides strain VS (26) revealed 43% sequence identity overall,
including low identity in the predicted promoter region (Figure 2.2A) which
contains a canonical –35 hexamer. Interestingly, there is a conserved stretch
of 15 nucleotides with a single T to C transition mutation located in
approximately the same location within the two leader regions (Figure 2.2B).
The region upstream of the predicted translation start site of pceA is ca. 80%
identical over 200 bases with that of CbcbA1588 from Dehalococcoides strain
CBDB1 with only five nucleotide differences found in the promoter region (data
not shown). The nucleotide and predicted amino acid sequences of the two
genes are 86.1% and 93.7% identical respectively.

**Figure 2.2.** (A) Regions upstream of transcription starts determined by 5’-
RACE for tceA, pceA, infA and DET1407 from D. ethenogenes strain 195, and
the region upstream of vcrA from strain VS (26). Underlined bases represent
potential –10 regions, TG motifs upstream of –10, and potential –35 regions.
(B) Transcription leader regions upstream of vcrA and tceA showing region of
high sequence identity.

**Detection of RDs by LC/MS/MS proteomics.** Proteomic analyses were
performed on PCE- and 2,3-DCP-grown cells to detect RD polypeptides.
Similar to previous results (24, 25), TceA and PceA RDs were detected with high peptide coverage in membrane-enriched fractions from PCE-grown cells, and were among the top five polypeptides in terms of peptide coverage, along with the products of three genes annotated as co-chaperonin GroEL (DET1428), BNR/Asp box repeat domain protein (DET1407), and formate dehydrogenase (DET0187) (Figure 2.3). Twenty-three unique peptides corresponding to TceA (54% coverage) and 25 unique peptides corresponding to PceA (55% coverage) were identified in PCE-grown membrane-enriched protein fractions, and no peptides from other RDs were detected. The same dominant proteins were identified in membrane-enriched fractions from 2,3-DCP-grown cells, with the notable exception of TceA. Although fewer proteins were detected and overall peptide coverage was lower in samples from 2,3-DCP-grown cells, which contained less protein than those from PCE-grown cells because the cultures reached lower cell densities, nineteen unique peptides corresponding to PceA (51% coverage) were identified, whereas no peptides from other RDs were detected.
Figure 2.3. *D. ethenogenes* protein coverage obtained from PCE-grown cells, white bars, and 2,3-DCP-grown cells, grey bars. Peptide fragments identified by ESI and MALDI MS/MS of the five most highly expressed proteins identified in cell membrane-enriched fractions.

**RD activity.** We examined cells harvested from cultures growing on either PCE or 2,3-DCP for the ability to utilize either substrate independent of growth. PCE-grown cells dechlorinated PCE to TCE and small amounts of DCEs and VC (Figure 2.4A), similar to previous results (27), indicating the presence of PceA and TceA activities. PCE-grown cells also converted 2,3-DCP to 3-MCP with no lag and at rates ca. 3-fold lower than PCE dechlorination (Figure 2.4B). 2,3-DCP-grown cells dechlorinated PCE to TCE, but less chlorinated ethenes were not detected (Figure 2.4C). These cells reductively dehalogenated 2,3-DCP ca. 5-fold slower than PCE (Figure 2.4D).
Figure 2.4. Reductive dehalogenation activities of resting cells of *D. ethenogenes* strain 195 grown on PCE or TCE. (A) PCE dechlorination by PCE-grown cells. (B) 2,3-DCP dechlorination by PCE-grown cells. (C) PCE dechlorination by 2,3-DCP grown cells. (D) 2,3-DCP dechlorination by 2,3-DCP grown cells. Graphs show a representative vial from experiments run in triplicate.
Discussion

From previous results (18, 19) it was expected that the genes encoding TceA and PceA would be highly expressed in PCE-grown cells of *D. ethenogenes*, and indeed the transcript levels of these two genes were higher than that for *rpoB* and orders of magnitude higher than genes encoding the other RDs, with the exception of DET0162, (discussed below). Moreover, peptide coverage for TceA and PceA was high in membrane-enriched fractions of PCE-grown cells, in agreement with previous results (25). PceA is not needed for growth on TCE, yet we found high transcript levels in TCE-grown cells; however, Maymó-Gatell *et al.* (20) found that TCE-grown cells of strain 195 showed high PCE dehalogenation activity, indicating that PceA is present in TCE-grown cells. Transcripts of DET1559 were detected at levels about 10-fold lower than those for *tceA* and *pceA* in PCE-grown cells (Figure 2.1.) but peptides corresponding to this potential RD were not detected in membrane-enriched cell preparations in this study. However, DET1559 peptides were detected with low coverage in a PCE-grown mixed culture containing *D. ethenogenes* (24) as were peptides from DET1545 in a pure culture preparation different from the one used in these studies. Rahm *et al.* (29) examined the temporal expression of a select group of RD genes from a mixed culture containing *D. ethenogenes* and found, similar to this study, that transcript levels of *tceA*, *pceA*, DET0162 and DET1559 increased during PCE reductive dehalogenation. In contrast, DET1545 transcripts were also detected though they did not reach similar maximum transcript levels like that of *tceA* until after all PCE was reductively dehalogenated to VC. Thus, there appears to be some variability in the detection of RDs expressed at lower levels than *tceA* and *pceA*. 
Transcript levels for *pceA* were higher relative to *rpoB* in 2,3-DCP-grown cells than they were in PCE-grown cells. Moreover, PceA was the only RD with detected in proteomic analyses of 2,3-DCP-grown cells. In contrast, *tceA* transcript levels were over two orders of magnitude lower than *rpoB*, peptides from TceA were not detected in 2,3-DCP-grown cells, and 2,3-DCP-grown cells did not have detectable TCE RD activity, indicating that *tceA* is not expressed in 2,3-DCP-grown cells.

It is not surprising that evidence for regulation of expression of RD-homologous genes in *Dehalococcoides* spp. is beginning to accumulate, since many are located adjacent to genes predicted to encode transcriptional regulators (15, 30). Johnson *et al.* (13), in their studies of a TCE-grown enrichment culture from Alameda Naval Air Station that contained *Dehalococcoides* spp., reported increased levels of *tceA* mRNA in starved cells given TCE, *cis*-DCE, *trans*-DCE, or 1,1-DCE, but not PCE or VC. These findings suggest that the molecular mechanism of control over *tceA* is finely tuned to recognize specific halogenated compounds. This culture did not use PCE and therefore could not produce TCE potentially needed to induce *tceA*. In strain 195, *tceA* was induced in cells growing on TCE or PCE (which is metabolized to TCE) but not in cells growing on 2,3-DCP. Curiously, unlike most other RD genes, there are no genes with strong resemblances to transcription regulators adjacent to *tceA*. Of the adjacent genes, DET0080 is annotated as having unknown function (www.tigr.org/tdb/mbd), but has similarity to the ArsR family of regulators below the noise cutoff in a hidden Markov model search. While this gene product is a possible candidate for
regulating *tceA* expression, it is also possible that regulatory circuits encoded elsewhere in the chromosome play a role in regulation.

These results also suggest that the PceA RD is responsible for dechlorination of 2,3-DCP. It has the highest transcript levels in 2,3-DCP-grown cells and it was detected with high peptide coverage, whereas no other potential RD was detected. Further bolstering this proposition is the finding that PCE-grown cells dechlorinated 2,3-DCP without a lag (Figure 4) indicating the appropriate reductive dehalogenase was already present. In an analogous experiment, 2,3-DCP-grown cells dechlorinated PCE to TCE without a lag but did not dechlorinate TCE. In *Desulfitobacterium* strain PCE1, a member of the *Firmicutes*, 2-chlorophenol and PCE dechlorination are carried out by distinct RD enzymes with little cross reactivity towards the other substrate (32). However, the 2-chlorophenol RD from this organism does not require adjacent chlorines, as are present in 2,3-DCP and PCE, so that it clearly has different substrate specificity from the 2,3-DCP-dechlorinating RD in strain 195, and its gene sequence is phylogenetically distinct from *pceA*. In light of the large number of potential RD genes present in the *D. ethenogenes* genome (30) with unknown function, it is surprising that PceA rather than one of the other RDs uses 2,3-DCP, but this finding does suggest an evolutionary route for *Dehalococcoides* spp. to take from utilizing chlorophenols, considered to be naturally occurring substrates, especially in soils (4), to utilizing PCE, considered a xenobiotic.

*Dehalococcoides* strain CBDB1 is adept at dehalogenating chlorinated aromatics (2), including chlorophenols (1). Similar to strain 195, strain CBDB1
reductively dehalogenates 2,3-DCP from the *ortho* position and reductively dehalogenates PCE to TCE and *trans*-dichloroethene (L. Adrian, personal observation). Strain CBDB1 can also reductively dehalogenate higher chlorinated chlorophenols such as 2,3,4-trichlorophenol from the *meta* position. In strain 195, the translated sequence of *pceA* has 93.7% amino acid identity with CbdbA1588 from strain CBDB1 (15). Downstream of cbdbA1588 are predicted histidine kinase (CbdbA1590) and response regulator (CbdbA1589) genes of a two component regulatory system that have 92.1% and 91.1% amino acid identity to their homologs associated with *pceA* in strain 195. Morris *et al.* (24) recently found that in cultures of strain CBDB1 grown on 2,3-DCP, CbdbA1588 peptides were detected with 31% coverage, while in contrast to strain 195, CbdbA080, homologous to DET1559, was also detected with 13% peptide coverage, and CbdbA088, with no homologs in strain 195, was detected with 10% peptide coverage. This result suggests that the Cbdb1588 serves as a 2,3-DCP reductive dehalogenase in strain CBDB1, but with the detection of multiple RDs the situation may be more complicated in this organism.

