FINAL REPORT

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Complete Detoxification of Short Chain Chlorinated Aliphatic Compounds:
Isolation of Halorespiring Organisms and Biochemical Studies of the Dehalogenating Enzyme Systems

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1. Executive Summary

The stimulation of halorespiring microorganisms is potentially the most promising and cost-effective technology for remediating sites contaminated with chlorinated solvents. Central to the successful implementation of such a bioremediation technology is the determination of the presence of halorespiring organisms and information on their physiology. Unfortunately, our knowledge of halorespiring microbes that detoxify chlorinated solvents is limited. Hence, our work focused on the isolation and characterization of halorespiring populations, and the initial investigation of the dechlorinating enzyme systems. In addition, tools to evaluate the presence/activity of halorespiring populations in the environment were developed.

Halorespiring enrichment cultures were obtained from a variety of contaminated and non-contaminated sites. Five enrichments completely dechlorinated tetrachloroethene (PCE) to ethene, and four different cultures dechlorinated 1,2-dichloropropane to propene. Hydrogen consumption threshold measurements and the determination of the fraction of electrons going towards reductive dechlorination confirmed the presence of halorespirers. Two halorespiring isolates were obtained: *Desulfuromonas ottawaensis* strain BB1, which dechlorinates PCE to *cis*-1,2-dichloroethene, and *Desulfitobacterium* sp. strain Viet1, which dechlorinates PCE to trichloroethene. *Desulfuromonas ottawaensis* strain BB1 dechlorinated PCE at rates of 150 nmol min^{-1} mg of $protein^{-1}$ with acetate as the electron donor. In cell-free systems PCE was dechlorinated at rates of at least 300 nmol min^{-1} mg of $protein^{-1}$ with methyl viologen as the electron donor. In addition, a defined mixed culture consisting of three populations including a *Dehalococcoides* species was obtained. This mixed culture completely dechlorinated PCE to the environmentally benign product ethene.

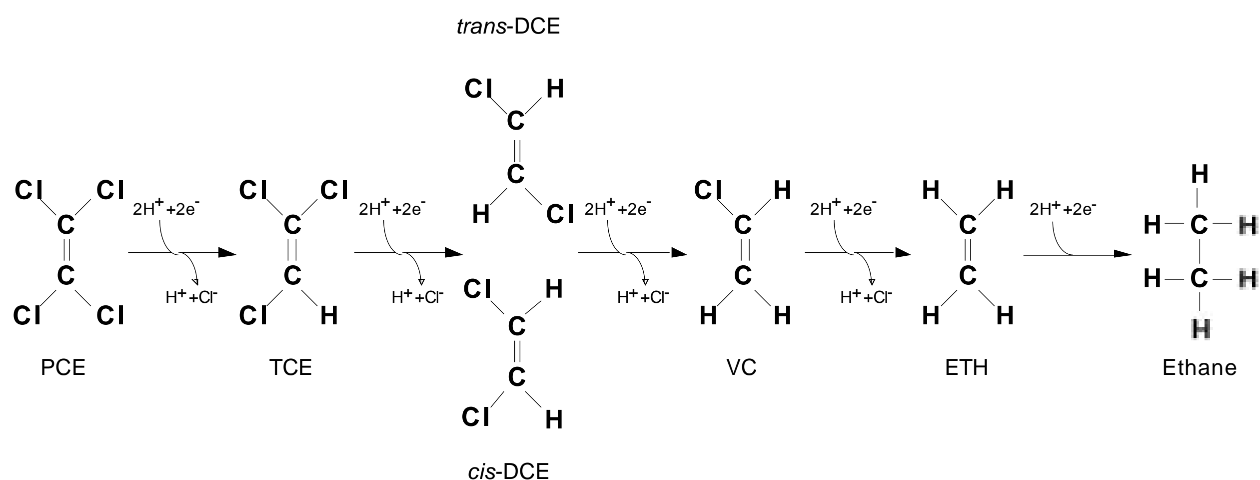
Specific primers based on 16S rDNA sequences of *Desulfuromonas ottawaensis* strain BB1 and the *Dehalococcoides* species were designed, and used in a nested PCR approach for the sensitive and specific detection of these organisms in environmental samples. Both dechlorinators were detected in three out of three river sediment samples tested, and two different contaminated aquifers tested positive with the *Desulfuromonas ottawaensis*- and *Dehaloccocoides*-targeted primers, respectively.

Natural attenuation and engineered bioremediation are often the most cost-effective corrective actions for addressing groundwater contaminated with chlorinated solvents. To decide on the most cost-effective and successful bioremediation strategy the presence or absence of halorespiring organisms must be confirmed. The tools developed in this work (measurements of hydrogen consumption thresholds, molecular probes) are relevant for regulatory agencies in order to facilitate decisions on which bioremediation technology (biostimulation or bioaugmentation) is most promising at a particular site. In addition, a better understanding of the physiology of the halorespiring organisms as well as the biochemistry of the dehalogenating enzyme systems enhances our knowledge of how these organisms can successfully be employed in the bioremediation of contaminated sites.

2. Research Objectives

Chlorinated solvents such as chlorinated ethenes (tetrachloroethene, PCE; trichloroethene, TCE) and chlorinated propanes are common ground water pollutants in the U.S. and other industrialized countries (1). Most are also problems at DOE facilities. In addition to their use as solvents, halogenated propanes, including 1,2-dichloropropane (1,2-D), have been extensively used as fumigants to control root parasitic nematodes for a variety of crops (2, 23, 30). These compounds are toxic and some of them are human carcinogens. Therefore, the presence of these chlorinated compounds in the environment is of public concern. Under anaerobic conditions the stepwise reductive dehalogenation of PCE to TCE, dichloroethenes (DCE's), vinyl chloride (VC), ethene (ETH) and ethane has been observed (3, 6, 9, 11-14, 17, 27, 29, 36, 38). ETH and ethane are considered environmentally acceptable products. The sequence of reductive dechlorination steps is depicted in Figure 1.

