Environmental Microbiology (2004) **6**(1), 45–54 doi:10.1046/j.1462-2920.2003.00525.x

Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and *cis*-DCE

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Summary

In order to study the effect of different chloroethenes (electron acceptors) on the bacterial composition of dechlorinating communities, two reductive dechlorinating enrichment cultures were developed that were able to reduce trichloroethene (TCE) and *cis*-1,2-dichloroethene (*cis*-DCE) to ethene using hydrogen as electron donor, respectively. The inoculum for the cultures was material from a methanogenic fluidized bed reactor (FBR), which was originally seeded with digester sludge and showed a stable capacity for tetrachloroethene (PCE) reduction to ethene for over six years. Molecular methods were used to determine and compare the microbial communities of these two enrichment cultures. A clone library of bacterial 16S rRNA genes was generated for each enrichment. The clones were screened into different groups by restriction fragment length polymorphism (RFLP) analysis using two different four base pair recognition restriction enzymes. A total of 12 sequence types were identified by phylogenetic analysis of nearly complete 16S rDNA sequences (~1450 bp). The sequences were affiliated with six recognized phyla of the domain Bacteria: Firmicutes (low G+C Gram-positives), Chloroflexi (green non-sulphur bacteria), Actinobacteria (high G+C Gram-positives), Bacteroidetes (Cytophaga-Flexibacter-Bacteroides), Nitrospira and Spirochaetes. The results led to the identification of an organism closely related to *Dehalococcoides ethenogenes* to be the presumptive dechlorinator in both enrichments. Different electron acceptors affected the bacterial diversity and the community profiles of the two enrichments. Most of the sequences identified in our dechlorinating enrichments shared high similarities with sequences previously obtained from other enriched dechlorinating cultures and chlorinated-compound-contaminated sediments or aquifers, suggesting these bacteria may have direct or indirect roles in reductive dechlorination.

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Introduction

The toxic chlorinated solvents PCE and TCE are among the most abundant groundwater contaminants. Under anaerobic conditions, PCE or TCE can be sequentially reduced to *cis*-DCE or to vinyl chloride (VC) and ethene by energy-yielding dehalorespiration or by cometabolic processes (Middeldorp *et al*., 1999). Because the reductive dehalogenation of PCE and TCE in respiratory processes is faster than anaerobic cometabolic processes, and because anaerobic conditions usually prevail in aquifers, reductive dechlorination has been the most studied and applied approach for bioremediation of PCE or TCE contaminated aquifers.

The reductive dechlorination process usually occurs in environments where methanogenesis and/or acetogenesis occurs (Holliger *et al*., 1999). The dechlorination function of a chloroethene-reducing microbial consortium, like any other anaerobic ecosystem, relies on the biochemical collaboration of different members in the microbial community. Many mixed cultures have been described that exhibit stable capacities to reduce chloroethenes completely to ethene after several laboratory transfers or during long-term performance in continuously operated reactors (Vogel and McCarty, 1985; DiStefano *et al*., 1991; Debruin *et al*., 1992; Holliger *et al*., 1993; Ballapragada *et al*., 1997; Flynn *et al*., 2000). However, the microbial compositions of such chloroethene-dehalogenating communities have not been studied extensively.

Most studies of chlorinated-ethene-dehalorespiring organisms have been based on traditional isolation-dependent methods, and have found that chlorinated-

ethene-dehalorespiring organisms can be categorized into two groups (Middeldorp *et al*., 1999). One physiological group, which reductively dechlorinates PCE and TCE only to *cis*-DCE, includes phylogenetically diverse groups of bacteria that lie in the Firmicutes, or δ and ε branches of the Proteobacteria. Another group, which is capable of further reductive dechlorination of *cis*-DCE to VC and ethene, is a specific cluster of bacteria that is closely related to *Dehalococcoides ethenogenes,* lying within the Chloroflexi (green non-sulphur bacteria). Isolation of dechlorinators that are capable of reducing *cis*-DCE or VC to ethene has been difficult (Ballapragada *et al*., 1997; Cupples *et al*., 2003). *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain FL2 are the only known isolates that are able to completely reduce PCE to ethene. However, unknown growth factors within a sludge supernatant are required by strain 195, suggesting that the dechlorinators may have a syntrophic interaction with other organisms in the consortium (Maymo-Gatell *et al*., 1997). Even though the *Dehalococcoides* group is often detected in environmental samples that perform complete dechlorination of chloroethenes (Löffler *et al*., 2000; Fennell *et al*., 2001; Hohnstock-Ashe *et al*., 2001; Hendrickson *et al*., 2002), it still remains to be seen whether there are other organisms in the environment that can catalyse the dechlorination of PCE or TCE to ethene besides *Dehalococcoides*.

A limited knowledge of dechlorinating microorganisms and of the diversity of microorganisms involved in the dehalogenation processes often hampers the understanding and the application of the complete reductive dechlorination technology for bioremediation of chlorinated ethenes. Fundamental information on the ecology and biophysical interaction of community members involved in the partial and complete dechlorination processes should help to better understand and design successful remediation strategies for different chloroethene-contaminated sites.

Culture-independent, 16S rDNA-based molecular approaches have been useful in studying microbial diversity and community structure of mixed enrichment cultures. Molecular methods have been applied to conduct phylogenetic analyses of a number of consortia that dehalogenate chlorinated aromatic compounds (Adrian *et al*., 1998; Pulliam-Holoman *et al*., 1998; von Wintzingerode *et al*., 1999; Zhou *et al*., 1999; Breitenstein *et al*., 2001). However, only a few microbial community analysis studies have been conducted with chloroethene-reducing enrichments or ecosystems (Dojka *et al*., 1998; Richardson *et al*., 2002).

In this study, we applied a culture-independent 16S rDNA-based molecular approach to characterize two dehalogenating mixed cultures, one that dechlorinated TCE to ethene and another that dechlorinated *cis*-DCE to ethene. Because reductive dechlorination can occur in the absence of methanogens (DiStefano *et al*., 1991) and all known chloroethene-respiring organisms belong to the domain Bacteria, only the bacterial community was characterized in this study. Possible roles of some populations present in the enrichments are discussed.