Since little is known about transcription initiation and regulation in *Dehalococcoides* spp., we determined the TSSs and examined upstream regions of two expressed RDs, *tceA* and *pceA*, and two "housekeeping" genes expected to be highly expressed, *infA*, and DET1407. The genome of strain 195 contains a gene predicted to encode a sigma"^70" homolog (DET0551) (17) as well as two smaller genes (DET0169 and DET1348) predicted to encode sigma W and extracellular sigma factor (ECF) homologs respectively, which usually regulate accessory functions. The predicted amino acid sequence of
DET0551 contains the conserved residues within its region 2.4 and 4.2 that are involved in nucleotide contact with the –10 and –35 hexamers in other sigma70 homologues (5, 9, 22), suggesting that its recognition sequences should resemble the canonical ones. Thus it is reasonable to expect some housekeeping and other genes to show sigma70 consensus binding sites. In all four genes, there was a reasonably good match to the consensus –10 sigma70 binding site, but only infA had a close match to the –35 consensus sequence, as does the vcrA gene from Dehalococcoides strain VS (Figure 2.2A). Both infA and DET1407 have potential extended –10 regions with a TGTG motif (33), and it is likely that this allows DET1407 to be transcribed in the absence of an acceptable –35 region. The region upstream of the tceA TSS has a TG motif extending its –10 region which may allow transcription despite the moderate match of its –35 region (23), whereas the pceA has no TG element and a poor –35 match. In both RD genes, binding by an activator may be needed for transcription, and in the case of pceA perhaps this function is provided by the response regulator of the two-component system predicted to be encoded by DET0315 and DET0316 adjacent to it. Finally, the conserved 15 bp sequence in the leader regions of tceA from strain 195 and vcrA from strain VS may bind homologous regulatory proteins.

Since DET0162 was not considered to encode a functional RD, it was surprising that it showed high transcript levels in cells grown in PCE, TCE, or 2,3-DCP; however, peptides from its translation product were not detected in any cells in this or a previous (25) study, suggesting that it is either not translated or that the translation product is unstable, and it is therefore a nonfunctional pseudogene in the process of degradation. It was recently
suggested that pseudogenes are a common feature of microbial genomes (28). While we were unable to identify a transcription start for DET0162 using 5'-RACE, there is an acceptable –10 region 108 bases upstream of the predicted translation start, similar to other genes (Figure 2), with TTGACA, a perfect –35 region match, 16 bases upstream of that, making its transcription possible. Why this presumably nonfunctional gene showed such high transcript levels is unclear.

The list of potential RD genes with unknown functions in *Dehalococcoides* spp. has increased rapidly over the past several years, while the list of halogenated substrates has increased at a slower pace. *Dehalococcoides* spp. grow to low densities making traditional protein purification techniques and genetic analyses of RDs difficult, but PCR-based measurements of transcripts and sensitive proteomic techniques allowed us to identify RD genes expressed in *D. ethenogenes* cells using different electron acceptors, and these techniques were supplemented by measuring RD enzymatic activity in cells. This approach should continue to be useful in identifying RDs involved in using other substrates by this microbial group important to bioremediation.

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CHAPTER THREE
REDUCTIVE DEHALOGENATION OF DICHLOROBENZENES AND MONOCHLOROBENZENE TO BENZENE IN MICRO COSMS

Abstract
Anaerobic microcosms were constructed using sediments from a historically chlorobenzene-contaminated site and were provided with yeast extract as an electron donor. In these methanogenic microcosms, all three isomers of dichlorobenzene (DCB) were reductively dehalogenated to monochlorobenzene (MCB) when added together or individually, with 1,2-DCB dehalogenation being the most rapid and 1,4-DCB the slowest. When nearly all of the DCBs were consumed, benzene was detected and its accumulation was concomitant with MCB disappearance. Small amounts of toluene were also detected along with benzene. Subsequent MCB doses were also converted to benzene, and benzene reached levels in excess of 5,000 µmol/L in some microcosms. An initial DCB dose stimulated, and in some cases was necessary for, MCB dehalogenation. Subsequent doses of DCB and MCB were dehalogenated more rapidly than previous ones, suggestive of a growth-related process. Addition of a ca. 4% inoculum from microcosms that had consumed DCBs or MCB stimulated DCB and MCB dehalogenation in fresh microcosms, also indicative of growth and suggests that the chlorobenzene-dehalogenating microorganisms in these microcosms are good candidates for bioaugmentation at anaerobic DCB or MCB contaminated sites.
Introduction

Dichlorobenzenes (1,2-, 1,3-, and 1,4-DCB) and monochlorobenzene (MCB) have been widely used in industry as chemical stocks, solvents or industrial surface cleansers, and over time have become common groundwater contaminants. In aerobic environments, microorganisms degrade DCBs and MCB by well-characterized pathways involving hydroxylation by oxygenases ultimately leading to substrates metabolized via the citric acid cycle (16, 30). However, DCBs and MCB are dense non-aqueous phase liquids (DNAPLs) and can migrate to anaerobic regions in the subsurface making them inaccessible to aerobic degradation. In anaerobic environments, halogenated compounds such as chloroethenes and chlorobenzenes can serve as terminal electron acceptors and be reductively dehalogenated by bacteria such as Dehalococcoides spp., Desulfitobacterium spp., Dehalobacter spp., and Sulfurospirillum spp. (18, 35). The complete reductive dehalogenation of DCBs and MCB may prove a practical means of bioremediation, since benzene, although toxic itself, can be transformed into non-toxic products such as CO$_2$ or CH$_4$ by anaerobic microbial communities (8, 9, 22). However, little is known about the microorganisms involved in the reductive dehalogenation of DCBs and MCB.

In the case of cultured microorganisms, only reductive dehalogenation of chlorobenzenes with three or more chlorines has been observed. Dehalococcoides strain CBDB1 reductively dehalogenates hexachlorobenzene (HCB), pentachlorobenzene (PeCB), all tetrachlorobenzenes (TeCB) and 1,2,3- and 1,2,4-trichlorobenzene (TCB), with a preference for doubly flanked chlorines, to a mix of 1,3- and 1,4-DCB (1, 19,
Dehalococcoides strain 195 (98% 16S rRNA gene identity to strain CBDB1), has a smaller chlorinated benzene substrate range with HCB, PeCB, 1,2,3,4-TeCB and 1,2,4,5-TeCB and no clear preference for the removal of doubly or singly flanked chlorines (14). The more distantly related bacterium DF-1 (89% 16s rRNA gene identity with strain 195) is able to reductively dehalogenate HCB, PeCB, and 1,2,3,5-TeCB to mainly 1,3,5-TCB (37).

Dehalococcoides and its relatives also dechlorinate other aromatics such as chlorophenols, polychlorinated biphenyls, and polychlorinated dibenzofurans and most likely play an important role in the attenuation of chlorinated aromatics in the environment (2, 5, 7, 14, 38). Other microorganisms such as Desulfitobacterium spp. and Desulfomonile, members of the Firmicutes and Proteobacteria respectively, reductively dehalogenate chlorinated aromatics such as chlorophenols and chlorobenzoates, but chlorinated benzene dehalogenation has not been demonstrated in these organisms (15, 28).

Dehalogenation of DCBs to MCB and MCB to benzene has only been demonstrated in microcosms, and no specific organisms have been identified as involved in these processes. In studies of HCB and PeCB reductive dehalogenation in sediments from Niagara Falls, New York, Ramanand et al. (33) demonstrated the reductive dehalogenation of all three DCB isomers to MCB in microcosms, though DCBs were never given as the sole substrates. Bosma et al. (6) showed that flow-through columns from Rhine River, Netherlands, sediments were able to reductively dehalogenate all three DCB isomers to MCB, with 1,2-DCB consumed first after 7 days. Masunaga et al. (24) found that estuarine sediments consumed 1,2-DCB at higher rates than the other two isomers. Microcosms from various contaminated US sites (32)
also favored 1,2-DCB, which was dehalogenated to MCB, and 1,4-DCB was
dechlorinated the most slowly, if at all. A microbial consortium derived from
Rhine River sediments was able to reductively dehalogenate 1,2,4-TCB to
MCB with 1,4-DCB being the major intermediate, and 1,3- and 1,2-DCB
produced in trace amounts (27).

Though thermodynamically favorable (10), reductive dehalogenation of MCB
to benzene has been rarely observed. In sediment microcosms from the
Saale River, Germany, fed DCBs, small amounts of benzene were produced
along with MCB, but only during DCB reductive dehalogenation in a seemingly
cometabolic process (31). Similar results were obtained with samples from
Robins Air Force Base, Georgia (32). More recently, Nijenhuis and colleagues
(21, 29) used natural carbon isotope fractionation, and $^{13}$C-MCB-fed biotrapsto obtain evidence for MCB dechlorination to benzene in the chlorobenzene-
contaminated Bitterfeld site in Germany.

In this study, sediment from a historically chlorobenzene-contaminated site
was used to establish microcosms and reductive dehalogenation activity of
DCBs and MCB was investigated. We describe the complete reductive
dehalogenation of DCBs and MCB to benzene under methanogenic
conditions.

**Materials and Methods**

**Chemicals.** All chlorobenzenes, benzene and toluene were purchased from
Sigma-Aldrich at the highest purity available. 1,4-dichlorobenzene was
solubilized in 99.5% ethanol (Aldrich) to make a 1M solution. Gases were purchased from Airgas East.

