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Identification of microorganisms involved in reductive dehalogenation of chlorinated ethenes in an anaerobic microbial community

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Abstract

In this study, we report on phylogenetic and physiological characterization of an anaerobic culture capable of reductive dehalogenation of tetrachloroethene (PCE) obtained from a PCE-contaminated site. The culture was enriched using different combinations of electron donors (hydrogen and acetate) and electron acceptors (PCE, *cis*-1,2-dichloroethene (cDCE) and controls without chlorinated ethenes). The resulting subcultures were analyzed using three different approaches: chemical analysis to document conversion of chlorinated ethenes; polymerase chain reaction (PCR) of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE) to compare community compositions; fluorescence in situ hybridization (FISH) to quantify specific groups of microorganisms using oligonucleotide probes previously designed or newly designed based on the sequences retrieved from sequence analysis of specific DGGE bands. Members of two genera which contain bacteria capable of reductive dehalogenation were detected in the culture: *Dehalococcoides* and *Desulfitobacterium*. The combined analyses suggested that *Dehalococcoides*-like bacteria are associated with complete dehalogenation of chlorinated ethenes to ethene with hydrogen as electron donor; and *Desulfitobacterium*-like bacteria, in contrast, are associated with incomplete PCE dehalogenation to cDCE and appear to be able to use acetate as electron donor. In addition, *Sporomusa*-like bacteria were identified, which most likely act as homoacetogens. The results demonstrated that combination of culture enrichment with different substrates, DGGE, and FISH allowed a detailed qualitative and quantitative characterization of the dominant microorganisms associated with reductive dehalogenation.

Keywords: Reductive dehalogenation; Bioremediation; *Dehalococcoides*; *Desulfitobacterium*; DGGE; FISH

1. Introduction

Anthropogenic chlorinated organic compounds, such as tetrachloroethene (PCE) and trichloroethene (TCE), are common groundwater contaminants due to their widespread use as solvents. Under anaerobic conditions, these compounds can be reductively dehalogenated to less chlorinated ethenes or innocuous ethene by microorganisms through dehalorespiration (McCarty, 1997).

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Because this process has the potential of completely detoxifying chlorinated ethenes, it is recognized as a promising approach for the cleanup of contaminated sites and has been extensively studied for in situ bioremediation. For the purpose of achieving effective PCE/TCE bioremediation, it is important to understand the factors controlling the rate and extent of reductive dehalogenation.

Among the factors affecting reductive dehalogenation are the metabolic capabilities of and interactions among the microorganisms involved. To date a number of bacteria have been cultivated that are capable of reductive dehalogenation of chlorinated ethenes (Gerritse et al., 1996; Krumholz, 1997; Maymo-Gatell et al., 1997; Miller et al., 1997; Holliger et al., 1999; He et al., 2003; Sung et al., 2003). Of these, most can only dehalogenate PCE to *cis*-1,2-dichloroethene (cDCE), such as *Dehalobacter restrictus*, *Sulfurospirillum multivorans* (previously *Dehalospirillum multivorans* (Luijten et al., 2003)), *Desulfuromonas chloroethenica*, *Desulfuromonas michiganensis*, and some *Desulfitobacterium* species; only *Dehalococcoides* species were identified as organisms capable of complete dehalogenation of PCE (*Dehalococcoides ethenogenes*) or cDCE/vinyl chloride (VC) (*Dehalococcoides* sp. Strain BAV1) to ethene. Most of these organisms can use hydrogen but not acetate as the direct electron donor (hydrogenotrophic dehalogenators), such as *Dehalobacter restrictus*, *S. multivorans*, *D. ethenogenes*, and some *Desulfitobacterium* species. In contrast, only a few can use acetate as electron donor (acetotrophic dehalogenators), including *D. chloroethenica* (Krumholz et al., 1996; Krumholz, 1997) and *D. michiganensis* (Loeffler et al., 1998; Sung et al., 2003). No acetotrophic dehalogenators cultivated so far are capable of complete dehalogenation of PCE to ethene, and hydrogen is generally assumed to be the major electron donor to sustain complete dehalogenation (Distefano et al., 1992; Smatlak et al., 1996; Ballapragada et al., 1997; Fennell et al., 1997; Yang and McCarty, 1998). Given the significant difference of these organisms in their capabilities to reduce chlorinated ethenes, it is necessary to identify those involved in reductive dehalogenation occurring at contaminated sites and to understand how they interact with each other and with other organisms (because almost always complex microbial communities are expected under field conditions) so as to optimize in situ reductive dehalogenation.

Culture-independent nucleic acid-based techniques have been providing interesting insights into the microorganisms in complex microbial communities in recent years (Muyzer, 1999; Amann and Ludwig, 2000; Dahllof, 2002). Nevertheless, there have been only few studies attempting to identify the organisms in complex microbial communities associated with reductive dehalogenation of chlorinated ethenes using these methods (Flynn et al., 2000; Loeffler et al., 2000; Fennell et al., 2001; Duhamel et al., 2002; Hendrickson et al., 2002; Lowe et al., 2002; Richardson et al., 2002; Aulenta et al., 2004). In these studies, either the organisms of interest, in most cases *Dehalococcoides* species, were detected in microcosms or field samples by amplifying specific 16S rRNA genes (rDNAs) with polymerase chain reaction (PCR); or universal primers were used to amplify all bacterial 16S rDNA sequences present, followed by cloning, sequencing or fingerprinting. The first approach only allows for specific detection of microorganisms with known sequence information. In contrast, the second approach may provide a more comprehensive understanding of the microbial community structure including identification of unknown organisms from environmental samples. However, due to the technical limitation of these qualitative PCR-based methods (Chandler et al., 1997; von Wintzingerode et al., 1997; Qiu et al., 2001), it is difficult to obtain quantitative information of the microorganisms in the samples, and usually only the dominant organisms in the community were retrieved, which were not necessarily the organisms actively involved in reductive dehalogenation. In addition, most of the previous studies did not provide direct correlations between identity and metabolic function of the organisms detected. Their physiological traits were usually inferred from sequence similarity to known organisms in public databases.

