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16S rRNA Gene-Based Detection of Tetrachloroethene-Dechlorinating *Desulfuromonas* and *Dehalococcoides* Species

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Members of the genera *Desulfuromonas* and *Dehalococcoides* reductively dechlorinate tetrachloroethene (PCE) and trichloroethene. Two primer pairs specific to hypervariable regions of the 16S rRNA genes of the *Dehalococcoides* group (comprising *Dehalococcoides ethenogenes* and *Dehalococcoides* sp. strain FL2) and the acetate-oxidizing, PCE-dechlorinating *Desulfuromonas* group (comprising *Desulfuromonas* sp. strain BB1 and *Desulfuromonas chloroethenica*) were designed. The detection threshold of a nested PCR approach using universal bacterial primers followed by a second PCR with the *Desulfuromonas* dechlorinator-targeted primer pair was 1×10^3 BB1 cells added per gram (wet weight) of sandy aquifer material. 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 yielded positive signals with the BB1- and the *Dehalococcoides*-targeted primers. One chloroethene-contaminated aquifer tested positive with the *Dehalococcoides*-targeted primers, and another contaminated aquifer tested positive with the *Desulfuromonas* dechlorinator-targeted primer pair. Restriction fragment analysis of the amplicons could discriminate strain BB1 from other known *Desulfuromonas* species. Microcosm studies confirmed the presence of PCE-dechlorinating, acetate-oxidizing *Desulfuromonas* and hydrogenotrophic *Dehalococcoides* species in samples yielding positive PCR signals with the specific primers.

Tetrachloroethene (PCE) and trichloroethene (TCE) are abundant groundwater pollutants (1). PCE and TCE can be transformed to less chlorinated ethenes in anaerobic cometabolic processes mediated by methanogenic, homoacetogenic, and sulfate-reducing microorganisms (6, 13). Other groups of bacteria, like *Desulfuromonas* sp. strain BB1 and *Desulfuromonas chloroethenica*, *Dehalospirillum multivorans*, *Dehalobacter restrictus* strains PER-K23A and TEA, *Enterobacter* sp. strain MS1, *Dehalococcoides ethenogenes*, and *Desulfitobacterium* sp. strain PCE-S (summarized in reference 8), can reduce PCE and TCE in terminal electron accepting processes (chlororespiration). Reductive dechlorination of PCE and TCE in respiratory processes is orders of magnitude faster than anaerobic cometabolic reduction. Hence, the stimulation of respiratory organochlorine reducing bacteria (OCRB) is a promising and cost-effective approach for the remediation of PCE-contaminated sites.

The only available pure culture to date that is capable of complete reductive dechlorination of PCE to ethene is the obligately hydrogenotrophic organism *D. ethenogenes* (16, 17). Hydrogen is generally considered the ultimate electron donor to stimulate the reductive dechlorination of chloroethenes. *Desulfuromonas* sp. strain BB1 and *D. chloroethenica* are unique dechlorinators in regard to their electron donor requirements: acetate, but not hydrogen, supports the reductive dechlorination of PCE and TCE (9; F. E. Löffler, J. Li, J. W. Urbance, and J. M. Tiedje, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998, abstr. Q-177, p. 450, 1998). Members of both groups of PCE dechlorinators are promising candidates to be used in engineered bioremediation approaches and are potentially relevant contributors to the natural attenuation of chloroethenes. Unfortunately, their distribution in anaerobic environments is unknown. Two engineered remediation approaches can be distinguished: (i) the stimulation of indigenous PCE dechlorinating organisms (biostimulation) and (ii) the introduction of organisms that manifest a desired activity which are not present at the contaminated site (bioaugmentation). Both approaches require methods to monitor the presence, distribution, and fate of the organisms of interest; hence, sensitive and specific monitoring systems need to be developed. 16S rDNA-based PCR methods have been used to detect and enumerate particular populations in the environment and to monitor bacterial species in bioaugmentation studies (3–5, 10, 21, 25). However, none of these methods has been applied to strict anaerobic chloroethene-respiring OCRB. The goal of this study was to develop reproducible, sensitive, and specific detection systems for PCE-dechlorinating *Dehalococcoides* and *Desulfuromonas* species and to evaluate the presence of these populations in environmental samples.