**Sediment samples.** Sediment samples were obtained from a water-saturated drainage ditch at the DuPont Chambers Works site adjacent to the Delaware River in Salem County, New Jersey, a chemical synthesis and waste treatment facility that has been historically contaminated with chlorinated benzenes and anilines as well as other chemicals. The samples were dark brown, and no chlorobenzenes or organic compounds other than methane were detected by gas chromatographic analyses (see below) of headspace samples from microcosms prepared from these sediments without additions. Sediment samples were stored in plastic 1-liter bottles aerobically at 4°C in the dark, and samples for microcosm studies were taken from layers below the surface and were presumably anaerobic as evidenced by methanogenesis. Sediments stored this way maintained anaerobic reductive dechlorination and methanogenic activity for over a year. Three batches of sediment samples of ca. 4 liters each were used, obtained in December 2005, March 2006, and June 2007.

**Microcosms.** Unless stated otherwise, all solutions were prepared anaerobically by flushing with high purity N₂ gas. Microcosms were prepared inside an anaerobic glove box in 125 ml serum vials and sealed with Teflon™-coated butyl rubber stoppers and aluminum crimps. To each vial, 20 g of sediment (wet wt) was added to 50 ml of anaerobic deionized water. For inoculated microcosms, 2 ml of slurry from an actively reductively dehalogenating microcosm (ca. 0.8 g sediment) was transferred using a
syringe with an 18 ga. needle into a microcosm containing 18 g fresh sediment in 50 ml of deionized water. Microcosms were amended with 0.2 g/L yeast extract to serve as an electron donor and provide nutrients, and 1.0 g/L sodium bicarbonate as a buffer. The headspace of all microcosms was flushed using 70% N₂/30% CO₂ to remove H₂ from the headspace atmosphere, and chlorinated benzenes were added by syringe either neat, or as a 1M ethanol solution for 1,4-DCB, and this ethanol also could be fermented and indirectly serve as an electron donor for reductive dehalogenation and methanogenesis. Subsequent doses of chlorobenzenes were accompanied by doses of yeast extract. Killed controls were autoclaved at 121°C for 45 minutes. Microcosms were incubated shaking at 30°C in the dark. Results presented are for individual microcosms, but all experiments were done in triplicate with similar results in replicates, and all experimental results were repeated at least once.

**Analytical Methods.** Dichlorobenzenes, benzene and methane were detected by headspace analysis using Perkin-Elmer 8500 gas chromatograph with flame ionization detector and Rtx-35 (35% diphenyl-65%dimethyl polysiloxane, 60 meters_0.53mm with 1.5 μm film thickness) capillary column (Restek). Both detector and injector temperatures were 210°C. The initial oven temperature was 75°C, and increased to 152°C at 12°C/min, then to 159°C at 3°C/min, and to 210°C at 20°C/min which was held for 2 minutes. Compounds were identified using retention times of chemical standards and peak areas were calculated using Peak Simple software.
Benzene and toluene identifications in initial studies were verified using a Hewlett Packard 6890 series GC system equipped with a HP 5973 Mass Selective Detector and HP 5 capillary column (Hewlett-Packard). The detector was kept at 280°C and injector at 255°C. The oven program had an initial temperature of 120°C held for 3 minutes followed by an increase to 220°C at 5°C/min, 220°C to 290°C at 10°C/min, and a final hold at 290°C for 2 minutes. Mass spectra were compared to those for authentic standards.

Results

Reductive Dehalogenation of DCBs. Anaerobic microcosms were initially fed a mixture containing approximately 400 µmol/L (nominal concentration - µmoles added per liter of liquid volume) of each DCB isomer. There was difficulty obtaining an accurate mass balance of chlorinated benzenes in microcosms due to their apparent adsorption, especially DCBs, to sediment material and syringes. Therefore, appearance of daughter products was considered more reliable for quantification than disappearance of substrates. Figure 3.1A shows results for a typical microcosm receiving all three DCB isomers. MCB was detected after 2 days of incubation and reached high concentrations at 7 days (note different scale for MCB), when benzene was also detected. 1,2-DCB was no longer detected after 7 days, 1,3-DCB after 9 days, and 1,4-DCB after 11 days (Figure 3.1A). The lag times for MCB appearance varied from 2 to 7 days in microcosms depending on sediment sample, and once activity started, the trends of reductive dehalogenations were similar, with disappearance of DCB isomers following the same order, and appearance of MCB followed by benzene. The identity of benzene was confirmed by gas chromatography/mass spectrometry. CH₄ was the only
product detected in headspaces of microcosms incubated with all additions except DCBs, and no dechlorination products were detected in autoclaved microcosms incubated with DCBs (data not presented).

Benzene slowly accumulated while DCBs were still present; however, once DCBs were consumed, its production increased considerably concomitant with MCB decrease (Figure 3.1B), indicating that MCB was reductively dehalogenated to benzene. Subsequent doses of DCBs to microcosms were consumed more rapidly than the initial dose, with transient MCB accumulation and further buildup of benzene. In microcosms that had begun producing benzene, a peak co-migrating with toluene was often detected in gas chromatograms, always in amounts less than 5% those of benzene. The identity of this peak as toluene was confirmed by gas chromatography/mass spectrometry (data not presented). The production of toluene by microcosms was unpredictable. For example, a duplicate microcosm to the one presented in Figure 3.1A produced comparable amounts of benzene, but less than 25% of the amount of toluene, and in some microcosms, toluene was barely detectable (data not presented).
Figure 3.1. Reductive dehalogenation of dichlorobenzene (DCB) isomers to monochlorobenzene (MCB) and benzene (ben) in (A) microcosm given 1,2-DCB, 1,3-DCB, and 1,4-DCB, and (B) the same microcosm given several doses of DCBs as indicated by the arrows showing increase production of benzene.

When added individually to microcosms, each of the three DCB isomers was converted to MCB followed by benzene (Figure 3.2A, B, C) with 1,2- and 1,3-DCB being the fastest and 1,4-DCB the slowest, a pattern found in multiple repetitions of this experiment. Both 1,2- or 1,3-DCB-fed microcosms reductively dehalogenated MCB to benzene with 1,3-DCB typically showing shorter lags than 1,2-DCB, whereas benzene production in 1,4-DCB-fed microcosms was inconsistent across sediment samples. When benzene production did occur in microcosms fed 1,4-DCB, it was slower compared to 1,2- or 1,3-DCB-fed microcosms, and reached lower levels.
Reductive dehalogenation of individual dichlorobenzene (DCB) isomers to monochlorobenzene (MCB) and benzene (ben) in (A) microcosm given 1,2-DCB and (B) microcosm given 1,3-DCB and (C) microcosm given 1,4-DCB.

**Reductive Dehalogenation of Monochlorobenzene.** Microcosms derived from the first two sediment samples we studied (see Methods) given solely MCB did not show any benzene production after monitoring up to 100 days (Figure 3.3A) in several attempts using different MCB concentrations. The third, most recently obtained, sediment sample exhibited MCB reductive dehalogenation activity (Figure 3.3B) and consumed the first feeding of 1,100 µmol/L MCB in 45 days and the second feeding in 10 days, followed by three more feedings, accumulating over 5,000 µmol/L benzene. Autoclaved control microcosms did not show MCB reductive dehalogenation activity (data not shown).

Microcosms from the first two sediment samples that had converted a dose of DCBs mainly to benzene were able to reductively dehalogenate subsequent additions of MCB (Figure 3.3C), in contrast to sediments receiving MCB alone (Figure 3.3A), thus demonstrating a stimulatory effect of prior consumption of DCBs on MCB dehalogenation.
Microcosm inoculation experiments. We examined the effect of addition of sediments that had consumed doses of DCBs and MCB on microcosms containing "naïve" sediments. Addition of 2 ml sediment slurry (ca.4% v/v) from a DCB-fed microcosm that had consumed three doses of DCBs accelerated the reductive dehalogenation of all three DCB isomers and MCB (Figure 3.4A). In microcosms fed all three DCBs, 400 µmol/L of 1,2-, 1,3- and 1,4-DCB were nearly undetectable in 3, 3, and 7 days, respectively. whereas in uninoculated controls, disappearance required 7, 9 and 11 days respectively (data not presented). After DCBs were nearly depleted, the rate of reductive dehalogenation of MCB to benzene increased. Inoculation of microcosms using individual DCB isomers also increased their consumption, including 1,4-DCB (Figure 3.4B), the complete reductive dehalogenation of which was not otherwise reliable.
To test whether addition of sediments adapted to MCB led to differences in performance, a culture that had consumed three doses of MCB following an initial dose of DCB (Figure 3.4C) was used as an inoculum. DCB disappearance was rapid in microcosms with these inocula (Figure 3.5A), and in contrast to DCB-fed ones, benzene accumulated rapidly from the beginning with little evidence of inhibition by DCBs. In microcosms fed MCB alone (Figure 3.5B), benzene accumulation was detected with little or no lag period, and subsequent MCB doses were consumed and produced benzene at increasing rates. Uninoculated control microcosms from the same batch of sediments, which was the second one, did not show benzene production after 60 days (data not presented). These MCB-dehalogenating microcosms were used to inoculate a subsequent series of MCB-fed microcosms, and this activity has been transferred several times subsequently (data not presented).

Figure 3.4. Reductive dehalogenation of dichlorobenzene (DCB) to monochlorobenzene (MCB) and benzene (ben) in inoculated microcosms where inoculum was given DCBs. (A) given 1,2-DCB, 1,3-DCB, and 1,4-DCB and (B) microcosms given 1,4-DCB.
Figure 3.5. Reductive dehalogenation of dichlorobenzene (DCB) to monochlorobenzene (MCB) and benzene (ben) in inoculated microcosms where inoculum was actively dechlorinating solely MCB. (A) given 1,2-DCB, 1,3-DCB, and 1,4-DCB and (B) microcosms given MCB and maintained on MCB as indicated by the arrows.