Fig. 1. Sequential reduction of PCE to ethane.



In addition to the intentional use of 1,2-D as a fumigant or as a solvent, this compound is formed as an undesired by-product in the chemical production of propylene oxide by the chlorohydrin process (37). In 1991 this process yielded about 165,000 metric tons of 1,2-D worldwide, of which 70,000 tons was produced in the U. S. This extensive output of 1,2-D creates a substantial waste management problem. Because 1,2-D is moderately soluble in aqueous systems and is recalcitrant to microbial degradation, this compound is found as a significant pollutant of ground water systems (4, 8, 20). Concentrations of 9 mg m^{-3} in ground water samples from areas in the Netherlands have been reported (4), and concentrations as high as 1.2 g m^{-3} have been detected in California (20).

The fate of 1,2-D in natural environments is poorly understood. Under laboratory conditions

1,2-D can be co-metabolically oxidized by some methanotrophic (31) and nitrifying (32) bacteria under aerobic conditions. These conversions are due to the fortuitous action of methane monooxygenase (MMO) and of ammonia monooxygenase (AMO), respectively. Both types of enzymes exhibit a broad range of substrate specificities. For example, whole cell suspensions of *Methylosinus trichosporium* OB3b expressing a soluble MMO, partially dechlorinated 1,2-D to 2,3-dichloro-1-propanol (31). In another study, the ammonia-dependent disappearance of 1,2-D was shown in cell suspensions of two soil nitrifiers, but no conversion products could be identified (32). In addition to co-metabolic processes, several aerobic bacteria available in pure culture are capable of using halogenated aliphatic compounds as a sole source of carbon and energy (15, 33). Hydrolytic and oxygenolytic dehalogenases have been characterized from these organisms but none of these enzymes transformed 1,2-D at significant rates, if at all (5, 15, 33, 34). Very limited information describing the degradation of 1,2-D under anaerobic conditions is available. Boesten et al. (4) studied the fate of 1,2-D in three anaerobic sandy subsoil materials. In one of these materials a 96% loss of 1,2-D was observed after two years of incubation, with propene and propane suggested as possible transformation products.

Cometabolic reductive dechlorination versus chlororespiration

Enzymes containing tetrapyrrole cofactors like factor F430, iron porphyrins, and cobalamins have been described to dehalogenate chloroethenes (18, 19, 35). Recent findings have also demonstrated that the reductive dechlorination of chloroethenes can be catalyzed by corrinoids and factor F430 without the involvement of proteins (7, 18, 19). Since many enzymes of methanogenic archaea and other anaerobic bacteria contain these tetrapyrrole cofactors in high amounts, co-metabolic dechlorination reactions are most likely catalyzed by these cofactors. These studies have also shown that the dechlorination rates drop by an order of magnitude with each chlorine removed (18). This might explain why cometabolic dechlorination of PCE and TCE has often been observed whereas a significant cometabolic conversion of DCE's and VC to ETH was never detected.

In summary, co-metabolic reductive dechlorination of chloroethenes does not allow complete detoxification of chloroethene-contaminated sites at acceptable rates. In addition, co-metabolic processes are difficult to manage in the field since they can not be directly stimulated. Hence, distinguishing between cometabolic and chlororespiring activities for assessment of applicable technologies is critical for a site.

All the dechlorination steps depicted in Figure 1 can be carried out by halorespiring organisms (25). These organisms use chloroethenes as terminal electron acceptors in their energy metabolism. The energy available from reductive dechlorination of aliphatic and aromatic chlorinated organic compounds is substantial, with $G^{0'}$ values ranging from -130 kJ to -175 kJ per dechlorination

reaction with H_2 as the electron donor (10). A few organisms capable of using chlorinated compounds as terminal electron acceptors in their energy metabolism have been isolated over the last few years. Organisms with this specialized process have dechlorination rates several hundred times faster than previously known for organisms that co-metabolize chlorinated ethenes. Recent findings have demonstrated that all dechlorination steps depicted in Figure 1 can be catalyzed by chlororespiring organisms, and support a terminal electron accepting process (TEAP). Hence, the chlororespiration process can lead to complete detoxification.

As a prerequisite for the implementation of a successful bioremediation strategy, cometabolic reductive dechlorination activity needs to be distinguished from chlororespiration, and the presence or the absence of chlororespiring bacteria must be confirmed. In addition, information on the physiology of the chlororespiring organisms and their dechlorinating enzyme systems are necessary in order to understand how the process can be best stimulated and managed under field conditions.

3. Methods and Results

Anaerobic microcosms were established using a variety of geographically distinct sediments. In several microcosms complete dechlorination of PCE to ETH, and 1,2-D to propene was observed. Upon subsequent transfers to anaerobic medium, five sediment-free, methanogenic enrichment cultures were obtained that dechlorinated PCE to ETH, and four cultures that dechlorinated 1,2-D to propene. 2-Bromoethanesulfonate (BES), a well known inhibitor of methanogens, did not inhibit the dechlorination of 1,2-D to propene or the dechlorination of PCE to *cis*-DCE. The complete dechlorination of PCE to VC and ETH, however, was severely inhibited. BES inhibited the dechlorination of chloroethenes in cultures in the absence of methanogens (Tab. 1). Therefore, BES should not be used to attribute dechlorination activities to methanogens (24).