In this study, we report on phylogenetic and physiological identification of dehalogenating organisms in a microbial community by combining culture enrichment procedures and molecular analysis techniques. The enrichment procedure was designed according to assumed metabolic requirements of dehalogenators using different combinations of electron donors (including hydrogen and acetate) and electron acceptors (including PCE, cDCE, and controls without chlorinated ethenes). Hydrogen and acetate were selected as electron donors because they were major products from anaerobic degradation of benzoate, which was the substrate used to maintain the studied culture. This selection is of universal significance as hydrogen and acetate are major intermediates in anaerobic decomposition of organic matter (Schink, 1997). The purpose of this step was to enrich the organisms possessing specific physiological traits. Subsequently, a biphasic full cycle rRNA analysis (Amann et al., 1995) was performed to identify and quantify the microorganisms involved in reductive dehalogenation by combining 16S rDNA-based PCR and denaturing gradient gel electrophoresis (DGGE) with fluorescence in situ hybridization (FISH). DGGE analysis followed by sequencing of specific gene fragments was applied to obtain information on the community composition under different culturing conditions and to identify enriched organisms. FISH was used for in situ quantification of selected

organisms with specific oligonucleotide probes previously or newly designed based on the sequences retrieved from sequencing analysis.

2. Materials and methods

2.1. Chemicals

Liquid PCE (>99.5%, GC grade, Fluka, Buchs, Switzerland), TCE (>99.5%, GC grade, Fluka), and cDCE (97%, GC grade, Fluka) were used for preparing stock feed solutions and analytical standards. VC (>99%, Scott Specialty Gases, Alltech Associates, Deerfield, IL), ethene (>99%, PanGas, Dagmersellen, Switzerland), and methane (>99%, PanGas) were used as analytical standards. Hydrogen (10% in nitrogen, PanGas), acetate (sodium salt, 99%, Fluka), and benzoate (sodium salt, 99%, Fluka) were used as electron donors and to develop analytical standards.

2.2. Culture and growth medium

The dehalogenating culture used in the study was maintained in an anaerobic chemostat (total volume 2.3 L, liquid volume 1.9 L, continuously stirred) inoculated with a culture from a chemostat described previously (Yang and McCarty, 1998) which was initially inoculated with aquifer material from a PCE-contaminated groundwater site in Victoria, TX. The culture in the daughter chemostat behaved similarly in physiology to the previous reactor following five months of incubation. It was maintained at 28 (±2) °C. A basal medium (Yang and McCarty, 1998) supplemented with 1.7 mM sodium benzoate, 20 mg/L yeast extract, and 0.98 mM PCE was added to the chemostat using a syringe pump at a rate of 50 mL/day, resulting in a 38-day retention time. Every day 50 mL of liquid was removed to bring the liquid volume back to 1.9 L. Under the described conditions, complete conversion of PCE to ethene was observed. Due to the presence of active methane-producing microorganisms, which can use hydrogen and acetate as electron donors, no accumulation of these intermediates of benzoate degradation was observed in the chemostat.

A pure culture of *D. ethenogenes* strain 195 was used as a control in PCR-DGGE analysis. The culture was grown on hydrogen and PCE as previously described (Maymo-Gatell et al., 1997).

2.3. Culture enrichment scheme

For culture enrichment, hydrogen and acetate were used as electron donors, PCE and cDCE as electron acceptors. With different combinations of electron donors and electron acceptors, six different types of subcultures were established including two controls with electron donors, but without chlorinated ethenes. Each combination was prepared in duplicates. All enrichment experiments were conducted at room temperature (22 °C) in 117-mL serum bottles. At the start of the experiment, 60 mL of the chemostat culture was added to each bottle anaerobically by gas purging with an 80% N_2/20% CO_2 mixture during the transfer. PCE, cDCE, hydrogen, and acetate were then added in amounts of 45, 15, 270, and 270 μmol, respectively, if required. Hydrogen and acetate were added in excess to ensure the completeness of dehalogenating reactions. The reason that different amounts of PCE and cDCE were used was because approximately the same amount of time was required to completely dehalogenate the PCE and cDCE added according to our previous experience. After about four weeks of incubation when 80% of the chlorinated ethenes was degraded (in terms of total chlorine removal) in the subcultures, these cultures were transferred (50% v/v) into the fresh basal medium. In total, six sequential transfers were made in a similar manner for each subculture. Chlorinated ethenes, ethene, methane, and acetate were measured every 5–7 days to monitor the physiological changes of the cultures. Samples were taken at the end of the 2nd, 4th, and 6th transfer for DGGE analysis and fluorescence in situ hybridization.

Bottles were sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) and aluminum crimp caps to maintain anaerobic conditions. Sorption of chlorinated compounds by the rubber stoppers was less than 5% after the first few days so that good mass balances could be maintained throughout the studies. All bottles were continuously mixed at 140 rpm on a shaker table (Adolf Kuhner, Basel, Switzerland).

2.4. Analytical methods

PCE, TCE, cDCE, VC, ethene, and methane were quantified by a Fisons HRGC Mega 2 Series gas chromatography (GC) (Fisons, Milan, Italy), using 250 μL headspace samples. The GC was equipped with a flame ionization detector and a GS-Q fused-silica capillary column (length, 30 m; inside diameter, 0.53 mm; Agilent Technologies, Basel, Switzerland). Analyses were performed by using a temperature program (40–180 °C, 20 °C/min). Compounds were identified by comparison of their retention times with that of external standards. Total amount of gaseous compounds in liquid were calculated by using published Henry's law constants (Gossett, 1987). Organic acid concentrations were determined with a DX-320 ion chromatography (IC) system, using 100 μL liquid samples. The IC was equipped with an electrical conductivity detector and an EG40 eluent gradient generator (Dionex, Sunnyvale, CA). The following

KOH eluent gradient was used: 0–7 min, 1 mM KOH; 7–25 min, 1–25 mM KOH; 25–28 min, 25–60 mM KOH; 28–28.1 min, 60–1 mM KOH; and 28.1–32 min, 1 mM KOH. Identification and quantification were made by comparison with external standards.