Discussion

These studies have demonstrated that microcosms constructed using Chambers Works sediments can reductively dehalogenate all three DCB isomers to MCB. Similar to other results (6, 31, 32), 1,2-DCB was used most readily and 1,4-DCB was used the slowest. It is common for adjacent chlorines in chloroaromatics to be more readily dehalogenated than those ortho or para to each other (2, 3, 6, 13). Subsequent DCB doses were consumed more rapidly than initial ones, which is evidence that DCB dehalogenation to MCB was a growth-related process. Further evidence for growth was the much higher activity in microcosms inoculated with material from DCB-dehalogenating microcosms. This behavior is consistent with DCBs serving as electron acceptors for anaerobic respiration similar to other chlorinated organic compounds including more highly chlorinated benzenes(3).
The greatest difference between these microcosm studies and previous ones is the large amount of benzene produced from DCBs and MCB and the timing of benzene production. Nowak et al. (31) and Quistorff (32) detected only 10-50 µmol/L benzene, which was only a small percentage to the MCB produced, and in both cases benzene production occurred only during DCB dechlorination to MCB, suggesting that it was a side reaction concurrent with DCB dehalogenation. In contrast, benzene in some of our samples accumulated to over 5000 µmol/L and its accumulation did not require concurrent metabolism of DCBs, and indeed seemed to be inhibited during DCB dehalogenation in some instances. There was no evidence for benzene consumption in microcosms where it accumulated in large amounts. Electron acceptors for benzene oxidation other than bicarbonate were unlikely to be available since the sediments were actively methanogenic, and the highly reducing conditions in these microcosms that received large amounts of electron donor are unlikely to be favorable for the syntrophic reactions involved in methanogenic benzene utilization (36). Moreover, the high benzene concentrations may have been toxic to organisms capable of metabolizing it further.

Small amounts of toluene accompanying benzene were detected in these microcosms. The significance of toluene production is unclear but it should be mentioned that small amounts of toluene were also found in methanogenic benzene-degrading microcosms (36), and may be a side-product of activated forms of benzene such as radicals that may be formed during reductive dehalogenation (34).
MCB dehalogenation was considerably slower and less robust than 1,2- or 1,3-DCB dehalogenation, and in some samples did not occur at all unless an initial dose of DCBs was added. While the increasing rates of benzene accumulation in MCB-fed microcosms and the ability to transfer MCB-dehalogenating activity to naïve microcosms suggest MCB dehalogenation is growth-related, the slower rate, stimulation by a previous dose of a more chlorinated substrate, and MCB utilization after DCBs are depleted are reminiscent of the co-metabolic reductive dehalogenation of vinyl chloride to ethene by *Dehalococcoides ethenogenes* strain 195 (26). The stimulatory effects of DCBs on MCB dehalogenation are most simply explained by positing that some or all of the organisms involved in DCB dehalogenation can also use MCB, and after their numbers increase while growing on DCBs, switch to the more slowly-utilized MCB upon DCB depletion. However, there may be more complex explanations, such as stimulation of growth of MCB dehalogenators by nutrients produced by DCB dehalogenators. As an example of nutritional crossfeeding, it was recently shown that growth of the DF-1 organism on PCBs required unknown factors from a *Desulfovibrio* culture (25). Moreover, the inability of some samples to use MCB unless a prior dose of DCBs was given is difficult to explain by a simple two-stage growth model.

The ability of the microorganisms in these microcosms to reductively dehalogenate DCBs and MCB relatively rapidly and our ability to transfer this activity to naïve microcosms suggests that organisms in these microcosms have potential as inoculants for bioaugmentation at anaerobic sites where these compounds are persistent, a situation analogous to the addition of
Dehalococcoides-containing cultures to chloroethene-contaminated sites apparently stalled at dichloroethene (12, 17, 23). However, benzene itself is toxic with a lower EPA-mandated drinking water limit than DCBs or MCB (http://www.epa.gov/safewater/contaminants/index.html). Thus it is imperative that treatment schemes also include benzene biodegradation. Moreover, our findings that significant amounts of benzene can be produced from MCB should be taken into account in attempts at modeling the environmental fates of DCBs and MCB.

More needs to be learned about the microorganisms dehalogenating DCBs and MCB in these microcosms, especially how well they perform at *in-situ* DCB and MCB concentrations that are typically well below those used in these studies. We are initiating microbiological and molecular ecological studies on these microcosms to begin to answer these questions.

**Acknowledgements**

We thank the DuPont Corporation for providing sediment and financial support. We are thankful to Clifford Fung for technical assistance in microcosm work. We also would like to thank Christopher DeRito and Anthony Hay for their assistance with the GC/MS.
REFERENCES


CHAPTER FOUR
PRELIMINARY CHARACTERIZATION OF MICROBIAL ENRICHMENT
CULTURES THAT REDUCTIVELY DECHLORINATE DICHLOROBENZENES
TO MONOCHLOROBENZENE

Abstract
Microbial reductive dechlorination of highly-chlorinated benzenes is well characterized, whereas little is known about reductive dechlorination of the important groundwater pollutants dichlorobenzenes (DCBs) and monochlorobenzene (MCB). I previously described sediment microcosms that reductively dechlorinated all three DCB isomers to MCB and benzene. From these microcosms, transfers (ca 4% v/v) were successfully made and maintained on a mineral salts medium supplemented with yeast extract, hydrogen, 2-bromoethanesulfonate, sodium sulfide, and vitamins. Cultures were given all three DCB isomers together or each individually. Transfers fed all three DCBs completely reductively dechlorinated 1,2-DCB to MCB with slight dechlorination of 1,3-DCB and no dechlorination of 1,4-DCB. In cultures fed 1,3-DCB alone, complete reductive dehalogenation to MCB was observed after 8 days and transfers with 1,4-DCB showed a longer lag of 19 days. In seventh transfer cultures reductively dehalogenating 1,2-DCB, low amounts of benzene were detected though never more than 0.44% of MCB produced. In transfers fed MCB, reductive dechlorination to benzene was not observed. Transfers into medium containing 0.2 mg/ml vancomycin showed no reductive dechlorination, indicating that Dehalococcoides spp., which lack a peptidoglycan cell wall and are vancomycin-resistant, were not the causative agent. These cultures have now been transferred 11 times and maintained.
the ability to dechlorinate 1,2-DCB to MCB. A 16S rRNA gene clone library of
the fourth transfer culture contained sequences matching >99% identical to
that of Dehalobacter restrictus, a known dehalogenator. Further analysis
using quantitative real time PCR and specific primers showed an increase in
Dehalobacter population during reductive dehalogenation. Characterization of
these cultures is underway with the goal of identifying and isolating the
organisms responsible for reductive dechlorination.

Introduction
Dichlorobenzenes (DCBs) and monochlorobenzene (MCB) have been used as
industrial solvents and made their way into the environment to become
prevalent contaminants. In aerobic environments, biomineralization of DCBs
and MCB can be carried out by organisms such as Pseudomonas (29). In
anaerobic environments, halogenated compounds such as chloroethenes and
chlorobenzenes can serve as terminal electron acceptors and be reductively
dehalogenated by bacteria such as Dehalococcoides spp., Desulfitobacterium
spp., Dehalobacter spp., and Sulfurospirillum spp. (17, 34). One potential
approach to DCB bioremediation is to stimulate organisms to carry out their
complete reductive dehalogenation to benzene. While benzene itself is toxic,
anaerobic microbial communities can convert it into non-toxic products like
methane or carbon dioxide (8, 20). However, little is known about
microorganisms that can reductively dehalogenate DCBs.

Dehalococcoides strain CBDB1, strain195 and the related strain DF-1 (89%
16s rRNA gene identity to strain 195) are the only isolated microorganisms
known to reductively dehalogenate chlorinated benzenes (1, 3, 12, 13, 39).
These three strains can dehalogenate higher chlorinated benzenes but are unable to reductively dehalogenate DCBs. Strain CBDB1 has a preference for the removal of double flanked chlorines and reductively dehalogenates hexa- to trichlorobenzenes (HCB, TCB) to a mix of 1,3- and 1,4-DCB. Strain 195 (98% 16S rRNA gene identity to strain CBDB1) has no clear chlorine preference and can reductively dehalogenate HCB, pentachlorobenzene (PeCB) and tetrachlorobenzenes (TeCB). Strain DF-1 can reductively dehalogenate HCB, PeCB, and 1,2,3,5-TeCB to mainly 1,3,5-TCB. Other microorganisms such as Desulfomonile spp., a member of delta-Proteobacteria, are capable of dehalogenating other chloroaromatics such as 3-chlorobenzoate and chlorophenols (27, 33).

Mixed culture studies have focused on tri- or higher chlorinated benzenes. Holliger et al. (19) established a microbial mixed culture enriched with 1,2,3-TCB from sediment columns derived from the Rhine River, Saale, Germany. The mixed culture was able to reductively dehalogenate HCB, PeCB, TeCB as well was 1,2,3- and 1,2,4-TCB to 1,3- or 1,4-DCB depending on initial substrate. A mixed culture described by Middeldorp et al. (26) was able to reductively dehalogenate 1,2,4-TCB to MCB via 1,4-DCB. Neither mixed culture was able to reductively dehalogenate 1,2- or 1,3-DCB.

Reductive dehalogenation of DCBs to MCB and MCB to benzene has been demonstrated only in sediment microcosms. Both Ramanand et al. (32) and Bosma et al. (5) showed that sediments were able to reductively dehalogenate all three DCB isomers to MCB, though DCBs were never given as the sole substrates. It has been shown in estuarine sediment microcosms that 1,2-DCB
was consumed at higher rates than the other two isomers (22). Microcosms from various contaminated US sites also favored 1,2-DCB, which was dehalogenated to MCB, and 1,4-DCB was dechlorinated the most slowly, if at all (31). Though thermodynamically favorable, reductive dehalogenation of MCB to benzene has been rarely observed. In DCB-fed fluidized bed reactors inoculated with sediments from the Saale River, Germany, small amounts of benzene were produced along with MCB, but only during DCB reductive dehalogenation in a seemingly cometabolic process (30). Similar results were obtained with samples from Robins Air Force Base, Georgia (31). Phylogenetic analysis of microbial communities in the Saale River reactors found a family of clones to be 98.8-99.4% identical to that of 16s rRNA gene of *Dehalobacter restrictus* (37).