Table 1. Influence of BES on reductive dechlorination of PCE in different nonmethanogenic cultures

Culture [a]		Total PCE added [µmol] [b]	TCE [µmol] [c]	*cis*-DCE [µmol] [c]	*trans*-DCE [µmol] [c]	VC [µmol] [c]	ETH [µmol] [c]
PM	+ BES	80	0	56.2	0	19.7	0
	- BES	80	0	12.6	0	36.8	27.3
AuS	+ BES	60	0	23.5	6.5	19.0	11.7
	- BES	60	0	0	6.0	0	51
RC	+ BES	40	22.7	10.4	2.2	0	0
	- BES	40	0	0	0	34.3	9.2
Parf	+ BES	40	38.6	<0.5	0.8	0	0
	- BES	40	13.9	5.7	19.1	0	0
TQ	+ BES	20	17.0	0	0	0	0
	- BES	20	1.3	3.3	10.7	0	0
BB1	+ BES	80	0	82.8	<0.5	<0.5	0
	- BES	80	0	78.0	<0.5	<0.5	0
Viet1	+ BES	80	76.2	<0.5	<0.5	0	0
	- BES	80	75.0	<0.5	<0.5	0	0

[a] Bottles were inoculated (1% [vol/vol]) from nonmethanogenic cultures obtained after the BES treatment. Glycerol (5 mM) and acetate (1 mM) were supplied as electron donors, except for *Desulfitobacterium* sp. strain Viet1 which was supplied with acetate (1 mM) and H_2 (0.4 mmol). PM, Père Marquette River MI; AuS, Au Sable River, MI; RC, Red Cedar River, MI; Parf, Parfume River, Vietnam; TQ, Tahquamenon River, MI.

[b] Head space analysis were performed weekly and PCE (20 µmol) was replenished when depleted until day 65. At day 79 PCE was completely consumed in all cultures. Cultures BB1 and Viet1 were measured every second day and PCE was replenished as depleted until day 17.

[c] The data are means obtained for duplicate cultures after 79 days. in one experiment. Similar values were observed in ±3 days in two subsequent experiments. Dechlorination in cultures of *Desulfuromonas ottawaensis* strain BB1 and strain Viet1 was monitored for 21 days.

The nonmethanogenic cultures obtained after the BES treatment were subsequently transferred to medium without BES, and complete dechlorination of PCE to ETH occurred as in the original microcosms. Subcultures were further enriched with PCE, *cis*-DCE, VC, or 1,2-D as the only available electron acceptor and with acetate, or acetate plus H_2 as the only available electron donor(s). To date, these cultures have undergone over 40 transfers. Three cultures that had

originally dechlorinated PCE to ETH, but were then enriched with *cis*-DCE or VC, lost their ability to dechlorinate PCE (16). This finding indicates that different populations are involved in the complete dechlorination of PCE to ETH in these cultures. Interestingly, the Red Cedar River sediment yielded cultures that maintained their ability to dechlorinate PCE even after enrichment with *cis*-DCE or VC. In order to identify the responsible dechlorinating population, a molecular analysis of this culture was performed. Restriction fragment analysis of the cloned 16S rDNA sequences amplified from the culture showed three distinct restriction patterns. Two of these restriction patterns corresponded to isolates, neither of which dechlorinated PCE. Based upon the complete 16S rDNA sequence of the third clone type, the dechlorinating population, was identified as a *Dehalococcoides* species (97% sequence similarity to *Dehalococcoides ethenogenes*).

The presence and activity of chlororespiring organisms in the different cultures was confirmed by determining H_2 consumption thresholds, and measuring fe values. The H_2 consumption threshold is inversely correlated to the changes in Gibbs free energy ($G^{0'}$) and the electrochemical potential of the H_2-consuming reaction (28). The steady-state threshold concentrations of H_2 will follow the following order (from least to more energetically favorable TEAP): acetogenesis > methanogenesis > sulfate reduction (sulfidogenesis) > Fe(III) reduction > Mn(IV) reduction > denitrification (25, 28). Hydrogenotrophic chlororespiring populations consume H_2 to threshold concentrations lower than those of acetogens, methanogens and sulfate reducers. Hence, the measurement of H_2 consumption thresholds is a useful tool to determine if chlorinated solvents are reduced in a TEAP. The H_2 consumption threshold concentrations in the different nonmethanogenic cultures were determined in the presence and in the absence of a chlorinated electron acceptor (Tab. 2).

Table 2. H_2 thresholds exhibited by cultures in the presence or in the absence of chlorinated aliphatic compounds supplied as electron acceptors

	H_2 concentration [ppmv] [a]	
Culture (enriched on)	In absence of chlorinated electron acceptor (± SD) [b]	In presence of chlorinated electron acceptor (± SD) [c]
KS (1,2-D)	117.3 (37.1; n=7)	0.30 (0.14; n=15)
RC (1,2-D)	343.2 (62.7; n=4)	0.25 (0.17; n=23)
PM (PCE)	319.3 (112.4; n=3)	0.21 (0.16; n=5)
PM (*cis*-DCE)	438.7 (84.7; n=3)	0.24 (0.07; n=6)
PM (VC)	379.3 (117.3; n=3)	0.20 (0.05; n=7)
AuS (PCE)	318.8 (85.7; n=5)	0.21 (0.16; n=11)
AuS (*cis*-DCE)	368.3 (88.3; n=4)	0.34 (0.25; n=6)
AuS (VC)	253.2 (60.9; n=4)	0.19 (0.13; n=8)
RC (PCE)	334.1 (24.5; n=7)	0.16 (0.07; n=10)
RC (*cis*-DCE)	413.3 (107; n=3)	0.21 (0.08; n=9)
RC (VC)	369 (61.7; n=3)	0.21 (0.11; n=13)

a H_2 concentrations were monitored until stable threshold values were measured. When no further decrease in the H_2 concentrations was observed, cultures were fed with H_2 (500 to 10,000 ppmv) and the consumption of H_2 to a constant threshold value was monitored again. Two or three threshold concentrations were obtained per culture, and n indicates the total number of analyses performed.

b Bicarbonate was supplied as the only available electron acceptor and an initial amount of 5 ml (83,000 ppmv) H_2 was added as the electron donor.