Results and discussion

Establishment of the TCE- and cis*-DCE-dechlorinating cultures*

Two methanogenic subculture enrichments were established, one degrading TCE to ethene and the other degrading *cis*-DCE to ethene. The enrichments were inoculated with material from a methanogenic, PCE-degrading FBR culture, which was originally seeded with activated sludge (Ballapragada *et al*., 1997), and was able to carry out complete reductive dechlorination of PCE or TCE to ethene in the temperature range of 15–35°C (Pietari, 1999). H_2 was found to be the direct electron donor for the reduction of chlorinated compounds. For the development of subcultures, lactate was used as the electron donor and carbon source initially. Dechlorination activity started after about 3–4 weeks. Later, lactate was replaced with H_2 as the electron donor and acetate as the carbon source to simplify the community. The reductive dechlorination rates slowly increased and seem to be stabilized after 6 months. After enrichment for over 8 months, the biomass concentrations (as volatile suspended solids, VSS) of the two enrichment cultures increased to about 300 mg l^{-1} and 500 mg l^{-1} for the *cis*-DCE and TCE-reducing culture respectively. Methane was produced in both cultures. The TCE-fed subculture was intended to enrich for bacteria carrying out reductive dechlorination of TCE to *cis*-DCE and for bacteria carrying out complete reductive dechlorination of TCE to ethene. The *cis*-DCE fed culture was intended to enrich for only bacteria that were able to carry reductive dechlorination of *cis*-DCE to ethene. However, after 8 months exposure to only *cis*-DCE, the *cis*-DCE-fed culture was still able to reduce TCE.

Chloroethene degradation characteristics of the enrichments

Degradation of TCE and *cis*-DCE and formation of corresponding dechlorination intermediate products by the TCE-reducing and *cis*-DCE-reducing enrichment cultures are shown in Fig. 1. Conversions of TCE to *cis*-DCE and of *cis*-DCE to VC were faster than the conversion of VC to ethene. Vinyl choride remained as the main intermediate product in the enrichment cultures until *cis*-DCE was completely consumed. Vinyl choride was degraded rapidly

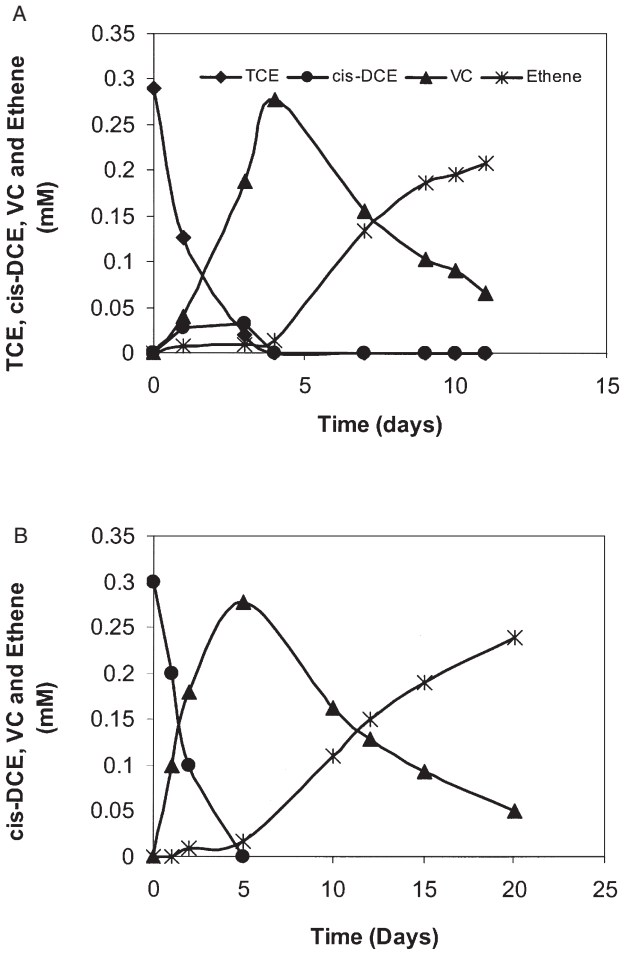

Fig. 1. Reductive dechlorination of TCE and *cis*-DCE and formation of intermediates by the TCE-reducing enrichment (A) and by the *cis*-DCE-reducing enrichment (B) at room temperature (22–25°C). Concentrations on the *Y*-axis refer to total amounts in the reactor divided by the liquid volume.

and corresponding ethene was produced after the disappearance of *cis*-DCE. These observations suggest that the final conversion of VC to ethene was inhibited by *cis*-DCE, which was consistent with the observations with the original FBR enrichment. The two subcultures could dechlorinate TCE and *cis*-DCE to ethene at an average rate of approximately 20 μmole l^{-1} day^{-1} and 12 μmole l^{-1} day^{-1} respectively.

Estimation of bacterial diversity

To compare the community structure and microbial diversity of the two dechlorinating enrichment cultures, two separate bacterial 16S rDNA clone libraries were established and screened by RFLP using two tetrametric restriction enzymes (*Msp*I and *Tsp*509I). Altogether, 28 unique RFLP patterns were found: five were present in both the TCE and *cis*-DCE clone libraries, 14 in the TCE reactor library only, and nine in the *cis*-DCE reactor clone library only. For each RFLP pattern that occurred more than once in either clone library, one or more representative clones were selected for 16S rDNA sequencing. As a result, a total of 12 sequence types were identified. An extrapolation method (Colwell and Coddington, 1994) was used to evaluate the representation of clones obtained and to estimate bacterial richness (diversity) of the two dechlorinating communities (Hughes *et al*., 2001). The results are shown in Fig. 2. The observed RFLP pattern accumulation data was fitted to a Michaelis-Menten type curve using non-linear regression analysis (Windows SSSP version 1.0). The bacterial richness expected at infinite effort (shown as the maximum number of unique RFLP patterns N_{max}) was then estimated. The maximum number of RFLP patterns was estimated to be 24 in the TCE and 17 in the *cis*-DCE reactor respectively. Therefore, approximately 79% (19/24) of the RFLP patterns in the TCE reactor and 82% (14/17) of those in the *cis*-DCE reactor were revealed in this study.