The goal of this study was to culture microorganisms involved in the reductive dehalogenation of DCBs. Enrichment cultures in growth medium containing DCBs were made from sediment microcosms able to reductively dehalogenate DCBs to MCB and MCB to benzene (14) and transferred multiple times. Here we report the reductive dehalogenation of 1,2-DCB by a mixed microbial culture.

**Materials and Methods**

**Chemicals.** All chlorobenzenes and chemicals were purchased from Sigma-Aldrich or Fisher Scientific at the highest purity available. 1,4-dichlorobenzene
was solubilized in either 99.5% ethanol or acetone to make a 1M or 5M solution respectively. Gases were purchased from Airgas East.

**Enrichments of 1,2-DCB Dehalogenators.** Unless stated otherwise, all solutions were prepared anaerobically by flushing with high-purity N₂ gas. Growth medium was dispensed into 125 ml serum vials (50 ml/vial) inside an anaerobic glove-box (95%N₂/5%H₂) and sealed with Teflon™-coated butyl rubber stoppers and aluminum crimps. The headspaces of all the vials were flushed using 70% N₂/30% CO₂ to remove H₂ from the headspace atmosphere, and chlorinated benzenes were added by syringe either neat, or as a 1M or 5M ethanol or acetone solution, respectively, for 1,4-DCB. The ethanol through fermentation could indirectly serve as an electron donor for reductive dehalogenation and methanogenesis.

A previously described, sediment slurry microcosms that reductively dechlorinated all three DCB isomers to MCB and benzene was used as the inoculum (14). From these microcosms, transfers (ca 4% v/v) were successfully made into a mineral salts medium developed for *Dehalococcoides* strain CBDB1 (1) supplemented with 0.2 g/L yeast extract, 1 g/L sodium bicarbonate, 0.8 mM Ti(III) citrate, and a vitamin solution containing 0.05 mg of vitamin B₁₂ per liter (25). Resazurin (0.001 g/L) was added to the medium as an indicator of oxygen contamination. During the course of this study, the medium supplements were changed and may have included one or more of the following (final concentrations): 5 mM 2-bromoethanesulfonate (BES), 0.8 mM Ti(III) NTA (replacing citrate), 0.2 g/L
sodium sulfide, 2 mM sodium butyrate, 2 mM sodium acetate, 3% hydrogen, and 0.2 g/L vancomycin. All cultures were incubated at 30°C in the dark.

Dichlorobenzenes, benzene and methane were detected by headspace analysis using Perkin-Elmer 8500 gas chromatograph with flame ionization detector as previously described (14). Results presented are for individual vials, but all experiments were done in duplicate with similar results in replicates, and all experimental results were repeated at least once.

**DNA Isolation and Quantitative PCR.** Genomic DNA was extracted using FastDNA for Soil kit (MP Biomedical) and resuspended in 55 µl of sterile deionized water according to the manufacturer’s instructions. Specific primers were used to target *Eubacteria* (Eub331F: 5’-TCCTACGGGAGGCAGCAGT-3’, Eub797R: 5’-GGACTACCAGGGTATCTAATCCTGTT-3’), *Dehalococcoides* (Dhc385 5’-GGGTTGTAAACCTCTTTTCAC-3’, Dhc692R: 5’-TCAGTGACAACCTAGAAAAC-3’), and *Dehalobacter* (Dhb477F: 5’-GATTGACGGTACTGCTTTACGG-3’, Dhb647R: 5’-TACAGTTTCCAATGCTTTACG-3’), populations (15, 16, 28). Real time PCR was performed as previously described using iQ SYBR Green Supermix and MyiQ Single Color Real Time PCR Detection System (Bio-Rad) (13). Copy number was quantified using plasmids containing PCR fragments of each individual 16S rRNA gene targets derived from pCR2.1 from TA Cloning Kit according to manufacturer’s instructions (Invitrogen).

**Clone library construction.** 16S rRNA gene clone libraries were constructed from DNA extracted from fourth-generation transfer cultures by PCR
amplification using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′) followed by library construction using pCR2.1 from TA Cloning Kit according to manufacturer’s instructions (Invitrogen). Clones were analyzed using restriction enzymes HhaI and HaeIII (New England Biolabs). Unique clones were sequenced at the Life Sciences Core Laboratory Center at Cornell University.

**Results**

**Development of a 1,2-DCB-Dehalogenating Enrichment Culture.** The first transfers from microcosms were made directly into medium with all three dichlorobenzene isomers added (1,2-, 1,3- and 1,4-DCB in ethanol), yeast extract as the electron donor, and Ti(III) citrate as a reducing agent. 1,2-DCB was the first DCB isomer to be reductively dehalogenated to monochlorobenzene (MCB), followed by small amounts of 1,3-DCB. No reductive dehalogenation of 1,4-DCB was observed (Figure 4.1A, data shown as nominal concentrations). Methanogenesis also occurred in these transfers and could potentially be a competing reaction with reductive dehalogenation for H\textsubscript{2}.

After the first transfer, the methanogen inhibitor, 2-bromoethanesulfonate, was always added to the medium. To favor reductive dehalogenation, subsequent growth conditions were designed to be electron-limiting by using butyrate as the primary electron donor, which is slowly fermentable and poises the H\textsubscript{2} concentrations low enough to allow reductive dechlorination to be competitive with methanogenesis (11). 1.4-DCB was solubilized in acetone, which was
nonfermentable, instead of ethanol and yeast extract concentrations were lowered (Table 4.1). These changes allowed for sustained reductive dehalogenation activity in transfers, although they did not completely eliminate methanogenesis.

After the third transfer, reductive dehalogenation of 1,4-DCB was still not observed and it was no longer added to further transfers. Since reductive dehalogenation of 1,2-DCB was the most rapid, after the fifth transfer the dehalogenating enrichment culture was maintained solely on 1,2-DCB (Figure 4.1B).

![Graph A](image1) ![Graph B](image2)

Figure 4.1. Reductive dehalogenation of dichlorobenzene isomers (DCB) to monochlorobenzene (MCB) in (A) first transfer given 1,2-DCB, 1,3-DCB, and 1,4-DCB with yeast extract as the electron donor and Ti(III)citrate as the reducing agent and (B) the sixth transfer given 1,2-DCB with butyrate as the electron donor and Ti(III)NTA as the reducing agent.
Table 4.1. Growth conditions used to establish 1,2-DCB mixed culture.

<table>
<thead>
<tr>
<th>Transfer No.</th>
<th>Dichlorobenzenes</th>
<th>Reducing Agent</th>
<th>Electron Donor</th>
<th>1,4-DCB (solvent)</th>
<th>yeast extract (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>1,2-, 1,3-, and 1,4-</td>
<td>Ti(III) citrate</td>
<td>yeast extract</td>
<td>ethanol</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>1,2-, 1,3-, and 1,4-</td>
<td>Ti(III) NTA</td>
<td>butyrate</td>
<td>acetone</td>
<td>0.02</td>
</tr>
<tr>
<td>4-5</td>
<td>1,2- and 1,3-</td>
<td>Ti(III) NTA</td>
<td>butyrate</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>1,2-</td>
<td>Ti(III) NTA</td>
<td>butyrate</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>1,2-</td>
<td>Na₂S</td>
<td>butyrate</td>
<td>---</td>
<td>0.02</td>
</tr>
<tr>
<td>8-11</td>
<td>1,2-</td>
<td>Na₂S</td>
<td>H₂</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

At the seventh transfer sodium sulfide replaced Ti(III) NTA as the reducing agent. Sodium sulfide is the reducing agent used in the cultivation of *Dehalococcoides ethenogenes* strain 195 but is toxic to *Dehalococcoides* strain CBDB1. In addition, studies have shown sodium sulfide to be inhibitory to some methanogens (4, 6, 7). Sodium sulfide did not inhibit 1,2-DCB-reductive dehalogenation to MCB and severely reduced methanogenesis.

**Benzene Production.** Reductive dehalogenation of MCB to benzene was not observed during the first six transfers though the originating sediment microcosm showed high benzene production from MCB. During the initial establishment of 1,2-DCB enrichments, transfers were also made into medium containing solely MCB. No reductive dehalogenation of MCB to benzene was observed after monitoring for 30 days. At the seventh transfer, in which sodium sulfide was used as reducing agent, trace amounts of benzene were detected, but never more than 0.44% of the MCB produced (Figure 4.2).
Figure 4. 2. Benzene production in the seventh transfer during 1,2-DCB reductive dehalogenation. Arrows indicate doses of 1,2-DCB.

**Vancomycin Sensitivity.** One of hallmarks of *Dehalococcoides* is the lack of a peptidoglycan cell wall making it vancomycin-resistant. Vancomycin was added to the second transfer and reductive dehalogenation activity was not observed indicating *Dehalococcoides* was not the causative agent (Figure 4.3).
Figure 4.3. Lost of reductive dehalogenation activity in second transfer in the presence of vancomycin.