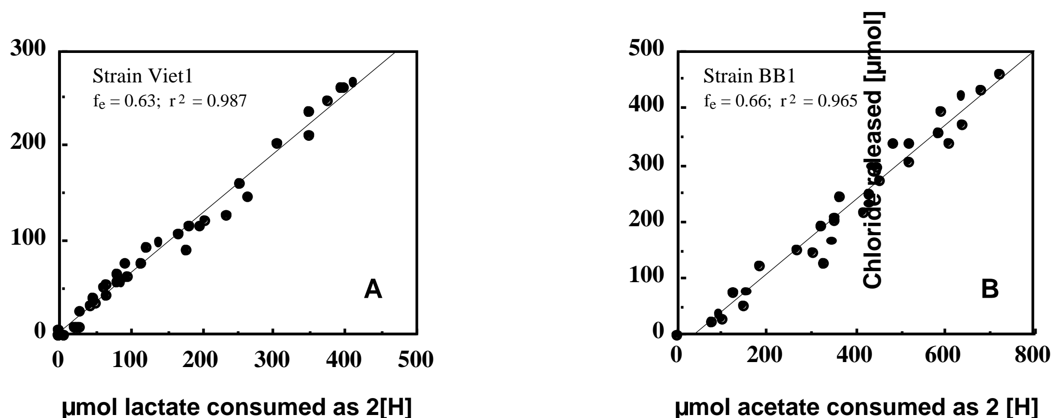
c Cultures were grown in a bicarbonate-buffered medium in the presence of the individual chlorinated aliphatic compound with 5 ml (83,000 ppmv) H_2 as the electron donor. Headspace analyses to monitor dechlorination were performed weekly and the chlorinated compounds were respiked before they were completely depleted.

The H_2 consumption threshold concentrations observed in dechlorinating cultures confirmed the presence and activity of chlororespiring cultures.

Another method to identify the activity of chlororespiring organisms is to determine f_e. The f_e value represents the fraction of electrons used for energy formation, i.e. the fraction of electrons released in the oxidation of the electron donor that are directed toward the reduction of the terminal electron acceptor. The f_e values were determined using linear regression analysis by plotting the electron pairs released in electron donor oxidation as 2 [H] against the amount of electron pairs

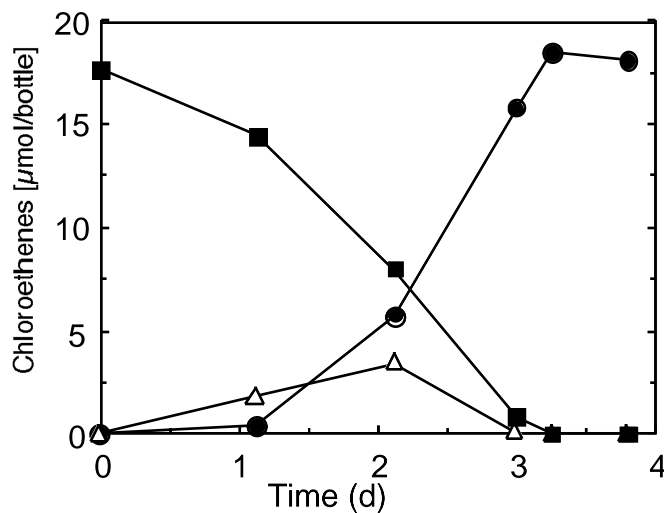
used to reduce the chlorinated electron acceptor (25). f_e values were determined for two PCE-respiring isolates obtained from the nonmethanogenic enrichment cultures (Fig. 2).

Fig. 2. Graphical determination of f_e. (A) *Desulfitobacterium* sp. strain Viet1 (grown with lactate and PCE); (B) *Desulfuromonas ottawaensis* strain BB1 (grown with acetate and PCE)



Desulfuromonas ottawaensis strain BB1 dechlorinated PCE to *cis*-DCE at high rates using acetate as the electron donor (Fig. 3).

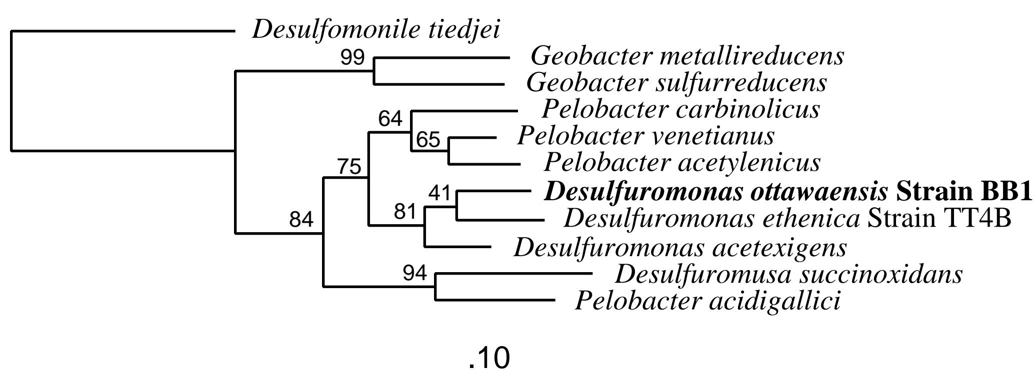
Fig. 3. Dechlorination of PCE to *cis*-DCE with the intermediate formation of TCE. Anaerobic 100-ml cultures with basal salts medium containing 5 mM acetate as the only available electron donor were inoculated with a 1% (vol/vol) inoculum. Dechlorination was followed by headspace analysis and data were averaged from duplicate cultures (squares, PCE; triangles, TCE; circles, *cis*-DCE).