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MATERIALS AND METHODS

Cultures and growth conditions. *Desulfuromonas* sp. strain BB1 was isolated from pristine freshwater sediment collected from the Père Marquette River in Michigan (12; Löffler et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998). Strain BB1 was grown in a basal salts mineral medium described previously (11, 13) and amended with 2.5 mM acetate and PCE (27 μl in 1 ml of hexadecane, resulting in an initial aqueous PCE concentration of 0.1 mM). Other *Desulfuromonas* strains, including *Desulfuromonas acetexigens* (DSM 1397), *Desulfuromonas acetoxidans* (DSM 684), *Desulfuromonas succinoxidans* (DSMZ 8964), and *Desulfuromonas thiophila* (DSM 8987) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; http://www.dsmz.de) and grown in media recommended by the DSMZ. A culture of *D. chloroethenica* was kindly provided by L. Krumholz, University of Oklahoma, Norman. E. Harris and D. Lovley, University of Massachusetts, Amherst, kindly provided cultures of *Geobacter metallireducens* and *Pelobacter acetylenicus*.

A highly enriched PCE-to-ethene dechlorinating mixed culture was obtained from Red Cedar River sediment (12, 13). A 16S rDNA clone library was established, and the 16S rDNA genes were cloned into the vector pCRII (Invitrogen, San Diego, Calif.) as previously described (19). Sequencing of the cloned 16S rDNA genes revealed the presence of three distinct populations: two *Spirochaete* species and a population that was related to *D. ethenogenes* (96.6% sequence

similarity). Both *Spirochaete* populations have been isolated in pure culture and were unable to dechlorinate PCE. Hence, the *Dehalococcoides* species, designated strain FL2, was inferred to be responsible for PCE-to-ethene dechlorination in this culture. The PCE-dechlorinating mixed culture was maintained in a basal salts mineral medium (11) amended with 0.5 mM acetate, 0.2 mM neat PCE, and hydrogen (10 kPa). Hydrogen and PCE consumption were followed by gas chromatography as previously described (13), and both substrates were replenished as they were depleted.

Primer design. Specific PCR primers were designed using Primer Selecter (DNASTAR, Inc., Madison, Wis.) based on the nearly complete 16S rDNA sequences of strains BB1 and FL2. The specificity of the selected primer combinations was examined with the PROBE-MATCH program of the Ribosomal Database Project (RDP) (14) and the PROBE-CHECK function of the ARB software (www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps). The BB1- and *Dehalococcoides*-targeted primers yielded amplicons of 835 and 434 bp, respectively. The nucleotide sequences of the *Desulfuromonas* dechlorinator-targeted forward primer was 5′AACCTTCGGGTCCTACTGTC3′ (*Escherichia coli* 16S rRNA positions 205 to 222), and the sequence of the reverse primer was 5′GCCGAACTGACCCCTATGTT3′ (1033 to 1015). The nucleotide sequences of the *Dehalococcoides*-targeted forward primer was 5′AAGGCGGT TTTCTAGGTTGTCAC3′ (728 to 750), and the sequence of the reverse primer was 5′CGTTTCGCGGGGCAGTCT3′ (1172 to 1155).

DNA isolation. Genomic DNA from pure cultures was obtained by following a standard protocol described by Maniatis et al. (15). Genomic DNA from *G. metallireducens* was kindly provided by J. Champine, Southeast Missouri State University, Cape Girardeau. Total DNA from aquifer and sediment material (0.25 g [wet weight]) was isolated with the UltraClean Soil DNA Kit from Mo Bio Laboratories, Inc. (Solana Beach, Calif.), by following the manufacturer's recommendations. DNA from the Bachman aquifer samples was also extracted from a larger sample of 5 g (wet weight) according to a previously described method (27). Purified DNA was dissolved in ultraclean water (Sigma, St. Louis, Mo.), and its concentration was determined spectrophotometrically and adjusted to 10 μg/ml.