**Dechlorination Range of the 1,2-DCB Enrichment.** To investigate the reductive dehalogenation potential of the 1,2-DCB enrichment, the sixth transfer cultures were given either 1,2-, 1,3- or 1,4-DCB as the sole electron acceptor. In cultures fed 1,3-DCB alone, complete reductive dehalogenation to MCB was observed after 8 days and transfers with 1,4-DCB showed a longer lag of 19 days (Figure 4.4A, B). However, upon change to sodium sulfide as a reducing agent the culture lost 1,3- and 1,4-DCB reductive dehalogenation activity (data not shown).
16S rRNA Gene Clone Library and Bacterial Population Quantification of a DCB Dehalogenating Culture. Genomic DNA was extracted from fourth transfer cultures grown either with or without DCBs and was used to construct clone libraries using universal bacterial primers targeting the 16S rRNA gene. Extractions were done after 36 days and 823 µmoles/L MCB was produced. From the culture given DCBs, 44% of the characterized clones (31/70) were of a restriction type, sequences from which were >99% identical to that from *Dehalobacter restrictus*, a known dehalogenator of chloroethenes (Table 4.2) (18). Other sequences found in the DCB-fed culture belonged to other members of the *Firmicutes*. *Dehalobacter* sequences were not found in culture not given DCBs. In either growth culture, *Dehalococcoides* sequences were not found. Clone library construction and analysis was performed by Dr. Hinsby Cadillo-Quiroz.

To quantify bacterial populations, real time PCR with SYBR green and specific primers targeting *Eubacteria*, *Dehalobacter*, and *Dehalococcoides* were used.
In the fourth transfer culture given DCB, the number of *Dehalobacter* 16S rRNA gene copies was $3.5 \pm 0.3 \times 10^6$ copies/ml, which was 100 times greater than in culture not given DCBs (Figure 4.5). *Dehalococcoides* 16S rRNA gene copies were detected both in the presence and absence of DCBs, $1.7 \pm 1.0 \times 10^4$ copy/ml and $2.1 \pm 1.4 \times 10^4$ copy/ml, respectively (Figure 4.5). In culture given DCB, *Dehalococcoides* 16S rRNA gene copies were 0.02% of the total *Eubacteria* 16S rRNA gene copies. The low copy number of *Dehalococcoides* may have been below the detection limit of the clone library. *Dehalobacter* 16S rRNA gene copy number was 3% of the total *Eubacteria* 16S rRNA gene copies in cultures given DCB.

Table 4.2. Phylogenetic groups identified in clone library from fourth transfer culture grown in the presence or absence of DCBs. Group assignment was determined using Ribosomal Database Classifier using an 80% confidence threshold (38).

<table>
<thead>
<tr>
<th>Library</th>
<th>Phylogenetic Assignment*</th>
<th>No. Clones (sequenced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DCB</td>
<td><em>Dehalobacter</em></td>
<td>31 (11)</td>
</tr>
<tr>
<td></td>
<td>Clostridiales</td>
<td>28 (7)</td>
</tr>
<tr>
<td></td>
<td>Clostridium</td>
<td>4 (3)</td>
</tr>
<tr>
<td></td>
<td>Unassigned</td>
<td>7 (0)</td>
</tr>
<tr>
<td>-DCB</td>
<td><em>Parabacteroides</em></td>
<td>18 (6)</td>
</tr>
<tr>
<td></td>
<td>Clostridiales</td>
<td>12 (4)</td>
</tr>
<tr>
<td></td>
<td>Clostridium</td>
<td>9 (2)</td>
</tr>
<tr>
<td></td>
<td>Spirochaetaceae</td>
<td>9 (5)</td>
</tr>
<tr>
<td></td>
<td><em>Peptostreptococcaceae incertae sedis</em></td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Ruminococcaceae</em></td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Bacteroidales</em></td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Bacteroides</em></td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>Desulfovibrio</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td><em>Firmicutes</em></td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>Unassigned</td>
<td>7 (0)</td>
</tr>
</tbody>
</table>
Figure 4.5. Comparison of 16S rRNA gene copy numbers from fourth transfer cultures grown with or without DCBs using primers that target \textit{Dehalococcoides} (Dhc), \textit{Dehalobacter} (Dhb) and \textit{Eubacteria} (Eub). Error bars represent standard deviation.

To examine changes in the bacterial population upon using sodium sulfide as a reducing agent and transferring solely on 1,2-DCB, genomic DNA was extracted from seventh transfer cultures in the presence and absence of 1,2-DCB after 0 days and 15 days when the culture given 1,2-DCB produced 933 μmoles/L of MCB. Using real time PCR analysis, the culture without 1,2-DCB showed a 10-fold decrease in \textit{Dehalobacter} 16S rRNA gene copies while the \textit{Eubacteria} population increased by 20-fold (Figure 4.6A). In culture given 1,2-DCB both \textit{Dehalobacter} and \textit{Eubacteria} 16S rRNA gene copies increased by 20- and 10-fold respectively, indicating growth of \textit{Dehalobacter} in dehalogenating conditions (Figure 4.6B). Based on 16S rRNA gene copies, \textit{Dehalobacter} was 14% of the total \textit{Eubacteria}. In both cultures, \textit{Dehalococcoides} PCR product was not detected. After the seventh transfer culture conditions were modified to enrich for \textit{Dehalobacter}. 

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Targeted Enrichment of *Dehalobacter*. As *Dehalobacter* is known only to utilize H$_2$ as an electron donor (18), the 1,2-DCB culture was serially diluted up to 10$^{-8}$ with H$_2$ as the electron donor. Repeated serial dilutions have not yielded 1,2-DCB reductive dehalogenation past 10$^{-4}$ dilution (Figure 4.7A). After 28 days, the number of *Dehalobacter* 16S rRNA gene copies was 1.6±0.3x10$^6$ copies/ml and 53% of the copies detected with eubacterial primers, while no PCR product was detected for *Dehalococcoides* (Figure 4.7B). The inability to transfer dechlorination activity to higher dilutions and the presence of other *Eubacteria* in the culture suggests a reliance upon another microorganism(s) to provide an unknown growth factor(s) similar to strain DF-1 whose growth relies on extracts of *Desulfovibrio* spp.(23).
Discussion

This chapter describes the establishment of a 1,2-DCB reductively dehalogenating mixed culture from sediment microcosms that can be successively transferred into growth medium. The mixed culture has been transferred 11 times over the course of ca. 1.4 years: the first three transfers were made in the presence of all three DCB isomers (ca. 0.75 year), 1,2- and 1,3-DCB for fourth and fifth transfer (ca. 0.25 year), and solely 1,2-DCB for the remaining transfers (ca. 0.4 year). In accord with other studies (1, 2, 10), the mixed culture showed a clear preference for the removal of the chlorine in the ortho position. Over the course of transfers, several changes were made to the growth conditions (Table 4.1), with the most notable being the addition of 2-bromoethanesulfonate (BES) to inhibit methanogens, sodium sulfide as a reducing agent, and the switch from yeast extract to butyrate and eventually hydrogen as the electron donor.
During the initial transfer, methanogenesis, a competitive reaction with reductive dehalogenation, was observed. BES has been used with limited success in reductive dehalogenation studies as it can also inhibit reductive dehalogenation as in *Dehalococcoides* strain 195 on chloroethenes (9) but not strain CBDB1 growing on chlorobenzenes (1). In this mixed culture, it did not inhibit the overall reductive dehalogenation activity, but it could have inhibited the activity of specific dehalogenating microorganisms. When examining TeCB and PeCB reductive dehalogenation to MCB, Middeldorp *et al* (26) found that adding BES inhibited several dechlorination pathways in their mixed consortium. Sodium sulfide is an alternative reducing agent from trivalent titanium but can be lethal to some dehalogenators such as strain CBDB1, but not others like *Dehalobacter restrictus* (1, 18, 25). While low levels of sulfide can be used as a sulfur source, it is also known that high levels of sulfide can be inhibitory to methanogens (6, 7). At the fifth transfer, sodium sulfide was used and methanogenesis was decreased even further with no effect on reductive dehalogenation.

The ability of the current mixed culture to reductively dehalogenate DCB isomers other than 1,2-DCB is unclear. While reductive dehalogenation of 1,3- and 1,4-DCB was observed at the fifth transfer, it was not detected at the seventh transfer when sodium sulfide was used as a reducing agent. It is possible that there were multiple dehalogenating populations with mixed sensitivity to sodium sulfide or that sodium sulfide itself inhibits the reductive dehalogenation of 1,3- and 1,4-DCB. It is common for the physiological properties of mixed cultures to change over the course of transfers. Holliger *et
al. (19) also observed a loss of substrate range with other isomers of TCB with the transfer of their mixed culture to solely 1,2,3-TCB. When Nowak et al. (30) adapted their FBR to single DCB isomers, they found that 1,2-DCB or 1,4-DCB-adapted FBRs could only dehalogenate that specific isomer and 1,3-DCB-adapted to dehalogenated both 1,2- and 1,3-DCB.

Benzene production was reminiscent of a cometabolic process in that it is only observed during the reductive dehalogenation of higher chlorinated benzenes and in minor amounts. This activity does not resemble that of microcosms used as original inoculum, which produced benzene at much higher concentrations (>3000 µmoles/L) and in the absence of DCB reductive dehalogenation (14). It could not be determined if benzene production occurred only after 1,2-DCB reductive dehalogenation ceased like ethene production from vinyl chloride in strain 195 (24) or only during the reductive dehalogenation of 1,2-DCB as previously observed in sediment microcosms (30, 31). It is likely that another microorganism was performing the MCB reductive dehalogenation observed in sediment microcosms.