Other electron donors used for reductive dechlorination were lactate and pyruvate, but not H_2 or formate. This is an important observation since it is generally believed that H_2 is the ultimate electron donor supporting chlororespiration in anaerobic environments.

As shown in Figure 4, phylogenetic analysis revealed that strain BB1 belongs to the genus *Desulfuromonas*, and its closest relative is another PCE to *cis*-DCE dechlorinating organism, *Desulfuromonas ethenica* strain TT4B (23).

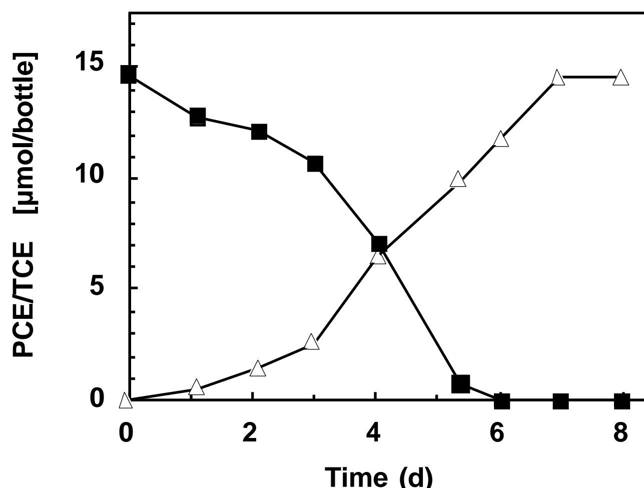
Fig. 4. Inferred phylogenetic relationships of *Desulfuromonas ottawaensis* strain BB1 and selected nearest relatives. The tree was generated by the neighbor-joining method using 1300 nucleotide positions. Bootstrap values are based upon 1000 replicates.



In addition to several key physiological features like electron donor and electron acceptor utilization, strain BB1's16S rDNA sequence is 5% different from the sequence of strain TT4B, indicating that strain BB1 is a different species. Strain BB1's ability to grow by chlororespiration was confirmed by showing PCE dependent growth with acetate, and a measured f_e value of 0.66.

The other isolate was identified as a new member of the genus *Desulfitobacterium*, and designated strain Viet1. *Desulfitobacterium* sp. strain Viet1 reduced PCE to TCE with H_2, formate, lactate, pyruvate, or yeast extract as electron donors (Fig. 5). Acetate, however, could not support reductive dechlorination.

Fig. 5. Dechlorination of PCE to TCE by *Desulfitobacterium* sp. strain Viet1. Anaerobic 100-ml cultures with basal salts medium containing 2.5 mM lactate as the only available electron donor were inoculated with a 1% (vol/vol) inoculum. Dechlorination was followed by headspace analysis and data were averaged from duplicate cultures (squares, PCE; triangles, TCE; circles, *cis*-DCE).



Growth of *Desulfitobacterium* sp. strain Viet1 on lactate was dependent on the presence of PCE, and no growth occurred in the absence of the chlorinated electron acceptor. Growth was also observed with H_2 as the only available electron donor. An f_e value of 0.63 was determined for growth with PCE as the elcectron acceptor, and H_2 was consumed below 0.4 ppmv only in the presence of PCE. These results confirmed that strain Viet1 is a chlororespirer, and can use PCE as an electron acceptor in a TEAP.

The culture conditions to obtain sufficient cell material to assess PCE dechlorinating activity in cell-free systems was optimized for both isolates. Strain BB1 was grown at 28°C in a mineral salts medium amended with 10 mM pyruvate, 20 mM fumarate and PCE. Strain Viet 1 was grown at 37°C in the same medium amended with 30 mM pyruvate and PCE. PCE was dissolved in hexadecane, and 0.5 ml of this solution were added per 10 ml of medium to give a final aqueous PCE concentration of 0.04 mM. Under these conditions 1 g (wet weight) and 1.5 g of cells were obtained per liter with strain BB1 and strain Viet1, respectively. Therefore, sufficient cell material for the investigation of cell-free dechlorinating activity was available. A test system to measure PCE dechlorination in cell-free systems using gas chromatography was developed. The reductive dechlorination of PCE was measured with reduced methyl viologen as the electron donor (22). Dechlorination activity was associated with the membrane fraction with less than 1% of the total activity remaining in the soluble protein fraction, which suggested that the dechlorinating enzyme systems are membrane-bound in both strains. The dechlorinating activity was inducible in both strains, and no PCE dechlorination was observed in extracts derived from cells that were grown in

the absence of PCE. Membrane fractions from PCE induced cells from *Desulfitobacterium* sp. strain Viet1 and *Desulfuromonas ottawaensis* strain BB1 dechlorinated PCE at rates of 210 nmol min^{-1} mg of $protein^{-1}$ and about 300 nmol min^{-1} mg of $protein^{-1}$, respectively. The membrane-bound reductive dechlorinases could be solubilized with 1% Triton X-100, and up to 80% of the total initial activity could be recovered in the solubilized fraction.