PCR. Amplification reactions were performed in a total volume of 20 μl. The reaction mixtures contained 2 μl of 10× reaction buffer (Boehringer GmbH, Mannheim, Germany), 2.5 mM $MgCl_2$, 250 nM concentrations of each primer, 250 μM concentrations of each deoxynucleoside triphosphate (Gibco BRL, Gaithersburg, Md.), 2.5 U of AmpliTaq polymerase (Gibco), 14 μg of bovine serum albumin (Boehringer Mannheim), and 10 ng of template DNA. Amplifications were carried out in a 9600 GeneAmp PCR system (PE Biosystems, Norwalk, Conn.). The following thermocycling program was used for the specific primers: 94°C for 3 min (1 cycle); 94°C for 45 s, 58°C for 30 s, and 72°C for 1.5 min (30 cycles); 72°C for 7 min (1 cycle). Annealing temperatures ranging from 48 to 68°C were tested, and a temperature of 58°C resulted in reproducible amplifications of the 16S rDNA sequences of the target organisms. For nested PCR, the initial amplification was performed with a pair of universal bacterial primers (8F [5′AGAGTTTGATCCTGGCTCAG3′, *E. coli* 16S rRNA positions 8 to 27] and 1541R [5′AAGGAGGTGATCCAGCCGCA3′, *E. coli* 16S rRNA positions 1541 to 1522]) (26) under the same conditions described above except that the annealing temperature was 55°C. The specific primers were then used in the second PCR, using the amplified products (0.2 to 10 μl) from the initial PCR as templates. Aliquots (3 to 5 μl) of the PCR products were resolved in 0.9% (wt/vol) agarose gels in Tris-acetate-EDTA buffer (15) and stained in aqueous ethidium bromide solution (0.5 μg/ml) for 25 min. After rinsing the gels with water, the bands were visualized by UV excitation and pictures were taken with a digital camera (Genomic Solutions Inc., Ann Arbor, Mich.). The 1-kb DNA ladder from Gibco was used as the size marker.

To perform PCR on cell lysates, bacterial cells from 1 ml of culture fluid were collected by centrifugation. The pellets were washed twice with 50 mM filter-sterilized potassium phosphate buffer (pH 7.5) and suspended in 10 μl of water. Aliquots (1 μl) or 10-fold dilutions in water of these suspensions were then used as templates for amplification. To estimate the detection threshold with the *Dehalococcoides*-targeted primer pair, strain FL2's 16S rDNA gene was cloned into vector pCR2.1 (TA Cloning Kit; Invitrogen, Carlsbad, Calif.). Plasmid DNA containing a single copy of strain FL2's 16S rDNA gene was isolated with the QIAGEN Plasmid Mini Kit (QIAGEN Inc., Valencia, Calif.) according to the manufacturer's protocol. Purified plasmid DNA was serially diluted with water and used as the template for nested PCR. The nested PCR approach was used for all experiments, unless indicated otherwise. When defined templates were used, the identities of the PCR products obtained with the BB1- and the *Dehalococcoides*-targeted primers were confirmed by double-stranded sequencing of the entire amplicons. Amplicons obtained from environmental samples were partially sequenced to verify their identity and further characterized by restriction fragment length polymorphism (RFLP) analysis.

In order to determine the sensitivity of detection with heterogeneous templates, PCR was performed with DNA extracted from aquifer solids amended with *Desulfuromonas* sp. strain BB1 alone or with strain BB1 and *E. coli* cells together. A sandy aquifer material that showed no PCE dechlorination activity under a variety of electron donor conditions and that never yielded amplification products with the specific primers was used in this experiment. *E. coli* JM109 was grown in Luria-Bertani LB medium at 37°C for 6 h, and *Desulfuromonas* sp. strain BB1 was grown with acetate and PCE. To quantify biomass, the cells were washed with phosphate buffer and diluted 10-, 20-, and 100-fold in 50 mM filter-sterilized potassium phosphate buffer (pH 7.5). The cells were stained with acridine orange (final concentration, 0.1% [wt/vol]) for 1 h in the dark. Samples were then filtered through 0.2-μm-pore-size polycarbonate membranes (Millipore, Bedford, Mass.), and the cells were counted by computer-assisted light microscopy (20). A 0.1-ml suspension of 0 to 10^6 BB1 cells was added to 0.25 g (wet weight) of nonsterilized aquifer material and incubated for 1 h at room temperature under aerobic conditions. In another experiment, 0.1 ml of 10-fold diluted suspensions of BB1 cells and 3×10^7 *E. coli* JM109 cells (0.1 ml) were mixed with 0.25 g of sterilized aquifer material. Total DNA was extracted from the samples by using the UltraClean Soil DNA Kit and used as the template for PCR.