Using a combination of culture independent methods, Dehalobacter sequences were identified and revealed to be a major component in the 1,2-DCB mixed culture and its growth to be linked to reductive dehalogenation. Its cultured representative, *Dehalobacter restrictus*, is a known dehalogenator of chlorinated ethenes but is not known to reductively dehalogenate chloroaromatics (18, 21). *Dehalobacter* strain TCA1 has been shown to reductively dehalogenate 1,1,1-trichloroethane (1,1,1-TCA) and can use either H₂ or formate as an electron donor (35). Grostern *et al.* (15) has shown
Dehalobacter to reductive dehalogenate 1,2-dichloroethane via dichloroelimination forming vinyl chloride. A Dehalobacter sp. has been identified in a coculture with Sedimentibacter sp. that is able to reductively dehalogenate β-hexachlorocyclohexane to MCB and benzene (36). Dehalobacter may have an unexplored diverse range of halogenated compounds but has been difficult to isolate due to its fastidious growth requirements. It is only known to use hydrogen as an electron donor (strain TCA1 being the exception) and halogenated compounds as electron acceptors.

Other identified bacteria in the DCB-fed clone library belong to known fermentors like Clostridia were most likely using the small amount of yeast extract present and providing hydrogen via fermentation reactions or other unknown essential nutrients. Since analysis was done when butyrate was provided as the electron donor a complex community with fermentors was expected. It is interesting to note the absence of Dehalococcoides and Chloroflexi sequences as they represent the only known groups with cultured chlorinated benzene dehalogenators. The addition of BES in culturing conditions may have selected against Dehalococcoides. Though no Dehalococcoides sequences were found in the 1,2-DCB mixed consortium, they still might play a larger role in the reductive dehalogenation within the original sediment microcosm, which is known to contain a diverse population of Chloroflexi (Haitjema, C., personal comm.).

This study is the first description of a 1,2-DCB reductive dehalogenating mixed culture in sediment-free medium. Dehalobacter sp. has been identified as a
member of the culture and may play a major role in reductive dehalogenation, as it was not present in clone libraries from cultures not fed 1,2-DCB. In chloroethane dehalogenating communities *Dehalobacter* and *Dehalococcoides* have been shown to play complementary roles in reductive dehalogenation (15). Further attempts to isolate the microorganism(s) responsible for 1,2-DCB dehalogenation are underway. Once the microorganism has been identified physiological parameters such as temperature and competing electron acceptors can be tested to better understand its capabilities as a bioremediation agent.
REFERENCES


CHAPTER FIVE
SUMMARY AND IMPLICATIONS IN THE FIELD OF BIOREMEDIATION

Summary and Conclusions: Chapter Two

*Dehalococcoides ethenogenes* strain 195 has 19 potential RD genes including *pceA* and *tceA*, whose gene products are responsible for the reductive dehalogenation of perchloroethylene (PCE) to trichloroethene (TCE) and TCE to vinyl chloride (VC) and ethene (ETH), respectively (33). Though strain 195 is able to dehalogenate a large range of other halogenated compounds, RD genes responsible have yet to be identified (3, 17, 24). RD gene, DET0162, contains a verified TGA stop codon that would cause the predicted gene product to be 59 amino acids rather than the typical size of 500 amino acids (19, 47). This truncation, and a shorter corresponding "B" gene (DET0163), thought to encode for a membrane anchoring protein, suggest it is no longer functional. Sixteen of the 19 RD genes in *D. ethenogenes*, including *pceA*, have predicted transcriptional regulator genes in close proximity suggesting they are under transcriptional regulation (47). The objective of Chapter Two was to investigate RD gene function and gain insight into its regulation.

*Dehalococcoides ethenogenes* strain 195 was grown on PCE, TCE or 2,3-dichlorophenol (2,3-DCP) and RD gene transcript levels was monitored using reverse transcription quantitative PCR. Resting cell assays and proteomic analysis was performed on PCE- and 2,3-DCP grown cells to verify RD gene products and physiological activity. When grown on PCE or TCE, *tceA* was expressed at levels several fold higher than that of *rpoB*, a housekeeping gene used as a marker of relevant transcript activity and 300-fold lower levels
when grown on 2,3-DCP. This was the first proof that \textit{tceA} was regulated on the transcriptional level, which was interesting as it does not have adjacent regulatory genes. Both \textit{pceA} and \textit{DET0162} were expressed under all growth conditions. Though it was known that TCE-grown cells reductively dehalogenated PCE (35), it was unknown at what level the activity could be ascribed to and this study showed \textit{pceA} was transcribed. While \textit{DET0162} was detected in transcript studies, proteomic analysis did not detect any associated peptides and most likely its transcript is not processed into a functional RD.

\textit{DET1559} transcript was detected at significant levels when cells were grown with PCE but its peptides were not detected in proteomic studies. In a PCE-dehalogenating mixed culture containing strain 195, \textit{DET1559} peptides were detected (37). Perhaps \textit{DET1559} was translated but at lower abundance than PceA or TceA making it difficult to detect. Using microarrays, Johnson \textit{et al.} (28) found \textit{DET1559} to be transcribed in TCE-fed cultures though under different growth condition (defined medium) then this study, which contained two undefined growth supplements (extract PCE-fed butyrate mix culture and supernatant of anaerobic sludge digestor) (36).

In this study \textit{pceA} was identified to be the 2,3-DCP reductive dehalogenase. In transcript and peptide analysis \textit{pceA} was the only RD gene detected when grown with 2,3-DCP. Providing additional evidence for PceA bifunctionality, PCE- and 2,3-DCP resting cell showed reductive dehalogenation activity for both PCE and 2,3-DCP. Located upstream of \textit{pceA} are two-component system (TCS) regulatory genes. Ten of the nineteen RD genes have
associated TCS genes and their histidine kinases (HK) lack the conserved membrane-spanning regions and are thought to be soluble (47). It would be of interest to discover how PCE, TCE or 2,3-DCP each acts as a ligand for the HK to activate transcription. An alternative scenario is that \textit{pceA} is constitutively expressed like that of \textit{Sulfurospirillum multivorans} (39). In strain 195, \textit{pceA} has been shown to be under temporal control with varying levels of transcript but never completely turned off (43).

\textit{Insight into RD Gene Evolution}

The bifunctionality of \textit{pceA} may reveal information of its genetic evolution. Though PCE can be produced by natural sources such as algae (1), the majority produced is anthropogenic (www.epa.gov). How did strain 195 and other bacteria evolve to utilize this manmade compound? 2,3-DCP represents a set of compounds commonly found in nature that can be produced by plants and fungi (21). It is possible that 2,3-DCP is the natural substrate of \textit{pceA} and genetic evolution has modified it to utilize PCE. This concept has been shown in \textit{Escherichia coli} and \textit{Klebsiella aerogenes} adaptation to novel five-carbon sugar, D-arabinose using the L-fucose pathway (7). The enzymes in the L-fucose pathway can use D-arabinose but is not expressed in its presence. Regulatory mutations are the first to occur which make the operon inducible by the new substrate or constitutively expressed. While it can be energetically costly to constantly produce proteins, selective advantages of constitutively expressed metabolic genes are a shorter lag time and improved activity by protein saturation. PceA might also be undergoing selection for improved catalytic capacity. In the case of \textit{E. coli} strain K12 acquiring ethylene glycol utilization, an anthropogenic substrate not metabolized by wild type, mutations
occur in lactaldehyde dehydrogenase, which improved substrate kinetics for an alternate substrate (5). It is possible that pceA is undergoing a similar evolutionary process. Alvarez-Cohen et al. (personal communication) have been transferring strain 195 continuously on TCE and recently discovered the loss of pceA transcription. In addition to insight into RD genetic evolution, mutations that cause loss of transcription or RD gene function would provide insight into its mechanism and regulation.

Given that over 80 homologous RD genes in Dehalococcoides spp. have been identified with strain CBDB1 harboring 32 and strain 195 with 19 (27, 31, 47), one can speculate on the evolutionary circumstances that caused such a proliferation within the genus and individual strains. Gene duplication is an efficient method for bacteria to develop new functions without losing existing function. Paralogous genes involved in metabolism are shown to undergo preferential retention and also, undergo faster mutation rates (20, 29). Strain 195 contains several RD genes with high amino acid sequence identity (>30%) which is a possible sign of past gene duplication events (Table 5.1) (20). What do these duplication events mean in terms of halogenated substrate range? The two vinyl chloride reductases, BvcA and VcrA, from strain BAV1 and VS, respectively, are 38% identical at the amino acid level and 39% and 36% with TceA of strain 195, respectively (30, 38). All three RDs reductively dehalogenate VC. TceA is able to reductively dehalogenate TCE, dichloroethene but not PCE. In comparison VcrA is able to reductively dehalogenate TCE at low rates and dichloroethenes but not PCE. The transcription and proteomic assay developed in Chapter Two can help address questions in evolution by identifying RD gene function. It would be of interest
to see if the RDs with higher sequence identity (>50%) such as DET1522 and DET1535 have distinct or overlapping substrate ranges.

Table 5.1. Comparison of reductive dehalogenases of strain 195. Sequence identities of 30% or greater are in bold.

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*homologs in strain CBDB1 with amino acid sequence identity between 86.4-95.4% (31)

**Improved Assessment of Reductive Dehalogenation Activity**

By correlating RD gene transcript levels with physiological activity, I have strengthened the biological understanding of reductive dehalogenation, which can be used for improved bioremediation applications. Using a combination of transcription, proteomics and resting cell assays, RD gene function in strain 195 can now be determined for other halogenated substrates such as
pentachlorobenzene. It is critical to use cultured microorganisms such as strain 195 to identify RD gene function, or RD analysis on uncharacterized mixed cultures and in situ field studies will not have relevance.