2.5. DNA extraction

From each subculture 10 mL of sample was transferred with sterile syringe into 15 mL polypropylene tubes and immediately centrifuged at 4500*g* for 10 min. After discarding the supernatant, the cell pellet was re-suspended in 1 mL DNA extraction buffer (50 mM NaCl, 50 mM EDTA, 50 mM Tris, 5% sodium dodecyl sulfate; pH 8) and DNA was extracted and purified according to the method of Sigler and Zeyer (Sigler and Zeyer, 2002). DNA samples were stored at −20 °C until further analysis.

2.6. 16S rDNA amplification and DGGE analysis

For Bacteria-specific PCR of 16S rDNA fragments, the primers BAC 341f-GC (5′-GC-clamp-CCT ACG GGA GGC AGC AG-3′) and BAC 534r (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al., 1993) were used. The PCR cocktail contained 0.2 μM of each primer, 0.2 mM of each deoxynucleotide (Invitrogen, Basel, Switzerland), 2 mM $MgCl_2$, 1.8 mg mL^{-1} bovine serum albumin (Sigma, Buchs, Switzerland), 1 U of *Taq* polymerase (Invitrogen), 1 μL of DNA extract, and 1 × PCR reaction buffer in a final volume of 50 μL. PCR amplification was carried out with a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). After initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 45 s and extension at 72 °C for 45 s was performed followed by a final extension step at 72 °C for 5 min.

DGGE analysis of 16S rDNA fragments was performed using DCode™ universal mutation detection system (BioRad Laboratories, Hercules, CA). Gels (16 cm × 16 cm) consisted of 10% bis-: acrylamide (37.5:1) and a denaturant gradient of 40–60%. Electrophoresis was performed in 1 × TAE buffer (0.04 M Tris base, 0.02 M sodium acetate, and 1 mM EDTA; pH 7.4) at 60 °C and 75 V for 15 h. Gels were stained for 30 min with a 1:10,000 dilution of GelStar® nucleic acid stain (Bio Whittaker, Denmark) and analyzed using the Gel Doc 2000 system (BioRad) and QuantityOne software (BioRad).

2.7. Cloning and sequence analysis

Portions of selected bands were excised from DGGE gels for sequence determination by coring the band of interest with a sterile pipette tip. Bands derived from different subcultures that appeared to share the same migration position were all excised in order to account for possible differences in the phylotypes that might have occurred during enrichment. Gel pieces were placed in 0.2 mL reaction tubes containing 100 μL of sterile water. After incubation for 3 h at 42 °C, 2 μL of the solution containing the eluted DNA was used for PCR and DGGE analysis described above. This procedure was repeated until signals of the desired bands were obtained without other co-migrating bands. Migration positions of plasmid inserts were checked and were in agreement with the excised bands in the community DGGE fingerprints. The "pure" DNA fragments were then excised and re-amplified using the primers described above, but without the GC-clamp. PCR products were cloned as described previously (Pesaro et al., 2003). Inserts of retrieved plasmids were amplified with BAC 341f-GC/BAC 534r primers and migration positions on DGGE gels were reconfirmed by directly comparing with the positions of the originally excised bands. Sequences of inserts were commercially determined on one strand (Microsynth, Balgach, Switzerland). A comparison of sequences obtained in this study with those of previous studies was performed through a BLAST (Altschul et al., 1997) search of the GenBank database.

2.8. Oligonucleotide probes and probe design for *Desulfitobacterium* species

A previously-described FISH probe, Dhe1259t was used here for in situ quantification of *Dehalococcoides* species (Yang and Zeyer, 2003). Probe Dsf440 and its helper probe Dsf475 (1:1, mol/mol) used for in situ quantification of *Desulfitobacterium* species were designed using the PROBE-DESIGN program of ARB software (Amann and Ludwig, 2000) based on five sequences retrieved from DGGE and sequence analysis. Their specificities were then checked with the PROBE-MATCH program of the Ribosomal Database Project (Maidak et al., 1997). The optimal hybridization stringency of Dsf440 and Dsf475 was determined with the acetate/PCE subculture by increasing the formamide concentration in the hybridization buffer in increments of 5% at a constant hybridization temperature of 42 °C. Probe-conferred signal intensity remained at the same level following the addition of formamide up to 5% and then decreased rapidly. Therefore, 5% formamide was used for all subsequent hybridization experiments with these probes. Samples were also analyzed with probe Non338 (Amann et al., 1995) to estimate the number of autofluorescent cells. Sequences and target sites of the probes used in this study are given in Table 1. Oligonucleotides synthesized with 5′-Cy3 labels were purchased from MWG-Biotech (Ebersberg, Germany).

Table 1
Probes used for FISH in this study

Probe	Target organisms	Sequence (5′–3′)	Position on 16S rRNA[a]	Formamide conc. (%)	References
Non338	Negative control	ACTCCTACGGGAGGCAGC	338–355	30	(Amann et al., 1995)
Dhe1259t	*Dehalococcoides* sp.[b]	AGCTCCAGTTCACACTGTTG	1259–1278	30	(Yang and Zeyer, 2003)
Dsf440	*Desulfitobacterium* sp.[c]	TACCGTTCGTCCCTGAAG	440–457	5	This study
Dsf475	Helper probe of Dsf440	CTCAGGTACCGTCATGTAAG	475–494	5	This study

[a] According to *E. coli* numbering.
[b] Including *D. ethenogenes*.
[c] According to Table 3.