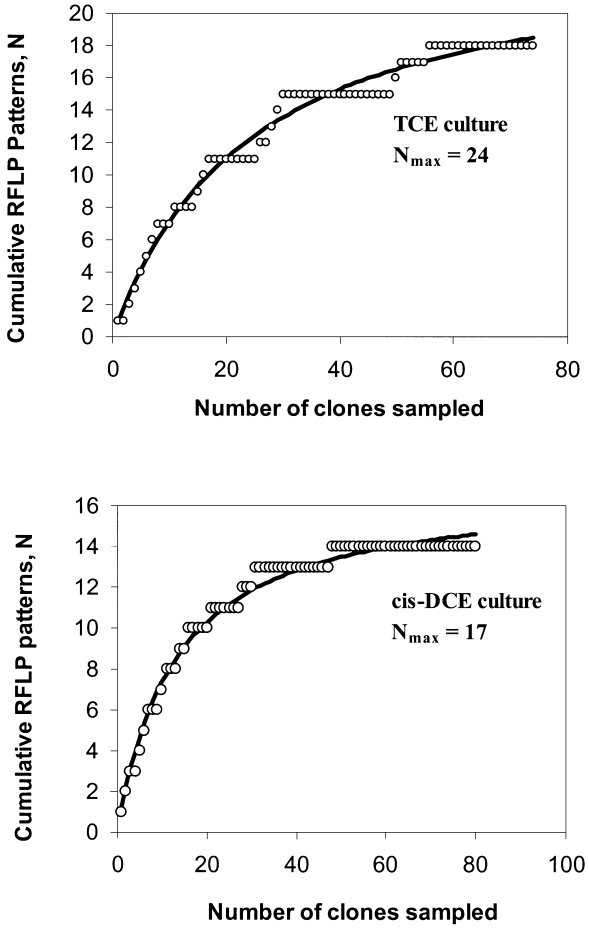

Fig. 2. Observed and estimated richness (RFLP pattern) of bacteria in the *cis*-DCE-reducing enrichment and TCE-reducing enrichment. Maximum richness is estimated by non-linear regression fitting of observed accumulation data to a Michaelis-Menten type equation.

Table 1. Phylogenetic affiliations of predominant RFLP types from TCE and *cis*-DCE-dechlorinating enrichments based on 16S rDNA sequences.

			Percentage in libraries	
RFLP type	Phylum	Closest phylogenetic relative (% similarity)	TCE	DCE
DCE5	Spirochaetes	1,2-dichloropropane-dechlorinating culture clone SHA-4 (98%)	–	13%
DCE17	Chloroflexi	*Dehalococcoides ethenogenes* (99%)	40%	34%
DCE25	Firmicutes	TCE dechlorinating culture clone 6A (97%)	–	8%
DCE29	Nitrospira	Chlorobenzene dechlorinating culture clone GOUTA19 (81%)	5%	3%
DCE33	Spirochaetes	*Spirochaeta* sp. Buddy from TCE dechlorinating culture (99%)	3%	3%
DCE47	Proteobacteria	*Afipia* sp. (99%)	–	5%
TCE4	Firmicutes	TCE-contaminated site clone FTLM142 (95%)	4%	3%
TCE5	Bacteroidetes	Chlorobenzenes dechlorinating culture clone IA-16 (93%)	5%	–
TCE8	Firmicutes	*Alkilaphilus cronoxidans* (93%)	7%	–
TCE16	Actinobacteria	*Microbacterium laevaniformans* (99%)	4%	–
TCE33	Firmicutes	Chlorobenzene dechlorinating culture clone IB-27 (99%)	12%	25%
TCE41	Firmicutes	Trichlorophenol-dechlorinating culture clone ZF2 (99%)	3%	–

Effect of electron acceptor (TCE versus cis-DCE) on community profiles

Table 1 shows the phylogenetic affiliations of the RFLP types that were sequenced as well as their percentage abundance in each clone library. It is worthwhile to note that these percentages may not represent the actual microbial abundance in the cultures as a result of the bias of PCR amplification of genes from communities (von Wintzingerode *et al*., 1997). Nevertheless, the apparent absence of certain RFLP types in one enrichment or the other indicates that the available electron acceptor does play a role in defining the structure of the dechlorinating communities. Two sequence types, DCE5 and DCE25, were found to be relatively abundant in the *cis*-DCE library; however, they were not detected in the TCE culture. Conversely, there were several RFLP types that were only detected in the TCE enrichment culture such as TCE8, TCE41, TCE5 and TCE16. In contrast, a number of RFLP types seemed to be present in both cultures including DCE17, TCE33, DCE33 and DCE29.

The notable differences between the two enrichments were the different electron acceptor applied and the various chloroethene (TCE, *cis*-DCE and VC) concentrations present during the batch feedings. The two enrichments were inoculated from the same source and were provided with identical electron donor and carbon source. As shown in Fig. 1, *cis*-DCE persisted in the *cis*-DCE-fed reactor at higher concentration than in the TCE-fed culture, in which *cis*-DCE was only present as an intermediate. Vinyl chloride reduction to ethene seemed to proceed only after the consumption of *cis*-DCE, and the VC reduction rate was slower in the *cis*-DCE-fed culture than in the TCE-fed culture. The lower feeding rate (15 μmole l^{-1} day^{-1}) of *cis*-DCE compared to the feeding rate of TCE (20 μmole l^{-1} day^{-1}), as well as the fact that one fewer chloride ion was removed per mole of cisDCE dechlorinated, compared with TCE, led to less biomass production for dechlorinators in the *cis*-DCE-fed reactor. This may explain the slower VC conversion to ethene observed in the *cis*-DCE culture. Some impurities such as chloroform have been found in commercially available *cis*-DCE (Maymo-Gatell *et al*., 2001), which might also have had some effect on the populations in the *cis*-DCE culture. Nevertheless, the results indicate that the presence of different electron acceptors affected not only the type of dechlorinators selected but also the community structure overall. These results are consistent with other researchers' observations. Different community profiles in dechlorinating cultures that were enriched with different chloroethenes as electron acceptors have also been reported by Flynn *et al*. (2000) and Duhamel *et al*. (2002), as shown by terminal restriction fragment length polymorphism or denaturing gradient gel electrophoresis patterns.

Phylogenetic analysis of sequence types from enrichment cultures

Phylogenetic analysis of the predominant RFLP types led to the identification of species in the two microbial communities; they belong to six recognized phyla: Firmicutes, Chloroflexi, Actinobacteria, Bacteroidetes, Nitrospira, Spirochaetes and α-Proteobacteria. The phylogenetic affiliation of predominant sequence types found is shown in Table 1. Figure 3 is an evolutionary tree that shows the bacterial 16S rDNA sequence types obtained from the TCE and the *cis*-DCE-reducing enrichments.