The ability for a contaminated site to undergo stimulated or natural bioremediation is usually accessed with DNA-based techniques by the determining the presence of *Dehalococcoides* spp. using 16s rRNA genes and specific RD genes such as *tceA* or *bvcA* (16, 25). However, neither of these markers actually denotes physiological activity. RD gene transcripts or peptides are dynamic markers that can be used to determine substrate inhibition, growth stage, or nutrient limitations. Lee et al. (32) monitored *Dehalococcoides* population and RD gene transcript of *vcrA*, *bvcA*, and *tceA* in a TCE-contaminated field site and found changes in RD gene transcript as chlorinated ethenes were dehalogenated. The *tceA* of strain 195 is under genetic regulation, though the nature of control is unknown. Its expression is not affected by the presence of 2,3-DCP. Superfund sites are contaminated with multiple organic substrates from chlorobenzenes to chloromethane as well as heavy metals. It would be of interest to see if the *tceA* expression of strain 195 is inhibited under these complex conditions.

RDs can be used to directly assess growth stage and reductive dehalogenase activity. In a study with strain 195 examining whole genome expression during the transition between exponential and stationary phase, unique RD genes (DET0173, DET0180, DET1535, and DET1545) were expressed during stationary phase (28). During stationary phase in strain 195, growth and reductive dehalogenation are uncoupled and these stationary phase-RDs can
be used to as indicators of growth state. Using a proteomic approach Morris et al. (37) was able to identify key RD peptides correlating with vcrA from dechlorinating cultures enriched from a TCE-plume at the heavily contaminated Department of Energy, Savannah River National Laboratory site. For site applications, this technique could be adapted to directly detect RD from sediment samples instead of enrichment cultures.

The transcript and proteomics approach can be expanded to correlate physiological states with RD activity. In ocean surface waters, a survey of community gene expression identified high levels of transcripts involved in carbon fixation which correlated with the carbon-limited environment (18). Using proteomics to analyze an acid mine drainage microbial community, peptides involved in oxidative stress and protein refolding were found to be abundant (44). These proteins reflect the environmental challenges microorganisms face living in highly acidic environment (pH<1). Transcriptome of strain 195 can be generated under different conditions such as nutrient limiting or in a mixed community and RD activity levels monitored. Specific conditions that effect RD activity can be identified which will provide a global perspective on the physiology of Dehalococcoides.

**Summary and Conclusions: Chapters Three and Four**

Reductive dehalogenation of dichlorobenzenes (DCBs) to monochlorobenzene (MCB) and MCB to benzene has only been demonstrated in microcosms, and no organisms have been identified to be involved in these processes (2). Several sediment microcosms and flow columns from a variety of
contaminated sites reductive dehalogenate DCBs to MCB with preferences for specific isomers (9, 42, 45). Though thermodynamically favorable (14), reductive dehalogenation of MCB to benzene has been rarely observed and only known to occur doing the reductive dehalogenation of higher chlorinated benzenes (42, 45). The objective of these chapters was to characterize the reductive dehalogenation of DCB and MCB in sediment from a historically chlorobenzene-contaminated site (Chapter Three) and a mixed culture developed from the DCB-dehalogenating sediment microcosms (Chapter Four).

In Chapter Three, I described the complete reductive dehalogenation of DCBs and MCB to benzene under methanogenic conditions in sediment microcosms. Sediment microcosms given all three DCB isomers reductively dehalogenated them all to MCB with a preference for 1,2- followed by 1,3- and 1,4-DCB. During the reductive dehalogenation of DCB, MCB was slowly dehalogenated to benzene, but once the DCBs were consumed, MCB turnover was rapid. This amount of reductive dehalogenation of MCB to benzene was an unprecedented amount of ca. 3500 \( \mu \)moles/L compared to 50 \( \mu \)moles/L observed by Nowak et al. (41). The reductive dehalogenation of MCB in the absence of DCBs was only observed in microcosms constructed from one of three sediment samples, indicating differences in dehalogenating activity in the microbial populations. Reductive dehalogenation was enhanced when sediment microcosms were inoculated with sediment slurry from a previously chlorinated benzene-dehalogenating microcosm. The addition of an inoculum eliminated the initial lag in MCB reductive dehalogenation, and inoculated microcosms immediately dehalogenated MCB concurrently with
DCBs. More importantly, inoculated microcosms could consistently dehalogenate MCB in the absence of DCB and this activity was maintained over several transfers into sediment microcosms.

The mechanism of MCB reductive dehalogenation remains elusive. The inoculation with dehalogenating sediment seemed to activate the MCB-dehalogenating population in some unknown manner. It was possible the inoculation provided a nutrient that the MCB-dehalogenating population could not generate or lacked the activation energy to initially produce. The reductive dehalogenation of MCB to benzene in the absence of higher chlorinated benzenes has never been observed before, and sediment microcosms in this study have potential uses in the field of bioremediation.

In Chapter Four, a 1,2-DCB-dehalogenating mixed culture was developed from DCB-dehalogenating sediment microcosms. Sediment slurry from a microcosm was initially transferred into mineral salts medium with yeast extract, and all three DCB isomers. Over the course of transferring, the mixed culture apparently lost its ability to reductively dehalogenate 1,3- and 1,4-DCB. Culturing conditions were modified over several transfers to minimize methanogenesis, a competing reaction, including the addition of 2-bromoethanesulfonate (BES). The DCB reductive dehalogenation activity was vancomycin sensitive indicating that *Dehalococcoides* was not the causative agent. The use of BES may have selected against *Dehalococcoides* in the mixed culture, as it can inhibit reductive dehalogenation activity in some strains. The 1,2-DCB-dehalogenating mixed culture was able to produce trace amounts of benzene but only if grown on DCB. This metabolism was not
similar to the robust benzene production found in the original sediment microcosm and may be a cometabolic process. To identify possible candidate dehalogenators, a 16S rRNA gene clone library was constructed from the fourth transfer cultures and sequences matching >99% similarity to \textit{Dehalobacter restrictus}, a known dehalogenator, was identified. At the seventh transfer growth of \textit{Dehalobacter} population was linked to reductive dehalogenation and accounted for 14% of the total bacterial population. Culturing conditions were modified to enrich for \textit{Dehalobacter}, and by the eighth transfer \textit{Dehalobacter} accounted for 53% of the total bacterial population.

Though identified over a decade ago (26), not much is known about the genus \textit{Dehalobacter}. The \textit{pceA} of \textit{Dehalobacter restrictus} has been identified (34) and using degenerate primers, two RD genes of unknown function were identified, rdA1-Dr and rdA2-Dr (46). Unlike its dehalogenating counterparts, \textit{Desulfitobacterium} and \textit{Dehalococcoides}, a representative genome for \textit{Dehalobacter} has not been sequenced though they are known to reductively dehalogenate chlorinated ethenes, ethanes, and hexanes (23, 26, 48, 50). Due to the lack of genomic and information the reductive dehalogenation capability of \textit{Dehalobacter} remains unknown and unexplored.

\textit{Potential for Bioremediation Application of MCB-Dehalogenating Microcosm}

In addition to anthropogenic introduction of DCB and MCB into the environment, they can accumulate as end products from the reductive dehalogenation of higher chlorinated benzenes. It has been observed that
higher chlorinated benzenes are more readily dehalogenated in anaerobic environments and pollutant profiles of sites can change over time (2, 10). Sediment analysis spanning several decades of Lake Ketelmeer, which receives water from the Rhine River in Germany, found an accumulation of DCBs resulting from the reductive dehalogenation of hexachlorobenzene, the original pollutant (8). While MCB oxidation and reduction has been demonstrated (40-42), the low concentration of end products does not alter the accumulative toxicity of the compounds found on site. However in this study, significant amounts of DCBs and MCB were reductively dehalogenated to benzene, a more toxic compound (49). Benzene has been shown to be recalcitrant in a number of environments (6, 15) and in those cases, the conversion of MCB to benzene is not desirable. The dynamics of DCB and MCB reductive dehalogenation needs to be taken into account for a better understanding of their fate in the environment.

The sediment microcosms in Chapter Three have a potential to be developed for field applications. The simple technique of inoculating sediment microcosms eliminated lag time of DCB and MCB reductive dehalogenation. It would be interesting to test if inoculating a site with actively dehalogenating sediment could stimulate sustained activity for in situ bioremediation. However, environmental factors that influence chlorinated benzene degradation are unknown. The Rhine River has been contaminated with MCB for decades with very little conversion to benzene (8). Environmental conditions may exist that inhibit its reductive dehalogenation, and experiments with mixed sediment microcosms would be informative of the types of
environments in which reductive dehalogenation of DCBs and MCB could function.

The sediment in this study can also be used to develop a broad range dehalogenating mixed cultures that degrade higher chlorinated benzenes to carbon dioxide or methane. The anaerobic degradation of benzene can occur by oxidation to carbon dioxide or reduction to methane (13). The MCB-dehalogenating sediment microcosms from this study can be mixed with a benzene-utilizing culture for complete remediation (11, 12). Mixing cultures can also relieve substrate inhibition if multiple halogenated substrates inhibit reactions. Grostern and Edwards (22) were able to overcome chloroethane inhibition of trichloroethene reductive dehalogenation by combining a trichloroethane dehalogenating mixed culture with one that dehalogenates chloroethenes. The sediment in this study has not been tested for its ability to reductively dehalogenate tetra- or higher chlorinated benzenes but has been shown to reductively dehalogenate trichlorobenzenes (Fullerton, H., personal communication). There are sediment sources and pure cultures, like strain CBDB1, that can reductively dehalogenate higher chlorinated benzenes that could be used to broaden the halogenated substrate range (2, 4). Due to its robustness and unique ability utilize DCBs and MCB, the reductive dehalogenating microorganisms found in the 1,2-DCB mixed culture and within sediment microcosms have the potential to be highly effective in bioremediation applications.
REFERENCES


Multiple nonidentical reductive-dehalogenase-homologous genes are common in *Dehalococcoides*. Appl Environ Microbiol 70:5290-7.