Sample collection. Samples were collected from three Michigan rivers and six contaminated aquifers (Table 1). The Au Sable River and the Père Marquette River sampling sites are located in quality fly fishing zones in National Forest Recreation Areas and are considered pristine environments. The Red Cedar River and the Au Sable River were sampled repeatedly at the same location. The Père Marquette River samples were collected twice from the same site (location PM 0) and once in September 1998 from three additional locations approximately 25, 75, and 150 m downstream (locations PM 1, PM 2, and PM 3, respectively). The aquifer samples from the Cape Canaveral site (Fla.), the Jacksonville site (Fla.), the Schoolcraft site (Mich.), and the B&J Industrial site (Mich.) were kindly provided by D. Fennell, G. Sewell, M. Dybas, and E. Petrovskis, respectively. Samples from the Jacksonville site were available from locations MW 510 (dissolved plume) and IW001 (source area). Samples from the Bachman Road site in Oscoda, Mich., were obtained from four different locations inside the chloroethene-plume (samples 1At, 1Bb, 2At, and 2Bb). Material was also collected from the noncontaminated area upstream of the plume (locations A and D). Individual samples were mixed by hand under sterile conditions to visual homogeneity and kept at 4°C under nitrogen.

Microcosms. Microcosms were established inside an anaerobic chamber with a nitrogen-hydrogen (97/3 [vol/vol]) atmosphere in 20-ml vials containing 2 g (wet weight) of aquifer or sediment material as previously described (11). One set of microcosms was flushed with sterile hydrogen-free dinitrogen to remove residual hydrogen, and then 5 mM acetate and 1.25 μl of PCE dissolved in 0.1 ml of hexadecane (resulting in a final aqueous PCE concentration of about 0.2 mM) were added by syringe. A second set of microcosms was amended with PCE dissolved in hexadecane, and 3 ml of hydrogen (30 kPa) was added as the electron donor. Hydrogen consumption and dechlorination were monitored by gas chromatography, and acetate oxidation was measured by high-performance liquid chromatography as previously described (11, 22). Negative controls included heat-treated (autoclaved on two consecutive days for 30 min) microcosms and cultures that did not receive an electron donor. Duplicate microcosms were established for each treatment.

RFLP analysis of amplified PCR products. The amplified fragments were digested with the restriction endonucleases *Sma*I or *Eco*RI (Gibco) according to the manufacturer's recommendations. The DNA fragments were resolved in 2% (wt/vol) Metaphor agarose (FMC Bioproducts, Rockland, Maine) in the presence of ethidium bromide (0.5 μg/ml) and fresh Tris-borate-EDTA buffer (15) at 4°C. Fragment sizes were estimated by using the DNA Molecular Weight Marker V (Boehringer).

Sequencing. 16S rDNA amplicons were purified (Wizard PCR Preps; Promega, Madison, Wis.) prior to sequencing by the fluorescent Dideoxy termination method at Michigan State University's sequencing facility. Automated fluorescent *Taq* cycle sequencing was performed with an ABI Catalyst 800-ABI 373A sequencing system (Applied Biosystems, Foster City, Calif.) using previously described bacterial-specific primers targeted to conserved regions of the 16S rDNA gene (26).

RESULTS AND DISCUSSION

Primer specificity. Specific primers directed against hypervariable regions of the 16S rRNA genes of *Desulfuromonas* sp. strain BB1 and *Dehalococcoides* sp. strain FL2 were designed. The former was developed following alignment of the selected sequences with the corresponding 16S rDNA regions of other *Desulfuromonas* species (Fig. 1). Direct and nested PCR performed with cell lysates of *D. acetexigens*, *D. chloroethenica*, and *Desulfuromonas* sp. strain BB1 yielded amplification products of the expected size and sequence, although *D. acetexigens* always produced only a faint band (Fig. 2). None of the other bacterial strains tested, including *D. thiophila*, *Desulfuromonas palmitatis*, *D. acetoxidans*, *D. succinoxidans*, *P. acetylenicus*, and *G. metallireducens*, resulted in amplification regardless of the template used (cell lysates or isolated genomic DNA). Hence, the primers were reasonably specific for the known PCE dechlorinators of this genus.