Even though the TCE-reducing enrichment was intended to enrich for both bacteria that dechlorinated TCE to *cis*-DCE (e.g. *Desulphuromonas* sp.) and bacteria that were capable of complete dechlorination of *cis*-DCE to ethene, no sequence type that was closely related to any known TCE- to *cis*-DCE dechlorinators was found in the TCE clone library. Instead, the predominant sequence

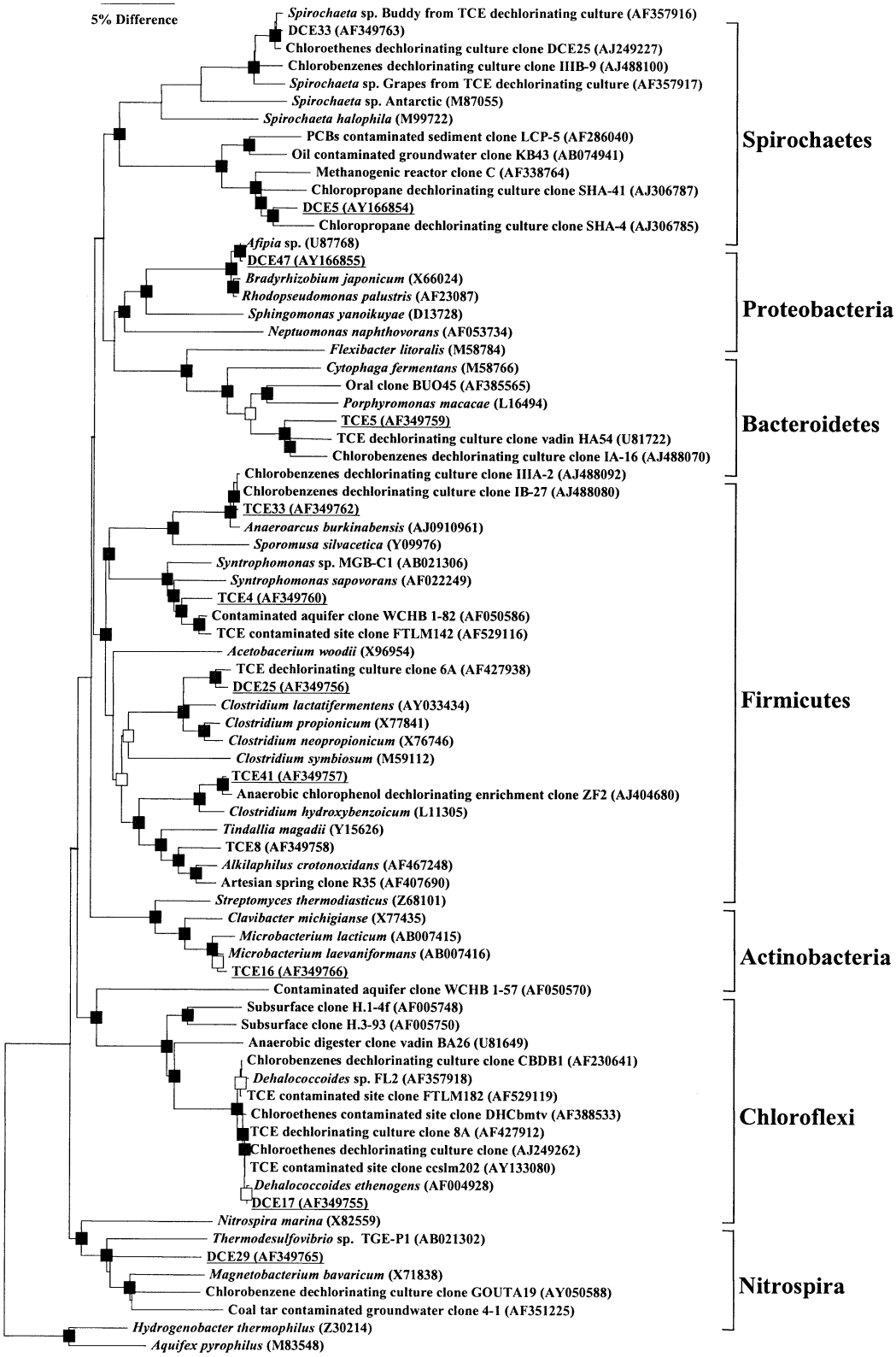

Fig. 3. Distance tree showing relationships between bacteria from the dechlorinating enrichments (underlined) and reference organisms. The tree was created using neighbour-joining and a Kimura 2 parameter correction. Solid squares denote nodes that were well-supported (>80% support for distance and parsimony with and without 50% mask); open squares were weakly supported (>60% all analyses); and empty nodes were essentially unresolved (<60% for at least one analysis).

type in both cultures, DCE17, was a close relative of two TCE to ethene dechlorinating isolates, namely *D. ethenogenes* strains 195 (99.1% identity) and CBDB1 (98.6% identity). DCE17 also shared high similarity to a number of environmental sequences found in other chloroethene-dechlorinating mixed enrichment cultures, contaminated sites and river sediments (Löffler *et al*., 2000; Hendrickson *et al*., 2002; Richardson *et al*., 2002). These closely related rDNA sequences form a unique phylogenetic cluster that is quite distant from all the other sequences belonging to the same bacterial division. Identification of DCE17 in our dechlorinating enrichments adds more evidence to several other researchers' observations that *Dehalococcoides* spp. are phylogenetically conserved and that they are prevalent in chloroethene-reducing environments. It is therefore inferred that the sequence type DCE17 found in this study represented a dechlorinating population in the TCE-reducing and *cis*-DCE-reducing enrichments.