2.9. FISH analysis

Approximately 10 mL of each subculture sample was immediately fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.2) at 0 °C for 12 h, washed twice in PBS and subsequently stored in 50% ethanol in PBS at −20 °C until further analysis (Zarda et al., 1998). Whole-cell hybridization was performed based on the protocol of Zarda et al. (Zarda et al., 1998). Fixed samples were applied to eight-well, teflon-coated slides (Erie Scientific Co., Portsmouth, NH), air-dried, and dehydrated in an ethanol series (50%, 80% and 96%) for 3 min each. A mixture of 1 μL probe (25–30 ng/μL), 9 μL hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS (pH 7.2)), and a specific amount of formamide depending on the probe used (Table 1) was applied to each well, followed by incubation for 2 h in a humid chamber at 42 °C. The slides were washed in pre-warmed washing buffer for 15 min at 48 °C, then rinsed with deionized water, and air-dried. The washing buffer contained 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% SDS, and 636 or 112 mM NaCl (when 5% or 30% formamide was used during hybridization, respectively). When probing for *Desulfitobacterium* species, which belong to the gram-positive bacteria, a pretreatment procedure with lysozyme (400 units/μL for 5 min at 0 °C) was included before hybridization to increase the permeability of these cells (Meier et al., 1999).

After hybridization, 20 μL of DNA intercalating fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, Sigma, Buchs, Switzerland) (1 ng/μL) was added to each well. After incubation for 3 min at room temperature in the dark, the slides were rinsed with deionized water and air-dried. DAPI staining was always included to detect total microorganisms present in the preparation. Microscopy was performed as described previously (Zarda et al., 1998). Bacteria were counted at 1000 × magnification. Forty fields selected at random covering an area of 0.01 mm^2 each were examined from a sample distributed over eight circular areas of 53 mm^2 each. Counting results were corrected by subtracting the numbers of autofluorescent cells. Complemented with cell concentration by centrifugation, reliable FISH detection of 10^4 cells per mL could be obtained in this study.

3. Results

3.1. Reductive dehalogenation with different electron acceptors and electron donors

PCE or cDCE was dehalogenated in similar fashion from one transfer to the other for each subculture

throughout the duration of the experiment. Depicted in Fig. 1 are time course plots of the total mass of chlorinated ethenes and ethene during the sixth transfer in different subcultures. Hydrogen was found to support complete dehalogenation of both PCE (Fig. 1a) and cDCE (Fig. 1b) to ethene. With acetate as electron donor, reductive dehalogenation occurred only when PCE was used as electron acceptor. cDCE was found to be the major degradation product of PCE dehalogenation (Fig. 1c). In acetate/cDCE subcultures, trace amounts of VC were detected (Fig. 1d), but this activity could not be sustained even after longer periods of incubation (data not shown). These results suggested that acetate was unable to support cDCE dehalogenation, which is consistent with previous observations with a culture from the same source (Yang and McCarty, 1998, 1999).

Rapid methane production was sustained during all transfers in all controls, but in the subcultures amended with either PCE or cDCE methane was only produced during the first two transfers and became undetectable thereafter (data not shown).

3.2. DGGE and sequence analysis

Samples from each subculture were subject to DGGE analysis to assess changes in the bacterial community throughout the experiment. Because the main purpose of this study was to identify the dominant microorganisms involved in reductive dehalogenation but not to perform full community analysis, only the DNA fragments which manifested considerable changes (especially enriched) after incubation were sequenced for further analysis. DGGE analyses of subculture samples after all transfers revealed similar banding patterns, but the most marked changes were observed after the last transfer.

With hydrogen as electron donor (Fig. 2a), DGGE fingerprints of the subcultures receiving PCE or cDCE clearly differed from the original chemostat culture and controls without chlorinated ethenes (bands 1–3; Fig. 2a). In comparison with the original chemostat culture, band 1, which was very faint in the controls, was enriched in H_2/PCE and H_2/cDCE subcultures. DNA from band 1 was found to share 100% sequence similarity to the 16S rDNA of uncultured *Dehalococcoides* species (Table 2), slightly different (1 bp difference in 170 bp) from that of *D. ethenogenes* strain 195. Band 2 did not appear to be present in the original chemostat culture profile, but was enriched to different extents in all hydrogen-receiving subcultures. The sequence of band 2 was most similar to the 16S rDNA of *Sporomusa sphaeroides* (99% sequence similarity). In DGGE fingerprints of H_2/PCE subcultures, a unique band, band 3, though fainter, was consistently detected. Although this band appeared to have the same migration position as the one derived from *D. ethenogenes* strain 195, sequence analysis of its DNA revealed