TABLE 1. Characteristics of sediment and aquifer samples used as starting material for DNA extraction and anaerobic microcosms and test results[a]

Site of sample collection	Contaminants	Date of sample collection (mo/yr)	Amplification of 16S rDNA fragments			End product(s) of PCE dechlorination in microcosms, with electron donor[b]:	
			Initial PCR, universal primers	Second PCR		Acetate	Hydrogen
				BB1-targeted primers	*Dehalococcoides*-targeted primers		
Bachman 1At	PCE, TCE, *cis*-DCE	8/1997	−[c]	−	−	*cis*-DCE	PCE[e]
Bachman 1Bb	PCE, TCE, *cis*-DCE	8/1997	−	+[d]	−	*cis*-DCE	PCE[e]
Bachman 2At	PCE, TCE, *cis*-DCE	8/1997	−	+[d]	−	*cis*-DCE	PCE[e]
Bachman 2Bb	PCE, TCE, *cis*-DCE	8/1997	−	+[d]	−	*cis*-DCE	PCE[e]
Bachman A	None	8/1997	−	−		PCE[e]	PCE[e]
Bachman D	None	8/1997	−	−	−	PCE[e]	PCE[e]
Cape Canaveral	Chloroethenes	9/1998	−	−	−	*cis*-DCE	
Jacksonville MW 510	PCE, TCE (dissolved plume)	6/1998	+	−	+	PCE[e]	VC, ETH
Jacksonville IW001	PCE, TCE (source area)	6/1998	−	−	−	PCE[e]	PCE[e]
B & J Industrial, sites C and D	PCE, TCE, BTEX[f]	7/1998	−	−	−	PCE[e]	PCE[e]
Schoolcraft	Chlorinated solvents	1995, 1998	−	−	−	PCE[e]	PCE[e]
Red Cedar River	None	7/1995, 5/1998, 11/1998	+	+	+	*cis*-DCE	ETH
Père Marquette River	None	4/1995, 9/1998	+	+	+	*cis*-DCE	ETH
Au Sable River	None	5/1995, 5/1997	+	+	+	*cis*-DCE	ETH

[a] DNA was isolated by using the UltraClean Soil DNA Kit from Mo Bio Laboratories, Inc. Microcosms were amended with PCE and acetate or hydrogen as the electron donor.

[b] Microcosms were analyzed after 10 weeks of incubation, and the major end products (>90% of the initial PCE concentration) are given. VC, vinyl chloride; ETH, ethene.

[c] −, no visible bands were obtained in the initial amplification with universal primers. The initial PCR, however, yielded PCR-amplifiable DNA, as confirmed in a control experiment with a pair of universal internal primers.

[d] Location 1Bb tested positive when DNA was extracted from fresh aquifer material. In addition, location 2Bb tested positive when an alternate DNA extraction method was used, and locations 2Bb and 2At tested positive after enrichment.

[e] Less than 5% of the initial amount of PCE was dechlorinated to TCE and *cis*-DCE.

[f] BTEX, benzene, toluene, ethylbenzene, and xylenes.

The genus *Dehalococcoides* forms a separate phylogenetic lineage, and its two known members (*D. ethenogenes* and strain FL2) are not closely affiliated with other known bacteria. The primer pair was designed to detect both strains and therefore be more comprehensive, yet remain specific for the group. Purified DNA from several other chlororespiring bacteria was used as template for the *Dehalococcoides*-targeted primers. As expected, amplification occurred only when DNA (or cell lysates) of the defined PCE-dechlorinating mixed culture or an *E. coli* clone containing the 16S rDNA gene of strain FL2 was used as a template.

Sensitivity. One to 10 pg of genomic DNA from strain BB1 was required to yield a visible band in direct PCR with the specific primers on ethidium bromide-stained agarose gels (data not shown). This amount corresponded to approximately 10^2 to 10^3 BB1 cells, assuming that a single BB1 cell contains about 8.8 fg of DNA (18). When whole cells were added as templates to the PCR reaction mixtures, 7×10^3 BB1 cells were required to yield a positive signal (data not shown). The nested PCR approach decreased the detection threshold by 3 orders of magnitude, and 1 to 10 BB1 cells were then sufficient to yield a visible band. The detection threshold for whole cells of strain BB1 was also evaluated by mixing a known number of BB1 cells with sterilized sandy aquifer material. An inoculum of 1×10^3 BB1 cells per g (wet weight) of aquifer material was required to yield a reproducible amplification with the *Desulfuromonas* dechlorinator-targeted primers in nested PCR. In the presence of 1.2×10^8 *E. coli* cells, the detection limit for strain BB1 decreased to about 3×10^5 BB1 cells per g (wet weight) of aquifer material when the specific primers were used directly on extracted DNA, and 3×10^3 BB1 cells were required to yield a signal in the nested approach (data not shown). Since *Dehalococcoides* strain FL2 is not available in pure culture, serially diluted plasmid DNA containing strain FL2's 16S rDNA gene was used to evaluate the sensitivity of the *Dehalococcoides*-targeted primers. One to 10 copies of strain FL2's 16S rRNA gene were sufficient to yield the expected PCR product in the nested PCR approach. Detection thresholds depend on various factors, such as the type of target organism (gram positive or gram negative), the type and composition of the matrix (aquifer, sediment, or soil), the number of other bacteria present in the sample material (competing target DNA in initial PCR), the quality and type of DNA-dependent DNA polymerase used, the additions made to relieve inhibition in PCR amplification (e.g., bovine serum albumin [BSA]), and the DNA extraction protocol used. The detection thresholds obtained with the methodology used in this study are consistent with the detection limits observed in other studies when 16S rDNA-based PCR approaches were used (3, 4, 5, 10, 21, 23, 24).