Even though the *Dehalococcoides* group of organisms seems to have the ability to perform dehalorespiration, phenotypic diversity exists among its members in terms of the different chlorinated compounds they can use as electron acceptors and the different dehalogenating enzyme(s) they possess. For example, CBDB1, which shared high similarity of 16S rDNA with *D. ethenogenes* strain 195, was an isolate that was able to thrive on the energy obtained from reductive dehalogenation of polychlorinated-benzenes (Adrian *et al*., 2000). CBDB1 was not able to dechlorinate PCE or TCE under the conditions studied. *Dehalococcoides* species lacking a TCE-reductase gene (tceA), which was found in *D. ethenogenes*, were detected in mixed cultures capable of reducing VC to ethene by Sung *et al*. (2002). Furthermore, the ability of the *Dehalococcoides* species to catalyse and conserve energy from the ultimate dechlorination step, VC to ethene, seems to be uncertain and may vary from strain to strain. The reduction of VC to ethene by *D. ethenogenes* strain 195 has been shown to be likely a cometabolic process (Maymo-Gatell *et al*., 2001). To date, no isolate has been obtained that can grow using the reductive dechlorination of VC to ethene as its only electron acceptor. However, there is evidence with laboratory mixed cultures that there might be organisms that can sustain the reduction of VC (Ritalahti *et al*., 2001; He *et al*., 2003; Cupples *et al*., 2003). In a separate study with the FBR culture and the two subcultures established in this study, all three cultures showed the ability to maintain VC reductive dechlorination in the absence of higher chlorinated-ethenes, with H_2 and acetate as the electron donor and carbon source respectively. In addition, the VC degradation rates increased 8–10 times during the 8 month period of the study (data not shown). These results indicate that the *Dehalococcoides* sp. identified in this study might represent an organism that can conserve energy from reductive dechlorination of VC.

One RFLP type, DCE 5, consisted of a significant number of clones in the *cis*-DCE-reducing culture clone library (13% of clones) and was only distantly related to any cultured bacteria (<80% homology). However, this sequence had high similarity with a number of uncultured bacteria that were found in other dechlorinating ecosystems including a 1,2-dichloropropane-dechlorinating microbial consortium, a sediment contaminated with polychlorinated biphenyls (PCB) and an oil-contaminated groundwater. The metabolic function of this organism and why it was present only in the *cis*-DCE-dechlorinating culture, but not in the TCE-reducing culture (in which *cis*-DCE was present as intermediate), is unclear. In addition, the close relatedness of DCE5 to sequences found in other dechlorinating ecosystems and its distance from other known bacteria indicates a possible association of DCE5 with dechlorination.

An abundant sequence cluster, designated as TCE33, present in both TCE and *cis*-DCE enrichments, was associated with the *Sporomusa* group of the *Firmicutes*. Five clones were sequenced in this group and the similarities of the sequenced regions ranged from 99% to 98% among these clones. The three database sequences most similar to TCE33, 99% to 98% identical, were from a stable dechlorinating bacterial consortium able to remove singly flanked chlorine substituents from chlorobenzenes. The known bacteria that shared the highest similarity with TCE33 found in GenBank included *Anaeroarcus burkinabensis* (98% identical) and *Anaeromusa acidaminophila* (97% identical). Other sequences within the *Sporomusa* group have significantly lower similarities (<92%). Both *Anaeroarcus burkinabensis* and *Anaeromusa acidaminophila* are anaerobic bacteria that are able to ferment lactate. Because these closely related bacteria share common metabolic properties and as lactate was fed as the electron donor and carbon source in their enrichment, it seems likely that this sequence type represents the population in our chloroethene-dehalogenating enrichments that fermented lactate. The presence of this group in our enrichments long after lactate was replaced by hydrogen and acetate is not understood.

Four other sequence types represented the *Firmicutes*: TCE4, DCE25, TCE8 and TCE41. The three sequences that matched TCE4 most closely (>94% identical) were clones from a hydrocarbon and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation (WCHB1-71, WCHB1-82), and from a TCE-contaminated site undergoing *in situ* bioremediation (FTLM142). Contaminants detected in the plume at the WCHB site included: benzene, toluene, ethylbenzene, xylenes, DCE and VC. Several known bacteria that share relatively high similarity (>90% identity) with this sequence include *Syn-*

trophomonas sp. MGB-C1 and S*yntrophomonas sapovorans*. These two *Syntrophomonas* species are proton-reducing and saturated fatty acid-β-oxidizing bacteria in syntrophic association with hydrogen-utilizing methanogens (Hansen *et al*., 1999). The enrichments in the present study were methanogenic and were fed lactate as the energy and carbon source. It is reasonable to hypothesize that the *Syntrophomonas* species found in our enrichments and in chloroethene-contaminated aquifers may share properties with related cultivated organisms. H_2 production is usually attributed partially to propionate metabolism in anaerobic cultures fed lactate. The proton-reducing acetogenic bacteria may play a role in providing H_2 as an electron donor for dehalogenation.

TCE41 was closely related (99% identity) to clone ZF2, which was present in significant numbers in a 2,4,6-trichlorophenol-dehalogenating enrichment culture that was enriched from river sediment (Breitenstein *et al.,* 2001). The known bacterium to which this sequence was most closely related (96% identical) was *Clostridium hydroxybenzoicum* str. JW/Z-1, which was also found to be involved in the dechlorination of chlorinated phenolic aromatic compounds (Huang *et al*., 1999).

Two other sequences, TCE8 and DCE25, which belong to the same *Clostridium* group, but quite distant from *C. hydroxybenzoicum,* were found in the TCE and *cis*-DCE enrichments. DCE25 shared the highest identity (97.4%) to clone sequence 6A, found in another TCE-reducing reductive dechlorinating enrichment that was inoculated with a sample from a chloroethene-contaminated site (Hendrickson *et al*., 2002). The *Clostridium* spp. that are most closely related to DCE25 include *C. lactatifermentans* (93.5% identity) and *C. propionicum* (93% identity). Both of these species are lactate fermenters (Kuchta and Abeles, 1985; van der Wielen *et al*., 2002). DCE25 and clone 6A might represent lactate-fermenting organisms in the two reductive dechlorinating enrichments as lactate was applied as an energy and carbon source to both of these cultures. A strict anaerobe isolated from the wastewater of a bean curd farm, *Alkaliphilus crontonoxidans* (93% identical), is the nearest relative of TCE8.