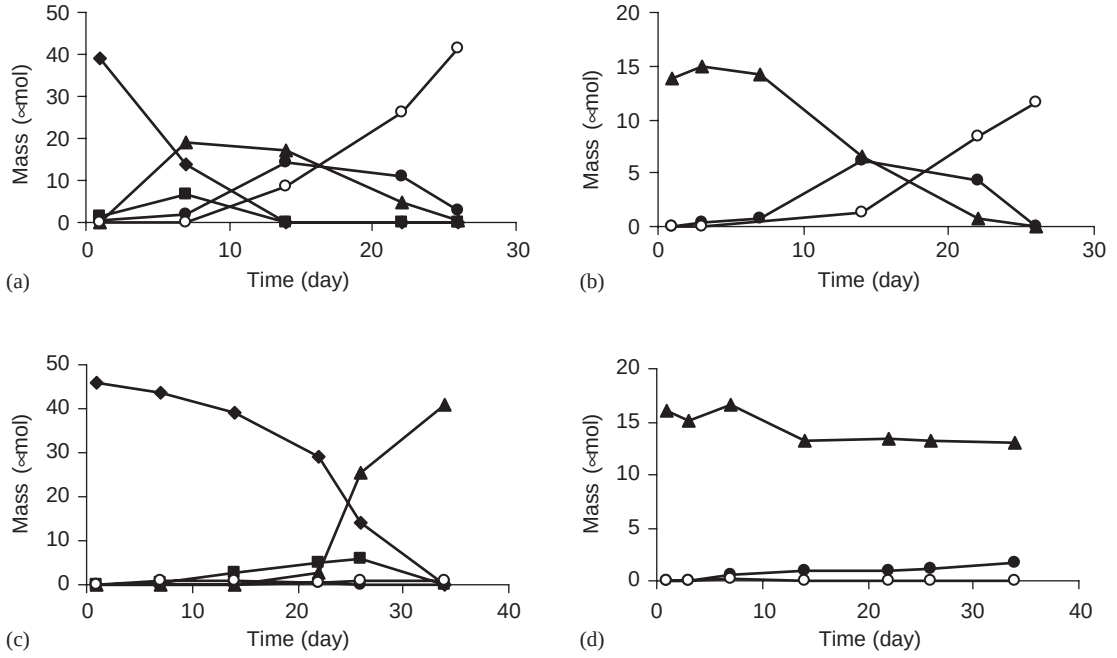

Fig. 1. Dehalogenation of PCE (a, c) and cDCE (b, d) with different electron donors including H_2 (a, b) and acetate (c, d) after 6 transfers. PCE (♦) and cDCE (▲) were transformed to different end products. Intermediates include TCE (■), VC (●) and ethene (○). Data are the average of duplicates (duplicates differed by < 10%).

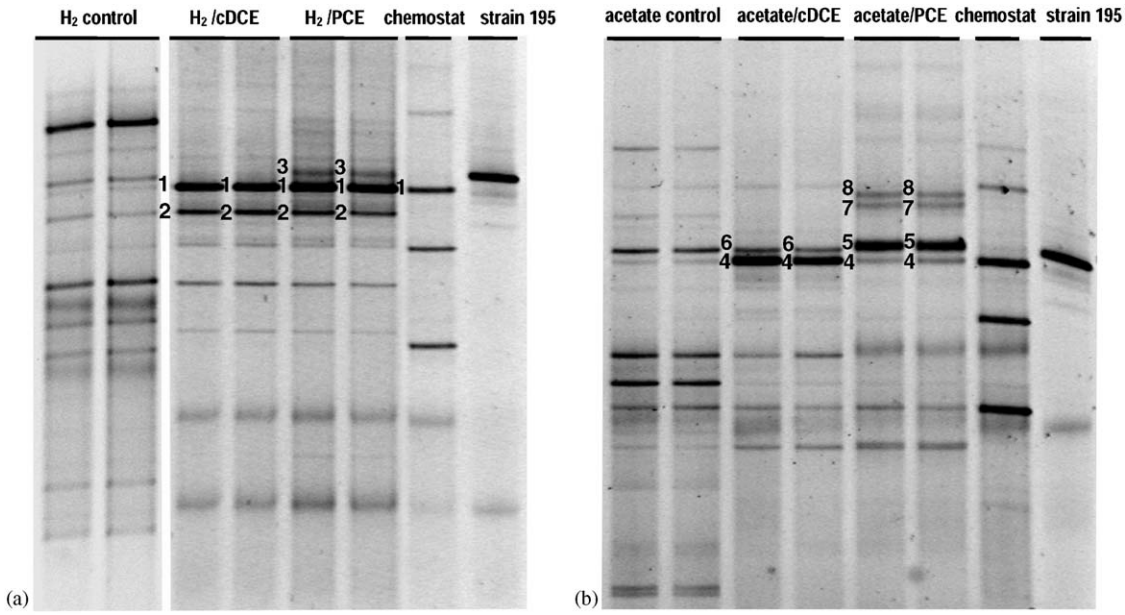

Fig. 2. DGGE patterns of 16S rDNA fragments of cultures grown with different electron acceptors and donors. The first six lanes of panel (a) and (b) are from subcultures enriched with hydrogen and acetate, respectively, which are shown in duplicates from independent microcosm. H_2 control and acetate control designate subcultures without chlorinated ethenes. Chemostat and strain 195 designate the original chemostat culture and *D. ethenogenes* strain 195, respectively, whose DGGE patterns were used for comparison in both cases. Bands subject to sequence analysis are labeled with numbers (refer to Table 2 for phylogenetic affiliations).

Table 2
Phylogenetic affiliations of 16S rDNA fragments retrieved in this study (band numbering refers to the DGGE gels in Fig. 2)

Band designation	Enrichment conditions (electron donor/acceptor)	Fragment length (bp)	Phylotype with highest sequence similarity; sequence accession number	Sequence similarity (%)
Band 1 (same as band 4)	H_2/PCE, H_2/cDCE acetate/PCE acetate/cDCE, chemostat culture	170	Uncultured *Dehalococcoides*; AF388533, AF388536, AF388542, AF388550	100
Band 2	H_2/PCE; H_2/cDCE	195	*S. sphaeroide*; AJ279801	99
Band 3	H_2/PCE	195	*D. hafniense*; X94975	100
Band 5	Acetate/PCE	195	*D. hafniense*; X94975	99
Band 6	Acetate/cDCE	195	*D hafniense*; X94975	96
Band 7	Acetate/PCE	195	*D. frappieri*; AJ276701	100
Band 8	Acetate/PCE	195	*D. hafniense*; X94975	99

that it was 100% similar to the 16S rDNA of *D. hafniense*.