Molecular approach to detect *Desulfuromonas* and *Dehalococcoides* species in environmental samples

Members of the genera *Desulfuromonas* and *Dehalococcoides* reductively dechlorinate tetrachloroethene and trichloroethene. Two designed primer pairs specific to hypervariable regions of the 16S rRNA genes of *Dehalococcoides* sp. and the acetate-oxidizing, PCE dechlorinating *Desulfuromonas* group (comprising strains BB1 and *chloroethenica*), yielded 434 and 835 basepair amplicons, respectively. A nested PCR protocol was developed with universal bacterial primers followed by a second PCR with the specific primer pairs (26). The detection threshold of this approach with the *Desulfuromonas*-specific primers was approximately $1x10^3$ BB1 cells added per gram (wet weight) of sandy aquifer material. *Eco*RI restriction fragment analysis (RFLP) of the amplicons could discriminate strain BB1 from strain *chloroethenica*. Total community DNA isolated from sediments of three Michigan rivers and six different chloroethene-contaminated aquifer samples was used as template in nested PCR. All river sediment samples tested yielded positive signals with the *Desulfuromonas*- and the *Dehalococcoides*-specific primers. One chloroethene-contaminated aquifer tested positive with the *Dehalococcoides*-specific primers, and another contaminated aquifer tested positive with the *Desulfuromonas*-specific primer pair.

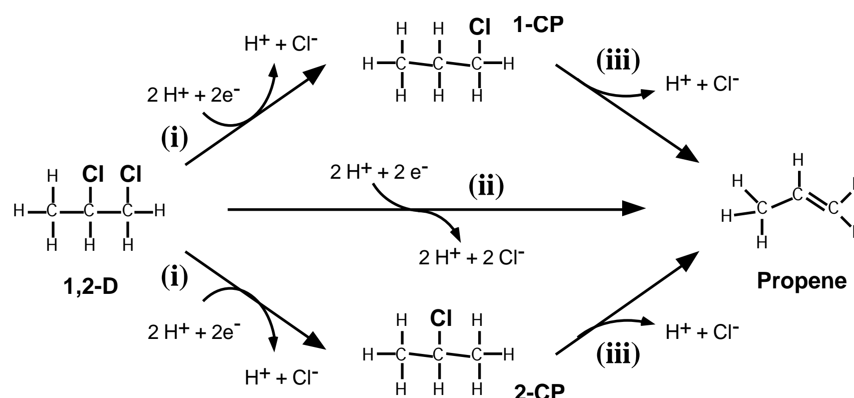
The results obtained with the BB1- and *Dehalococcoides* specific primers were supported by microcosm studies. All sediment and aquifer materials that gave a positive signal with the BB1-specific primers dechlorinated PCE to *cis*-DCE with acetate as the only available electron donor. Microcosms established with materials that showed a positive signal with the *Dehalococcoides*-specific primers dechlorinated PCE to VC and ETH with H_2 as the electron donor. Materials that exhibited no PCE dechlorinating activity in the microcosm studies did not result in amplification with either pair of the specific primers. Hence, no false positive results were obtained with the nested PCR methodology developed in this study (26).

The nested PCR protocols developed for the detection of PCE dechlorinating *Dehalococcoides* and *Desulfuromonas* species are useful to detect these populations in environmental samples, and to monitor their fate in bioaugmentation approaches.

Reductive dechlorination of 1,2-D

Four microcosms that were enriched with 1,2-D as electron acceptor dechlorinated 1,2-D to propene with the intermediate formation of monochlorinated propanes (1-chloropropane and 2-chloropropane). Sediment-free, nonmethanogenic cultures that completely dechlorinated 1,2-D to propene were derived from these microcosms. These enrichments could be indefinitely transferred to mineral salts medium containing acetate as a carbon source, H_2 as the electron donor and 1,2-D as the electron acceptor. Interestingly, in all sediment-free enrichment cultures only dichloroelimination occurred and the intermediate formation of monochloropropanes was never observed. Obviously, the organisms that carried out a single hydrogenolysis step of 1,2-D to monochlorinated propanes in the microcosms were either not present or not active in the sediment-free cultures. Similarly, dehydrochlorination of monochlorinated propanes to propene was observed only in the microcosms and not in sediment-free cultures (Fig. 6).

Fig. 6. Anaerobic transformation of 1,2-D. (i) Hydrogenolysis of 1,2-D resulting in the formation of monochlorinated propanes (1-CP and 2-CP), (ii) dichloroelimination (vicinal reduction) of 1,2-D resulting in the formation of propene, and (iii) dehydrochlorination of monochlorinated propanes resulting in the formation of propene. Hydrogenolysis of monochlorinated propanes and dehydrochlorination of 1,2-D, a reaction that would result in the formation of monochlorinated propenes, were not observed. Note that in sediment-free cultures only reaction (ii), dichloroelimination, occurred.



H_2 threshold measurements suggested that chlororespiring organisms were responsible for the conversion of 1,2-D to propene (see Tab. 2). ARDRA analysis of a clone library from the enrichment culture derived from Red Cedar River sediment showed seven different patterns. Partial sequence analysis of the cloned 16S rDNA genes revealed that six of the seven clones had identical sequences with only one distinct sequence.