A *Spirocheata* sequence (DCE33), which was found in both of our enrichments, shared high identity (>94%) with a number of isolates and sequences from other chloroethene-dechlorinating consortia, and they form a unique cluster that is distant from other sequences (<87% identity) in the same division. *Spirochaeta* sp. buddy and *Spirochaeta* sp. grapes were isolated from a highly enriched and defined TCE-dechlorinating consortium, which was found to contain three distinct populations, including the two spirochetes mentioned above and *D. ethenogenes* FL2. Clone III B-9 was identified from the same dechlorinating consortium from which the sequences that shared the highest identity with TCE33 were found. Physiological characterization of these anaerobic dechlorination cultures is not available. The fact that these five closely related *Spirochaeta* sequences were found in four different highly enriched dechlorinating cultures with different inocula suggests that these bacteria may have specific association with dechlorinating consortia and they may perform an important function in the dehalogenating communities.

Two similar sequence types DCE29 and TCE65 (>99% identical) were found in the TCE enrichment (5% of clones) and the *cis*-DCE enrichment (3% of clones). These sequences were members of the phylum Nitrospira. However, they were distantly (<90% identical) related to other sequences in the database. Many *Nitrospira* species are known to be aerobic nitrifying bacteria. Because the sequence type found in our enrichments was distinct from any known *Nitrospira* species, the functions of the microorganisms that clones TCE65 and DCE29 represented are unknown.

In summary, screening and analysis of 16S rDNA libraries from community DNA from a TCE-dechlorinating and a *cis*-DCE-dechlorinating culture revealed similarities and differences. Both enrichments were dominated by an organism that was closely related to *D. ethenogenes* and was presumed to be a dechlorinator. Several other bacterial groups that were present in both reactors include: *Anaeroarcus burkinabensis*, *Syntrophomonas* sp., *Spirochaeta* sp. and a *Nitrospira*. A number of sequences were present in only one culture but not the other, including a sequence that branched deeply within the phylum Spirochaetes that was found only in the *cis*-DCE clone library. Another important and interesting finding of this study is that most of the sequences found in our dechlorinating enrichments (nine out of 12 sequence types identified) shared high identify almost exclusively with sequences found in other dechlorinating consortia or environments, suggesting their specific association with dechlorinating ecosystems. This association may be due to physiochemical conditions that favour dechlorination (such as pH, Cl^- concentration, redox potential, methanogenic or acetogenic conditions, etc.) and/or, they may have syntrophic or biochemical collaborations with dechlorinators. It is also possible that some of these sequences represent bacteria with yet to be discovered dechlorinating properties. The discovery of these sequence clusters specifically from dechlorinating communities justifies future attempts to elucidate the roles these bacteria play in dechlorination processes. Those that are essential to dechlorination can be monitored to help determine whether active dechlorination is occurring in the environment. Detection of these signature members, together with dechlorinators can help predict and explain whether complete or partial dechlorination may or may not occur as well as various degradation patterns observed at sites.

Experimental procedures

Culture enrichments

The dechlorinating enrichments investigated in this study were enriched under anaerobic conditions at room temperature (20–25°C) in 2-litre flasks equipped with gas collection and pressure monitoring devices. The reactor contained 1 L of reduced anaerobic mineral media (RAMM) (Ballapragada *et al*., 1997) and 1 L headspace of anaerobic gas (80% N_2 and 20% CO_2). Increasing amounts of TCE (10–20 μmole l^{-1} day^{-1}) or *cis*-DCE (5–15 μmole l^{-1} day^{-1}) were fed by syringe through septum valves (Mininert, VWR) in batch mode after each dose of TCE or *cis*-DCE was mostly converted into ethene. For the first 8 weeks, lactate (2.2 mM) was fed as the electron donor and carbon source after each batch feed of chlorinated-compounds. Later, lactate was replaced by H_2 (2 mmole) as the electron donor and acetate (1 mM) as the carbon source to simplify the community. After the enrichment was grown under these conditions for 8 months at a solids retention time of 120 days, 0.5 ml of the liquid culture from each reactor was used for DNA extraction (biomass of approximately 300–500 mg l^{-1} as VSS). Batch tests of the ability of the enrichment to maintain VC reduction in absence of highly chlorinated compounds were conducted in 27 ml Balch tubes. Ten millilitres of RAMM media and 0.2 ml FBR culture, or 10 ml liquid TCE or *cis*-DCE culture were transferred into Balch tubes and VC (4.4 μmole), H_2 (0.22 mmole) and acetate (1.7 mM) were added.

Vinyl chloride, *cis*-DCE and TCE were analysed by manual injection of headspace gas into an SRI 8600 series gas chromatographer equipped with a flame ionization detector (FID) and a capillary column (Supelco SPB624, 60 m × 0.5 mm ID), helium as carrier gas (11.2 ml min^{-1}), using the following temperature programme: 40°C for 2 min, followed by 5°C min^{-1} ramping to 65°C, then 25°C min^{-1} to 190°C. Ethene and methane were determined by manual injection of the headspace gas onto a Hewlett Packard 5830 A GC, equipped with a FID and a 6-foot HayeSepQ packed column (Supelco, PA), isothermal at 95°C, with N_2 as the carrier gas (50 ml min^{-1}). Concentrations were calculated using external standards. The Henry's constants used to calculate liquid concentrations were 1.1 for VC, 0.2 for *cis*-DCE and 0.45 for TCE (Gossett, 1987).

DNA extraction

A lysozyme and phenol-chloroform based DNA extraction method was used to extract DNA from the dechlorinating enrichments. 0.1–0.2 mg (as VSS) of culture was resuspended in buffer solution (25 mM Tris, pH 8, 100 mM EDTA, 50 mM glucose, 1 mg ml^{-1} lysozyme) in a 1.5 ml centrifuge tube and incubated for 1 h at 37°C. Sodium dodecyl sulphate (1% wt/vol) was added and the mixture was gently inverted several times. Lysates were extracted with phenol-chloroform two to three times until the top layer was clear. Sodium acetate was added to 0.3 M. Community nucleic acids were precipitated from the solution by adding two volumes of 95% ethanol, incubating at −20°C for 1 h, and then centrifuged at 14000 r.p.m. (Eppendorf, model 5415C) for 20 min.