Detection in environmental samples. The specific primers were used to detect PCE-dechlorinating *Desulfuromonas* and *Dehalococcoides* species in six different aquifer samples and three Michigan river sediment samples. (i) River sediments. Initial PCR with universal bacterial-specific primers yielded products of the expected size from all river sediment samples. Most interestingly, all river sediment samples yielded amplification products with the *Desulfuromonas* dechlorinator-targeted (Fig. 3A) and the *Dehalococcoides*-targeted (Fig. 3B) primer sets. These results were independent of the sampling season (spring, summer, fall and winter) and were not influenced by a storage period of up to 4 years at 4°C. The activities of one or more hydrogenotrophic PCE-to-ethene dechlorinating populations, as well as acetate-oxidizing PCE-to-*cis*-1,2-

Strain	*E. coli* position 5' 210 215 220 3'
D. sp. strain BB1 Forward	AAC CTT CGG GTC CTA CTG TC
D. chloroethenica	··· ··· ··· ··· ··G ·C· ··
D. acetexigens	·-· ··· ··· ·-· ··· ·C· ··
D. thiophila	CCT ··· ·CT ·AA AGC T·C ·G
D. palmitatis	·-· ··· ··· ·-· ··· ··· ··
D. acetoxidans	CCT ··· ·TT ·AA AGC TAT ·G

Strain	*E. coli* position 5' 1020 1025 1030 3'
D. sp. strain BB1 Reverse	GCC GAA CTG ACC CCT ATG TT
D. chloroethenica	··· ··· ··· ··· ··· ··· ··
D. acetexigens	··· ··· ··· ··· T·· C·· ··
D. thiophila	··· ··· ··· ··· AA· ··· ··
D. palmitatis	··· ··· ··· ··T ·TC C·C ·C
D. acetoxidans	··· -·· ··· N·· ·AA ··· ··

FIG. 1. Alignment of parts of *Desulfuromonas* sp. strain BB1's 16S rRNA gene sequence with corresponding regions of phylogenetically related *Desulfuromonas* species. The sequences shown stem from variable regions and were used to generate *Desulfuromonas* dechlorinator-targeted oligonucleotide primers. A hyphen indicates a gap, and a dot indicates sequence identity to the 16S rDNA sequence of *Desulfuromonas* sp. strain BB1.

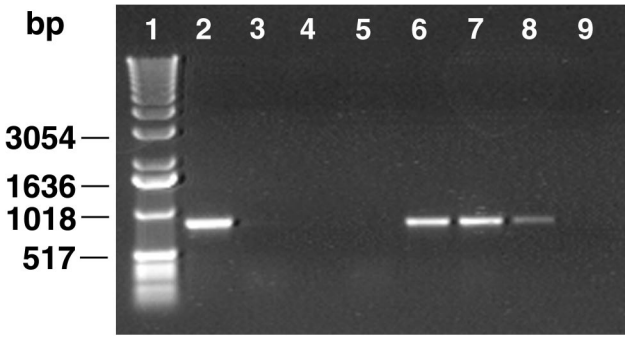

FIG. 2. Primer specificity. Cell lysates of phylogenetically related *Desulfuromonas* species were used as templates for the *Desulfuromonas* dechlorinator-targeted primer pair. Lanes: 1, 1-kb ladder marker; 2, BB1 genomic DNA (positive control); 3, *D. acetoxidans*; 4, *D. thiophila*; 5, *D. succinoxidans*; 6, *Desulfuromonas* sp. strain BB1; 7, *D. chloroethenica*; 8, *D. acetexigens*; 9, no target DNA (negative control).