4. Relevance, Impact and Technology Transfer

Natural attenuation and engineered bioremediation are often the most cost-effective corrective actions for addressing groundwater contaminated with chlorinated solvents. To decide on the most cost-effective and successful bioremediation strategy the presence or absence of halorespiring organisms must be confirmed. The tools developed in this work (measurements of H_2 consumption thresholds, molecular probes) are relevant for site owners and regulatory agencies in order to facilitate decisions on which bioremediation technology (biostimulation or bioaugmentation) is most promising at a particular site. We are currently testing these methods at several state and DoD sites. In addition, a better understanding of the physiology of the halorespiring organisms as well as the biochemistry of the dehalogenating enzyme systems enhances our knowledge of how these organisms can be most successfully employed in the bioremediation of contaminated sites.

5. Project Productivity

Significant progress was made to more comprehensively characterize the physiology of micro-organisms that can use chlorinated solvents as terminal electron acceptors in their energy metabolism. H_2 consumption threshold measurements and the determination of f_e values were shown to be reliable tools to detect the activity of halorespiring populations. In situ measurements at sites contaminated with chlorinated compounds are required to validate these findings. This, however, was beyond the scope of this research. The development of specific molecular probes gave a preliminary insight into the distribution of two PCE respiring populations, however, further research is required to fully understand the distribution and abundance of halorespirers in sediments and aquifers. The complete purification and characterization of the dechlorinating enzyme systems from *Desulfuromonas ottawaensis* strain BB1 could not be completed because of time constraints. The focus of the proposed research was shifted to the H_2 consumption threshold measurements and the determination of f_e values because we felt that the development of such tools is of greater practical use to DOE, DoD and other site owners. We will, however, seek additional funding from DOE or other agencies to complete the investigations on the dechlorinating enzyme systems.

6. Personnel Supported

Dr. James M. Tiedje was the lead PI but not salaried by the project, Dr. Frank E. Löffler, Co-PI, was supported by this project, and, for a 1-year effort, Ms. Qing Sun was supported as a graduate student.

7. Publications

The following publications stem from this research effort:

1. **Löffler, F. E., K. M. Ritalahti, and J. M. Tiedje.** 1997. Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. Appl. Environ. Microbiol. **63:**4982-4985.
2. **Löffler, F. E., J. M. Tiedje, and R. A. Sanford.** 1999. The fraction of electrons consumed in electron acceptor reduction (f_e) and hydrogen thresholds as indicators of halorespiratory physiology. Appl. Environ. Microbiol. **65:**4049-4056.
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Results from this research were presented at national and international meetings (see under 8, Interactions).

8. Interactions

Results from this research were presented at the EMSP Workshop in Chicago, July 1998, and at national (General Meetings of the American Society of Microbiology in Atlanta and Chicago; the 5th International Symposium on In situ and on-site Bioremediation in San Diego, CA; the 1999 International Symposium on Subsurface Microbiology in Vail, CO), and international meetings (8th International Symposium on Microbial Ecology in Halifax, Canada; general meeting of the VAAM in Hamburg, Germany).

Collaborations were established with Dr. Babu Fathepure, Department of Civil and Environmental Engineering, The University of Michigan, Dr. R. A. Sanford, Department of Civil and Environmental Engineering, University of Illinois at Urbana/Champaign, and with Dr. Erik Petrovskis who is a scientist at HSI GeoTrans, Ann Arbor, MI.

9. Transitions

The knowledge gained from this research is useful to detect chlororespiring populations in environmental samples, and to demonstrate and to monitor their dechlorinating activity. The molecular approach developed in this research was used detect PCE respiring populations at the PCE contaminated Bachman Road site in Oscoda, MI in a project funded by the Michigan Department of Environmental Quality. H_2 threshold measurements and determinations of f_e values confirmed the presence and activity of chlororespiring organisms at this site.

In a collaborative effort with HSI GeoTrans in Ann Arbor, MI, these techniques were applied to demonstrate the presence/absence of PCE respiring populations in aquifer samples collected from a variety of contaminated sites in the U.S.

10. Patents

No patents were filed from this work.

11. Future Work

Future work will focus on the isolation of *cis*-DCE and VC degrading populations to more comprehensively understand how complete detoxification of chloroethenes can be achieved. Also, the complete purification of the dechlorinating enzyme systems will be pursued. It will be interesting to compare the properties of the PCE to TCE dechlorinase and the PCE to *cis*-DCE dechlorinase, and to understand why the desulfitobacterium enzyme removes only one chlorine whereas the dechlorinase from *Desulfuromonas ottawaensis* strain BB1 removes two chlorines. The community analysis of the 1,2-D dechlorinating enrichment cultures based on the cloned 16S rDNA sequences will be completed. This will allow the development of more efficient procedures to isolate the 1,2-D dechlorinating populations, and to gain more detailed knowledge of the microbial strategies to dechlorinate chlorinated propanes.

12. Literature Cited

1. **Abelson, P. H.** 1990. Inefficient remediation of ground-water pollution. Science **250:**733.
2. **Babich, H., D. L. Davis, and G. Stotzky.** 1981. Dibromochloro-propane (DBCP): a review. Sci. Total Environ. **17:** 207-221.
3. **Bagley, D. M., and J. M. Gossett.** 1990. Tetrachloroethene transformation to trichloroethene and *cis*-1,2-dichloroethene by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. **56:**2511-2516.
4. **Boesten, J. J. T. I., L. J. T. van der Pas, M. Leistra, J. H. Smelt, and N. W. H. Houx.** 1992. Transformation of ^{14}C-labelled 1,2-dichloropropane in water-saturated subsoil materials. Chemosphere **24:**993-1011.
5. **Bosma, T., E., Kruizinga, E. J. De Bruin, G. J. Poelarends, and D. B. Janssen.** 1999. Utilization of trihalogenated propanes by *Agrobacterium radiobacter* AD1 through heterologous expression of the haloalkane dehalogenase from *Rhodococcus* sp. strain m15-3. Appl. Environ. Microbiol. **65:**4575-4581.
6. **de Bruin, W. P., M. J. J. Kottermann, M. A. Posthumus, G. Schraa, and A. J. B. Zehnder.** 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58:**1996-2000.