PCR and cloning

Community 16S rDNAs were PCR amplified in a reaction mixture containing 1 × PCR buffer (Pharmacia), 200 μM dNTPs (deoxynucleotide triphosphate), 500 nM of each forward and reverse primer, and 0.025 U of Taq polymerase (Pharmecia) per μl. The PCR mixture was incubated in a model PTC-100™ programmable thermal controller (MJ Research). The PCR program for 16S rDNA amplification was 96°C for 4 min (for initial denaturation), followed by 32 cycles at 94°C for 1.5 min, 42°C for 1 min, 72°C for 4 min and a final extension at 72°C for 10 min. One bacteria clone library was prepared for each dechlorinating community. For both clone libraries, rDNAs were amplified with forward oligonucleotide primer 8F (specific for Bacteria, 5′-AGA GTT TGA TCC TGG CTC AG-3′) and universal reverse oligonucleotide primer 1492R (5′- GT TAC CTT GTT ACG ACT T-3′). 16S rDNA PCR products (5 μl) were run on a 1% agarose gel to check for the size and purity of the DNA before they were used for cloning. Cloning was performed with a TOPO TA Cloning kit (Invitrogen Corp.) following the manufacturer's instructions. Plasmids were purified form *E. coli* transformants using the Easypreps protocol (Berghammer and Auer, 1993).

Screening clones by RFLP analysis

16S rDNA inserts from recombinant clones were re-amplified from 2 μl of plasmid DNA by PCR using primers 8F and 1492R as previously described. The re-amplified insets were run on a 1% agarose gel to check for the size and purity before they were used for digestion. Aliquots of crude PCR products from each clone were separately digested with two tetrameric (4-base-specific) restriction enzymes, *Msp*I and *Tsp*509 I. For *Msp*I, 5 μl aliquots of PCR products were digested in mixtures containing 1× NEB (New England Biolabs) buffer 2 and 10 U of restriction enzymes in a final volume of 20 ml at 37°C for 1.5 h. For *Tsp*509 I, 5 μl of PCR products were digested in mixtures containing 1× NEB (New England Biolabs) buffer 1 and 10 U of restriction enzymes in a final volume of 20 ml at 65°C for 1.5 h. DNA fragments were separated on 2% agarose gel (Sigma type I-A), which was stained with 0.5 μg ml^{-1} ethidium bromide in a 1× TAE buffer. Two ladders (50 bp ladder and 1 kb ladder) were used as standards to determine the band sizes. The RFLP patterns of each clone library were grouped visually, and one or more representatives from each group were selected for sequencing.

Sequencing representative16S rDNA clones

For representative clones, rDNA inserts from were sequenced using the BigDye kit (ABI) with four primers including: M13 forward, M13 reverse, SP3 (5′-ACT CCT ACG GGA GGC AGC AG-3′) and SP11R (5′-AGG GTT GCG CTC GTT-3′). The products were cleaned with Ultrafree-MC 30 000 NMWL filter unit (Millipore) and then separated on an ABI 377 automated sequencer.

Phylogenetic analysis

Sequence contigs were created using the GeneDoc editor.

Initially, the newly generated sequences were analysed using SEQUENCE MATCH (Ribsome Database project II, Maidak *et al*., 2001) and BLAST (Basic Local Alignment Search Tool) to determine their phylogenetic affiliations. The sequences were screened for possible chimeras using CHECK-CHIMERA (Maidak *et al*., 2001). Two were found. Reference sequences were downloaded from the RDP or NCBI databases, and NCBI sequences were aligned using the RDP's ALIGN SEQUENCE program. The alignment was proofread manually using GENEDOC (Nicholas and Nicholas, 1994) and analysed using distance, parsimony, and maximum likelihood. Distance trees were constructed by using TREECON software (Kimura correction; neighbour joining algorithm) (Van de peer and de Wachter, 1994). Parsimony analyses were done using PAUP 4.0 beta (branch and bound algorithm) (Swofford, 1998). Maximum likelihood analyses were done using FASTDNAML (Olsen *et al*., 1994). Also, bootstrap analyses were done for distance and parsimony.

GenBank accession numbers

The sequences from the rDNA clones in this study were submitted to GenBank and they have GenBank accession no. AF349755 to AF349766, AY166854 and AY166855.

Acknowledgements

We thank Dr Cheryl Jenkins for many helpful instructions on using GENEDOC and TREECON. We are grateful to Dr Jaana M.H. Pietari for providing the seed of FBR culture, which she had been maintaining. We also thank Professor John Ferguson for valuable discussions. This work was supported by Department of Energy, CRESP Award No. E-FG26–00NT40938 and NIEHS Award No. P42-ES-04696.

References

Adrian, L., Manz, W., Szewzyk, U., and Gorisch, H. (1998) Physiological characterization of a bacterial consortium reductively dechlorinating 1, 2, 3- and 1, 2, 4-trichlorobenzene. *Appl Environ Microbiol* **64:** 496–503.

Adrian, L., Szewzyk, U., Wecke, J., and Gorisch, H. (2000) Bacterial dehalorespiration with chlorinated benzenes. *Nature* **408:** 580–583.

Ballapragada, B.S., Stensel, H.D., Puhakka, J.A., and Ferguson, J.F. (1997) Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ Sci Technol* **31:** 1728–1734.

Berghammer, H., and Auer, B. (1993) 'Easypreps': Fast and easy plasmid minipreparation for analysis of recombinant clones in *E. coli*. *Biotechniques* **14:** 527–528.

Breitenstein, A., Saano, A., Salkinoja-Salonen, M., Andreesen, J.R., and Lechner, U. (2001) Analysis of a 2,4,6-trichlorophenol-dehalogenating enrichment culture and isolation of the dehalogenating member *Desulfitobacterium frappieri* strain TCP-A. *Arch Microbiol* **175:** 133–142.

Colwell, R.K., and Coddington, J.A. (1994) Estimating terrestrial biodiversity through extrapolation. *Phil Trans R Soc London B* **345:** 101–118.

Cupples, A.M., Spormann, A.M., and McCarty, P.L. (2003) Growth of a *dehalococcoides*-like microorganism on vinyl chloride and cis-dichloroethene as electron acceptors as determined by competitive PCR. *Appl Environ Microbiol* **69:** 953–959.

Debruin, W.P., Kotterman, M.J., Posthumus, M.A., Schraa, D., and Zehnder, A.J. (1992) Complete biological reductive transformation of tetrachloroethene to ethene. *Appl Environ Microbiol* **58:** 1996–2000.

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

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R. (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64:** 3869–3877.

Duhamel, M., Wehr, S.D., Yu, L., Rizvi, H., Seepersad, D., Dworatzek, S., *et al.* (2002) Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, cis-dichloroethene and vinyl chloride. *Water Res* **36:** 4193–4202.