Distinct community changes were also observed with the subcultures receiving acetate as electron donor (Fig. 2b) compared to the original chemostat culture. Band 4 was present in fingerprints of both acetate/PCE and acetate/cDCE subcultures and its DNA sequence was 100% similar to an uncultured *Dehalococcoides species* and was affiliated with the same phylotype as band 1 from communities grown with hydrogen as electron donor (Table 2). Band 5 was enriched in fingerprints of acetate/PCE subcultures compared with the original chemostat culture. Although this band appeared to share the same migration position with *D. ethenogenes* strain 195, it showed 99% similarity to *D. hafniense*. Besides band 5, the appearance of two fainter bands, 7 and 8, were specifically associated with DGGE patterns of acetate/PCE subcultures. Band 7 showed 100% sequence similarity to *D. frappieri*, whereas band 8 revealed 99% match to the sequence of *D. hafniense*.

Band 5, 7, and 8, differred by 1–2 bases in their sequences, all had high similarity to known *Desulfitobacterium* species. It is hard to judge at this point whether these different sequences represented different species or only different copies of the rRNA operon of the same species. Band 6 was observed in DGGE patterns derived from acetate/cDCE subcultures and was attributed to *D. hafniense* with 96% sequence similarity.

3.3. FISH detection of Dehalococcoides- and Desulfitobacterium-like bacteria

Because the identification of *Dehalococcoides*- and *Desulfitobacterium*-like bacteria by DGGE analysis was based on the sequences of DNA fragments of less than 200 base pairs in length, FISH was used as a complement for verification and, in addition, for quantification of the numbers of metabolically active cells of these organisms in the subcultures and the original chemostat culture. One of the two probes designed previously for *Dehalococcoides* species, Dhe1259t (Yang and Zeyer, 2003), was used in the current study. As shown in Fig. 3a, these bacteria were found mostly as small coccoids, consistent with the morphology description for *D. ethenogenes* (Maymo-Gatell et al., 1997). Only 4% of DAPI-stained bacteria were detected as *Dehalococcoides*-like bacteria in the original chemostat culture (Fig. 4). In comparison, clear changes occurred during enrichment. In two substrate controls without chlorinated ethenes, the number of *Dehalococcoides*-like bacteria decreased to undetectable

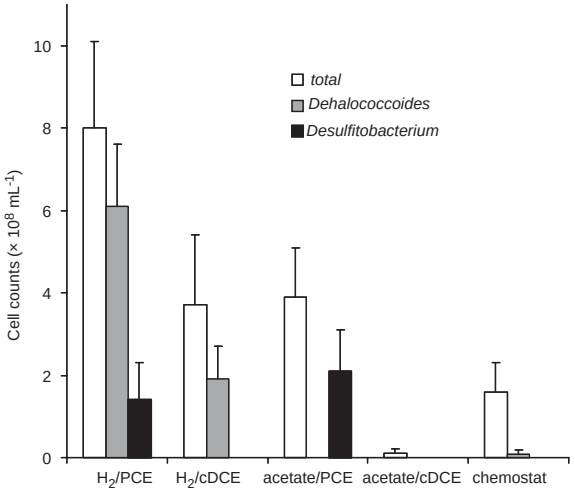

Fig. 4. FISH quantification of *Dehalococcoides*- and *Desulfitobacterium*-like bacteria in the culture enriched with different electron acceptors and donors. Error bars indicate one standard deviation ($n = 40$).

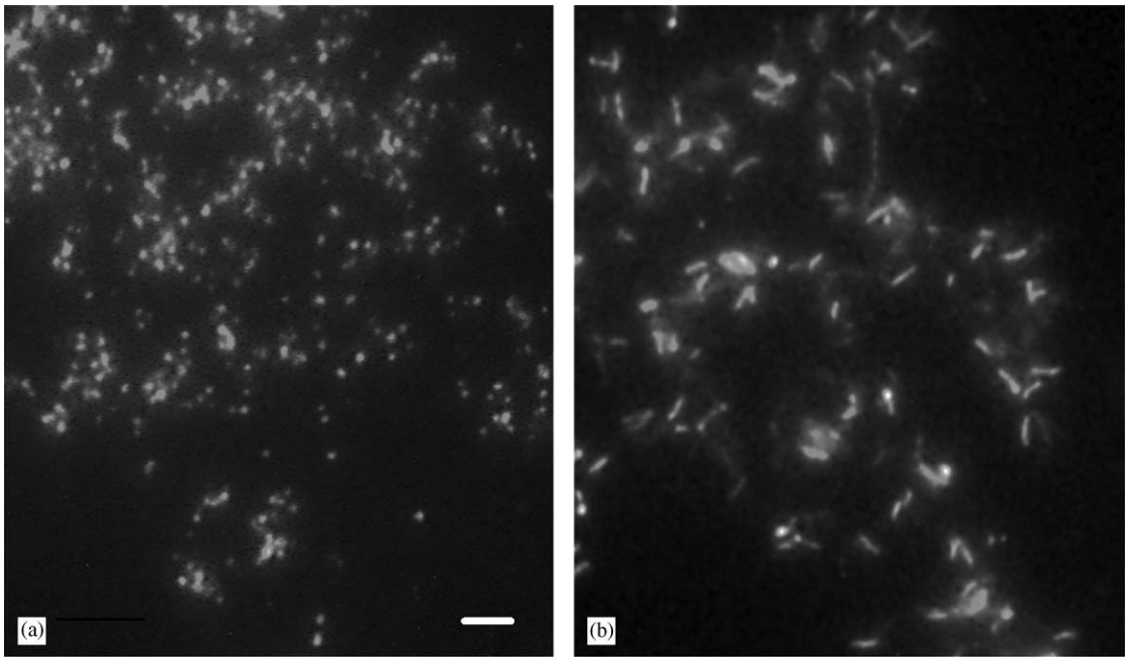

Fig. 3. Specific detection of *Dehalococcoides*- and *Desulfitobacterium*-like bacteria by FISH. Epifluorescence micrographs of in situ hybridization with probe Dhe1259t for H_2/PCE subcultures (a) and probe Dsf440 for acetate/PCE subcultures (b) are presented. Scale bar, 5 μm.

level (not shown). However, in the presence of PCE or cDCE, when hydrogen was used as electron donor, a significant increase in *Dehalococcoides*-like bacteria was observed, which reached 52% and 76% of the total bacteria in H_2/cDCE and H_2/PCE subcultures, respectively, at the end of the sixth transfer. In contrast, *Dehalococcoides*-like bacteria dropped to undetectable level when acetate was used as electron donor.