dichloroethene dechlorinating populations, were confirmed in the microcosm experiments (Table 1). In the direct PCR approach with the BB1- and *Dehalococcoides*-targeted primers, only two sediment samples collected from the Père Marquette River (locations PM 1 and PM 2) resulted in positive signals. (ii) Aquifer materials. In contrast to the sediment materials, the Jacksonville MW510 sample was the only aquifer material that resulted in visible amplification in the initial PCR with the universal primers. To ensure that the samples that did not yield a visible band of amplified product were suitable for a second round of PCR, a second pair of universal primers (342F [5′CTACGGG(AG)(GC)GCAGCAG3′, *E. coli* 16S rRNA positions 342 to 357] and 1115R [5′AGGGTTGCGCTCGTTG3′, positions 1115 to 1100]) was used to amplify an internal region of the 16S rDNA fragments amplified in the initial PCR. Amplicons from all samples, including those that did not yield a visible band in the initial PCR with the universal primers 8F and 1541R, were amplified in this control experiment (data not shown). Hence, the initial PCR yielded DNA fragments from all samples that were suitable for PCR amplification with the specific primers. Nested PCR with the *Dehalococcoides*-targeted primers yielded a positive signal with the Jacksonville MW510 sample, but no amplification occurred with any other aquifer material (Fig. 3B). The microcosm studies confirmed the results obtained with the molecular approach. The Jacksonville MW510 microcosms were the only aquifer material-based microcosms that indicated the presence of a hydrogenotrophic PCE-dechlorinating population. Vinyl chloride and ethene accumulated in hydrogen-fed microcosms, whereas acetate-amended cultures showed only negligible dechlorination. Such dechlorination patterns are expected for *Dehalococcoides* species that depend on hydrogen as the electron donor and cannot couple PCE dechlorination to acetate oxidation. Amplification with the *Desulfuromonas* dechlorinator-targeted primers was observed in one aquifer sample and occurred with the DNA preparation extracted from aquifer material collected at location 1Bb of the Bachman Road site (Fig. 3A). No amplification, however, was seen with DNA isolated from Bachman locations 1At, 2At, and 2Bb, even though the microcosm experiments indicated the activity of acetate-oxidizing, PCE-dechlorinating populations at all locations inside the plume (Table 1). With an alternate DNA extraction method (27) that used a larger amount of fresh aquifer solids (5 g), an additional sample (location 2Bb) tested positive with the *Desulfuromonas* dechlorinator-targeted primers prior to enrichment. After 4 months of incubation with acetate and PCE, the microcosms established with Bachman material were sacrificed and the extracted DNA was used as a template in nested PCR. Again, PCR with the universal primers did not yield visible amplification products on agarose gels; however, three out of four PCE-dechlorinating microcosms tested positive with the *Desulfuromonas* dechlorinator-targeted primers, indicating the presence of a BB1-like population (Table 1). Samples A and D, which were collected upstream of the plume, did not show PCE-dechlorinating activity and never yielded amplification products in PCR. None of the other aquifer samples yielded amplification products with the *Desulfuromonas* dechlorinator-targeted primers. With the exception of the enrichments obtained from the Cape Canaveral site material, this observation agreed with the microcosm studies. Microcosms established with Cape Canaveral aquifer material dechlorinated PCE to *cis*-DCE with acetate as the only electron donor, but no amplification was observed with the *Desulfuromonas* dechlorinator-targeted primers. The false negative results obtained with Cape Canaveral aquifer material could be explained by the presence of other, as-yet-unidentified, acetate-oxidizing, PCE-dechlorinating populations.

RFLP analysis of amplicons. RFLP analysis performed on the amplicons obtained with the *Desulfuromonas* dechlorinator-targeted primers could distinguish between *Desulfuromonas* sp. strain BB1, *D. chloroethenica*, and *D. acetexigens*. According to published sequence data for *D. chloroethenica* (GenBank accession no. U49748), the amplified fragments of strain BB1 and *D. chloroethenica* should be distinguishable by their unique RFLP patterns generated by *Sma*I or *Eco*RI digestion. RFLP patterns of *Sma*I-digested amplicons, however, failed to distinguish the two strains (data not shown). Partial sequencing of the 16S rDNA of *D. chloroethenica* revealed two *Sma*I sites which were also present in strain BB1's 16S rDNA gene. No *Eco*RI sites, however, were present in the *D. chloroethenica* and *D. acetexigens* amplicons, and RFLP patterns obtained with *Eco*RI could distinguish strain BB1 from *D.*