Fennell, D.E., Carroll, A.B., Gossett, J.M., and Zinder, S.H. (2001) Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environ Sci Technol* **35:** 1830–1839.

Flynn, S.J., Löffler, F.E., and Tiedje, J.M. (2000) Microbial community changes associated with a shift from reductive dechlorination of PCE to reductive dechlorination of *cis*-DCE and VC. *Environ Sci Technol* **34:** 1056–1061.

Gossett, J.M. (1987) Measurement of Henry's law constants for C1 and C2 chlorinated hydrocarbons. *Environ Sci Technol* **21:** 202–208.

Hansen, K.H., Ahring, B.K., and Raskin, L. (1999) Quantification of syntrophic fatty acid-β-oxidizing bacteria in a mesophilic biogas reactor by oligonucleotide probe hybridization. *Appl Environ Microbiol* **65:** 4767–4774.

He, J., Ritalahti, K.M., Aiello, M.R., and Löffler, F.E. (2003) Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as *Dehalococcoides* Species. *Appl Environ Microbiol* **69:** 996–1003.

Hendrickson, E.R., Payne, J.A., Starr, M.G., Perry, M.P., Fahnestock, S., Ellis, D.E., and Ebersole, C. (2002) Molecular analysis of *Dehalococcoides* 16S ribosomal DNA From chloroethene-contaminated sites throughout north America and Europe. *Appl Environ Microbiol* **68:** 485–495.

Hohnstock-Ashe, A.M., Plummer, S.M., Yager, R.M., Baveye, P., and Madsen, E.L. (2001) Further biogeochemical characterization of a trichloroethene-contaminated fractured dolomite aquifer: electron source and microbial communities involved in reductive dechlorination. *Environ Sci Technol* **35:** 4449–4456.

Holliger, C., Schraa, G., Stams, A.J.M., and Zehnder, A.J.B. (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol* **59:** 2991–2997.

Holliger, C., Wohlfarth, G., and Diekert, G. (1999) Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol Rev* **22:** 383–398.

Huang, J., He, Z., and Wiegel, J. (1999) Cloning, characterization, and expression of a novel gene encoding a reversible 4-hydroxybenzoate decarboxylase from *Clostridium hydroxybenzoicum*. *J Bacteriol* **181:** 5119–5122.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., and Bohannan, B.J.M. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* **67:** 4399–4406.

Kuchta, R.D., and Abeles, R.H. (1985) Lactate reduction in *Clostridium propionicum*, purification and properties of lactyl-CoA dehydratase. *J Biol Chem* **260:** 13181–13189.

Löffler, F.E., Sun, Q., Li, J., and Tiedje, J.M. (2000) 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl Environ Microbiol* **66:** 1369–1374.

Maidak, B., Cole, J., Lilburn, T., Parker, C., Saxman, P., Farris, R., *et al.* (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29:** 171–173.

Maymo-Gatell, X., Chien, Y.T., Gossett, J.M., and Zinder, S.H. (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* **276:** 383–398.

Maymo-Gatell, X., Nijenhuis, I., and Zinder, S.H. (2001) Reductive dechlorination of cis-1,2-dichloroethene and vinyl chloride by ‘‘*Dehalococcoides ethenogenes*. *Environ Sci Technol* **35:** 516–521.

Middeldorp, P.J.M., Luijten, M.L.G.C., van de Pas, B., Van Eekert, M.H.A., Kengen, S.W.M., *et al.* (1999) Anaerobic microbial reductive dehalogenation of chlorinated ethenes. *Bioremed J* **3:** 151–169.

Nicholas, K., and Nicholas, H. (1994) GENEDOC: a full featured multiple sequence alignment editor, analyzer and shading utility for Windows. [www document]. URL http://www.psc.edu/biomed/genedoc/

Olsen, G.J., Matsuda, H., Hagstrom, R., and Overbeek, R. (1994) FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10:** 41–43.

Pietari, J.M.H. (1999) Development and characterization of a psychotropic dechlorinating culture and temperature response of a mesophilic dechlorinating culture. MSc Thesis. University of Washington, WA, USA. pp 46–58.

Pulliam-Holoman, T.R., Elberson, M.A., Cutter, L.A., May, H.D., and Sowers, K.R. (1998) Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Appl Environ Microbiol* **64:** 3359–3367.

Richardson, R.E., Bhupathiraju, V.K., Song, D.L., Goulet, T.A., and Alvarez-Cohen, L. (2002) Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. *Environ Sci Technol* **36:** 2652–2662.

Ritalahti, K.M., krajmalnik-Brown, R., Tiedje, J.M., and Löffler, F.E. (2001) Dehalococcoides species are the dominant vinyl chloride-dechlorinating bacteria in the anaerobic environments. In: *Sixth International Symposium on In-situ and On-site Bioremediation*, Platform Abstracts C1, June. San Diego, CA, USA.

Sung, Y., He, J., Krajmalnik-Brown, R., Padilla, E., Ritalahti, K.M., and Löffler, F.E. (2002) Distribution and Ecology of Chloroethene-Dechlorinating Populations in Subsurface Environments. In: *American Society for Microbiology 102nd General meeting*, Abstracts Q15, May. Salt Lake City, UT, USA.

Swofford, D.L. (1998) *paup*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sunderland, MA, USA: Sinauer Associates.

Van de peer, Y., and de Wachter, R. (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Appl Biosci CABIOS.* **10:** 569–570. [www document]. URL http://www.evolutionsbiologie.uni-konstanz.de/peer-lab/treeconw.html

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

van der Wielen, P.W.J.J., Rovers, G.M.L.L., Scheepens, J.M.A., and Biesterveld, S. (2002) *Clostridium lactatifermentans* sp. Nov., a lactate-fermenting anaerobe isolated from the caeca of a chicken. *Int J Syst Evol Microbiol* **52:** 921–925.

von Wintzingerode, F., Gobel, U.B., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21:** 213–229.

von Wintzingerode, F., Selent, B., Hegemann, W., and Gobel, U.B. (1999) Phylogenetic analysis of an anaerobic, trichlorobenzene-transforming microbial consortium. *Appl Environ Microbiol* **65:** 283–286.

Zhou, J., Palumbo, A.V., and Strong, J.M. (1999) Phylogenetic characterization of a mixed microbial community capable of degrading carbon tetrachloride. *Appl Biochem Biotech* **80:** 243–253.