7. **Burris, D. R., Delcomyn, C. A., Smith, M. H., and Roberts, A. L.** 1996. Reductive dechlorination of tetrachloroethene and trichloroethene catalyzed by vitamin B12 in homogeneous and heterogeneous systems. Environ. Sci. Technol. **30:**3047-3052.

8. **Cohen, D. B., D. Gilmore, B. S. Fischer, and G. W. Bowes.** 1983. Water quality and pesticides: 1,2-dichloropropane (1,2-D) and 1,3-dichloropropene (1,3-D). California State Water Resources Control Board, Sacramento.

9. **Distefano, T. D., J. M. Gossett, and S. H. Zinder.** 1991. Reductive dechlorination of high concentrations ot tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. Appl. Environ. Microbiol. **57:**2287-2292.

10. **Dolfing, J., and D. B. Janssen.** 1994. Estimates of Gibbs free energies of formation of chlorinated aliphatic compounds. Biodegradation **5:**21-28.

11. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53:**2671-2674

12. **Fathepure, B. Z., and S. A. Boyd.** 1988. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. Appl. Environ. Microbiol. **53:**2976-2980.

13. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. **49:** 149-156.

14. **Fathepure, B. Z., and J. M. Tiedje.** 1998. Anaerobic bioremediation: microbiology, principles, and application. *In* D. C. Adriano, J.-M. Bollag, and W. T. Frankenburger (ed.), Bioremediation of soils, American Society for Agronomy. In press.

15. **Fetzner, S., and F. Lingens.** 1994. Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. Microbiol. Rev. **58:** 641-685.

16. **Flynn, S. J., Löffler, F. E., and J. M. Tiedje.** 2000. Microbial community changes associated with a shift from reductive dechlorination of PCE to reductive dechlorination of cis-DCE and VC. Environ. Sci. Technol. In press.

17. **Freedman, D. L., and J. M. Gossett.** 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55:**2144-2151.

18. **Gantzer, C. J., and J. M. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. **25:** 715-722.

19. **Glod, G., W. Angst, C. Holliger, and R. Schwarzenbach.** 1997. Corrinoid-mediated reduction of tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogeneous aqueous solution: reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31:**253-260.

20. **Holden, P. W.** 1986. Pesticides and groundwater quality - issues and problems in four states. National Academy Press, Washington, D.C.1.

21. **Krumholz, L. R., R. Sharp, and S. Fishbain.** 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethene dehalogenation. Appl. Environ. Microbiol. **62:**4108-4113.

22. **Löffler, F**. E., R. A. Sanford, and J. M. Tiedje. 1996. Initial characterization of a reductive dehalogenase from *Desulfitobacterium chlororespirans* Co23. Appl. Environ. Microbiol. **62:**3809-3813.

23. **Löffler, F. E., J. E. Champine, K. M. Ritalahti, S. J. Sprague, and J. M. Tiedje.** 1997. Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. Appl. Environ. Microbiol. **63:**2870-2875.

24. **Löffler, F. E., K. M. Ritalahti, and J. M. Tiedje.** 1997. Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. Appl. Environ. Microbiol. **63:**4982-4985.

25. **Löffler, F. E., J. M. Tiedje, and R. A. Sanford.** 1999. The fraction of electrons consumed in electron acceptor reduction (f_e) and hydrogen thresholds as indicators of halorespiratory physiology. Appl. Environ. Microbiol. **65:**4049-4056.

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27. **Löffler, F. E., J. Li, J. W. Urbance, and J. M. Tiedje.** 1998. Description of *Desulfuromonas ottawaensis* strain BB1, a novel tetrachloroethene-reducing, acetate-oxidizing anaerobe from pristine freshwater sediment. Manuscript in preparation.

28. **Lovley, D. R., and S. Goodwin.** 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta. **52:**2993-3003.

29. **Maymó-Gatell, X., Y.-T., Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276:1568-1571.

30. **McKenry, M. V.** 1983. The nature, mode of action, and biological activity of nematicides, p. 59-73. *In* D. Pimentel (ed.), CRC handbook of pest management in agriculture, vol. 3. CRC Press, Boca Raton, Fla.

31. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55:**2819-2826.

32. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1990. Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. Appl. Environ. Microbiol. **56:**2568-2571.

33. **Slater, J. H.** 1994. Microbial dehalogenation of haloaliphatic compounds, p. 379-421. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

34. **Swanson, P. E.** December 1994. U.S. patent 5,372,944.

35. **Terzenbach, D. P. and M. Blaut.** 1994. Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. FEMS Microbiol. Lett. **123:**213-218.

36. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49:**1080-1083.

37. **Weissermel K., and H.-J. Arpe.** 1993. Industrial organic chemistry, 2nd ed. VCH, New York, N.Y.

38. **Wild, A. P., G. Schwarz, W. Winkelbauer, A. M. Cook, S. A. Braus-Strohmeyer, and T. Leisinger.** 1992. Trichloroethene dechlorination to ethene or ethane. *In* Anaerobic dehalogenation and its environmental implications. Athens, Georgia, American Society for Microbiology.