Probe Dsf440 was designed according to five *Desulfitobacterium*-like sequences retrieved from sequence analysis of excised bands. Due to the weak signal obtained with this probe, a helper probe Dsf475 was designed to improve the in situ accessibility of probe Dsf440 and thus enhance fluorescence (Fuchs et al., 2000). The new probe was found to be fully complementary to four of the five sequences obtained from this study with the exception of one band (band 8), which exhibits one mismatch with the probe. In addition, it is also fully complementary to or exhibits only one mismatch to the 16S rDNA sequences of several *Desulfitobacterium* species (Table 3). All other 16S rDNA sequences in public databases have at least three mismatches with the probe.

Desulfitobacterium-like bacteria appeared mostly rod-shaped (Fig. 3b). These bacteria were not detected in the original chemostat culture, but were greatly enriched in all cultures receiving PCE as electron acceptor, reaching 53 and 17% of total bacteria counts with acetate and hydrogen as electron donor, respectively (Fig. 4). In all the other subcultures, in contrast, *Desulfitobacterium*-like bacteria remained undetected.

4. Discussion

With a combination of culture enrichment, DGGE fingerprinting, and FISH analysis, *Dehalococcoides*- and *Desulfitobacterium*-like bacteria were found to be involved in reductive dehalogenation of chlorinated ethenes in a complex microbial community. Several lines of evidence provided in the study suggested that the *Dehalococcoides*-like bacteria detected in this culture are capable of complete dehalogenation of chlorinated ethenes to ethene using hydrogen as electron donor. Based on physiological characterization, only the subcultures enriched with PCE or cDCE as electron acceptor, and hydrogen as electron donor maintained the original chemostat culture's capability of complete PCE or cDCE dehalogenation, indicating the existence of hydrogenotrophic complete dehalogenators. Subsequent FISH analysis revealed that under these conditions *Dehalococcoides*-like bacteria were greatly enriched compared with the original chemostat culture. In contrast, the relative and absolute abundance of these bacteria clearly decreased under all the other enrichment conditions. This correlation of *Dehalococcoides*-like bacteria with complete dehalogenation using hydrogen as electron donor is in agreement with previous reports (Maymo-Gatell et al., 1997). With respect to electron acceptors, it is certain that the *Dehalococcoides*-like bacteria detected in this culture are capable of cDCE dehalogenation. Indeed, other studies with this culture also indicated the presence of cDCE-dehalogenating *Dehalococcoides* (Cupples et al., 2003). However, we suspect that the *Dehalococcoides*-like bacteria were also

Table 3
Alignment of 16S rRNA target regions of *Desulfitobacterium* species

Sequence/Organisms[a]	Target sequence (5′−3′)
Band 3	CTTCAGGGACGAACGGTA[b]
Band 5	–––––––––––––––––
Band 6	–––––––––––––––––
Band 7	–––––––––––––––––
Band 8	––––––––––––––T–––
D.hafniense str. DCB–2 (X94975)	–––––––––––––––––
D. frappieri str. PCP–1 (U40078)	–––––––––––––––––
D. sp. Str. TCE1 (X95742)	–––––––––––––––––
D. sp. (X95972)	–––––––––––––––––
D. chlororespirans str. Co23 (U68528)	–––––––––––––––––
D. frappieri str. G2 (AF320982)	–––––––––––––––––
D. frappieri str. TCP–A (AJ404686)	–––––––––––––––––
D. frappieri str. DP7 (AJ276701)	–––––––––––––––––
D. hafniense str. GBFH (AJ307028)	––––––––––––––N–––
D. sp. Str. PCE1 (X81032)	––––––––––––––––C–
D. dehalogenans str. JW/IU–DC1 (L28946)	––––––––––––––––C–

[a]Refer to Fig. 2 for the naming of bands. In parenthesis are GenBank accession numbers of the corresponding organisms or sequences.

[b]Identical to the target sequence of probe Dsf440.

involved in PCE dehalogenation because enrichment of these organisms was observed in H_2/PCE subcultures (Fig. 4). One can argue that *Desulfitobacterium*-like bacteria were responsible for PCE dehalogenation in those subcultures because they were also enriched under the same condition. However, active *Desulfitobacterium*-like bacteria were not detected by FISH in the chemostat, where complete PCE dehalogenation occurred. Nevertheless, we can not exclude the possibility that different species of *Dehalococcoides* might be present in this culture. Further studies are necessary to verify our hypothesis and define the physiology of these bacteria in this culture.

On the other hand, *Desulfitobacterium*-like bacteria detected in this culture appeared to be capable of incomplete PCE dehalogenation to cDCE because enrichment of these bacteria (compared with the original chemostat culture) was observed only in PCE-amended subcultures but not in cDCE-amended ones. With respect to electron donor requirements, *Desulfitobacterium*-like bacteria might be able to use both hydrogen and acetate because they were enriched in both H_2/PCE and acetate/PCE subcultures. However, it is more likely that acetate served as the direct electron donor since acetate was detected in H_2/PCE subcultures during all transfers (0.5–1.0 mM). Additional evidence is that *Sporomusa*-like bacteria, which are capable of converting hydrogen and carbon dioxide to acetate (Moeller et al., 1984), were found in all hydrogen-receiving subcultures. The role of *Desulfitobacterium*-like bacteria as acetotrophic dehalogenators in this culture is an interesting finding because no *Desulfitobacterium* species identified so far can use acetate as electron donor for reductive dehalogenation, although a number of these bacteria have been reported performing dehalogenation of either chlorinated ethenes or chlorinated aromatic compounds (Utkin et al., 1994; Bouchard et al., 1996; Christiansen and Ahring, 1996; Gerritse et al., 1996, 1999; Sanford et al., 1996). To date *Desulfuromonas* species are the only known organisms capable of using acetate as electron donor for PCE dehalogenation to cDCE (Krumholz et al., 1996; Krumholz, 1997; Sung et al., 2003). The affiliation of *Desulfitobacterium* species with acetotrophic dehalogenators suggests that these bacteria might have a more versatile physiology than previously reported.

In addition to *Dehalococcoides* and *Desulfitobacterium*, *Sporomusa*-like bacteria were also detected in this culture. It is known that these bacteria are capable of homoacetogenesis (Moeller et al., 1984). The *Sporomusa*-like bacteria present in this culture may also act as homoacetogens because enrichment of these bacteria was detected in all hydrogen-receiving subcultures (Fig. 2), where concurrent acetate formation occurred (data not shown). *Sporomusa*-like bacteria may also play a role in reductive dehalogenation because it was previously reported that *Sporomusa ovata* was able to reductively dehalogenate PCE to TCE (Terzenbach and Blaut, 1994). However, the dehalogenation activity observed in that study depended on concomitant acetogenesis from methanol and carbon dioxide (Terzenbach and Blaut, 1994). Further physiology studies are necessary to assess the role of *Sporomusa*-like bacteria in reductive dehalogenation.

In terms of methodology, this study demonstrated the usefulness of culture enrichment to characterize microorganisms involved in reductive dehalogenation. The enrichment scheme in this study allowed enrichment of the organisms directly accomplishing dehalogenation, possibly due to the elimination of organisms involved in benzoate degradation in the original culture. However, this procedure may also enrich organisms that may not use chlorinated compounds, such as *Sporomusa*-like bacteria in this study.

Additionally, our results confirmed the importance and necessity of using a complementary quantification technique, such as FISH, when performing community structure analysis with PCR-DGGE analysis. Several limitations were observed with PCR-DGGE during this study. First, PCR-DGGE fingerprinting alone could not provide quantitative information with respect to the abundance of bacteria. This is largely due to the potential intrinsic bias of the conventional end-point PCR, which lowers the value of band intensity as a measure for species abundance (von Wintzingerode et al., 1997). Second, PCR-DGGE could lead to misleading results. For example, although the results obtained from DGGE were largely in agreement with FISH analysis, discrepancies were observed with acetate/cDCE and acetate/PCE subcultures. DGGE analysis indicated that both *Dehalococcoides*- and *Desulfitobacterium*-like bacteria were present in acetate/cDCE subcultures, and that *Dehalococcoides*-like bacteria were present in acetate/PCE subcultures. However, opposite results were obtained from FISH. One possible explanation to this is that these bacteria were present, but not metabolically active thus could only be detected by DGGE but not by FISH because DGGE analysis in this study was carried out with 16S rDNA of the microorganisms, while FISH targeted 16S rRNA. Theoretically this limitation can also be eliminated by performing 16S rRNA-targeted DGGE analysis. Further, DGGE fingerprints of the dehalogenating microbial community was to a great extent dependent on the primer pairs used for PCR amplification. Among the several primer pairs we experimented with, BAC 341f and BAC 534r was the only one clearly reflecting the shift of microbial community structure under different enrichment conditions, and the other primer pairs tested resulted in similar banding patterns among different treatments. As a result, we were only able to obtain DNA fragments of less than 200 base pairs in

length. Although the rDNA sequences retrieved contain much specific phylogenetic information (V3 region), they did not allow a highly robust phylogenetic analysis.

In summary, this study provided molecular evidence for the presence of both hydrogenotrophic complete dehalogenators, *Dehalococcoides*-like bacteria, and acetotrophic incomplete dehalogenators, *Desulfitobacterium*-like bacteria, in one microbial community. This is one of the few reports on the presence of different types of dehalogenators in one microbial community capable of PCE dehalogenation. Previously, Schlotelburg et al. (Schlotelburg et al., 2002) reported on the presence of *Dehalococcoides* and *Dehalobacter restrictus* in an anaerobic bioreactor dehalogenating 1,2-dichloropropane. While evidence is increasing that *Dehalococcoides* are widely distributed in microbial communities associated with complete dehalogenation and thus play a predominant role in this process (Fennell et al., 2001; Hendrickson et al., 2002), one wonders about the role of incomplete dehalogenators, such as *Desulfitobacterium* in this study. Do they have an ecological niche? How do they interact with *Dehalococcoides*-like bacteria and further impact the rate and extent of reductive dehalogenation? Moreover, do similar interactions also occur in environmental microbial communities? Further investigations to characterize the interactions between *Dehalococcoides* and *Desulfitobacterium* are required to address these questions.

5. Conclusions

- Molecular evidence was presented to indicate the presence of both *Dehalococcoides*- and *Desulfitobacterium*-like bacteria in one microbial community capable of reductive dehalogenation.
- *Dehalococcoides*-like bacteria in the studied community are associated with complete dehalogenation of chlorinated ethenes to ethene using hydrogen as electron donor.
- *Desulfitobacterium*-like bacteria are associated with incomplete PCE dehalogenation to cDCE and appear to be able to use acetate as electron donor.
- Combination of conventional enrichment techniques with DGGE and FISH was found a powerful tool for identification of key microbes involved in PCE dehalogenation, correlation of functions of the microbes with their phylogenetic identities, and in situ detection of their activity.

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