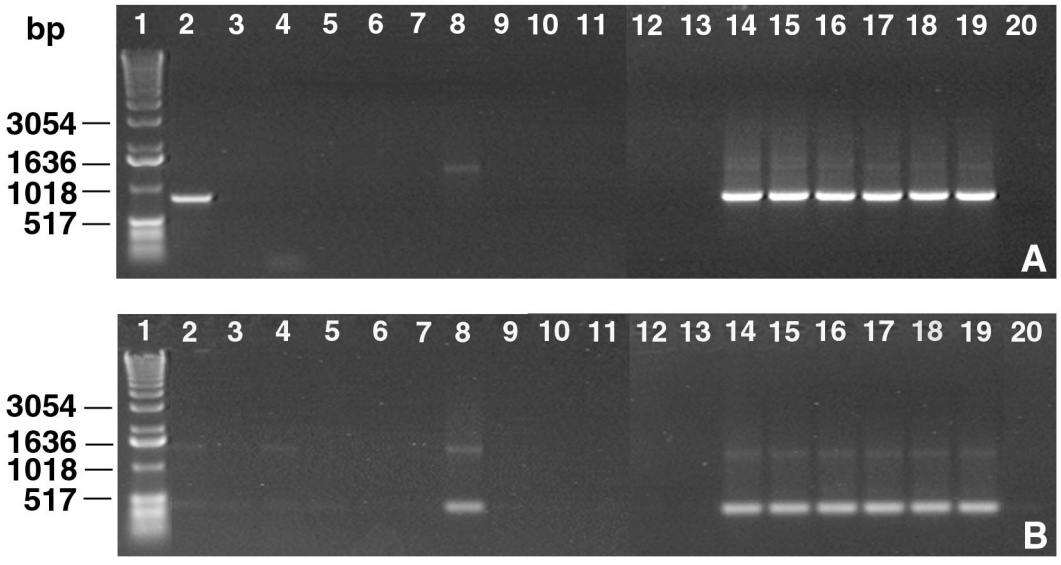

FIG. 3. Detection of PCE dechlorinators in environmental samples. Total DNA was isolated from 0.25 g of aquifer or sediment material and used as template DNA in a first-round amplification with universal bacterial-specific primers. The *Desulfuromonas* dechlorinator- and *Dehalococcoides*-targeted primers were then used in a second amplification phase (nested PCR approach). (A) Nested PCR with the *Desulfuromonas* dechlorinator-targeted primer pair. (B) Nested PCR with the *Dehalococcoides*-targeted primer pair. Lanes 1, 1-kb ladder markers; lanes 2 to 6, Bachman aquifer samples 1Bb, 1At, 2Bb, 2At, and 4A, respectively; lanes 7 to 12, aquifer samples from Cape Canaveral, Jacksonville MW510 and IW001, B&J Industrial site C and D, Schoolcraft, respectively; lanes 13, sandy aquifer material used in *E. coli* experiment; lanes 14 to 19, freshwater sediment samples from Red Cedar River (collected July 1995, May 1998, and November 1998), Père Marquette River (collected April 1995 and September 1998), and Au Sable River, respectively; lanes 20, negative controls.

chloroethenica and *D. acetexigens* (Fig. 4). Figure 4 shows the RFLP patterns after *Eco*RI digestion of the amplicons obtained with the *Desulfuromonas* dechlorinator-targeted primers from the different river sediments. All *Eco*RI-digested amplicons yielded RFLP patterns identical to those of *Desulfuromonas* sp. strain BB1, suggesting that this type of organism is more commonly distributed in the environment. As expected from computational analysis, the amplicons obtained with the *Dehalococcoides*-targeted primers could not be distinguished by RFLP analysis. Computer alignment of the amplicons from both strains revealed a 9-bp duplication at the 5′ end in the *D. ethenogenes* 16S rDNA fragment. If this duplication is not a sequencing error, it would result in a 9-bp-longer 443-bp amplicon with 16S rDNA from *D. ethenogenes*.

The results obtained with the BB1- and *Dehalococcoides*-targeted primers were supported by the microcosm studies, and no false positive results were obtained with the nested PCR approach. Hence, the primers may be useful in assessing the type of bioremediation that may be productive at contaminated sites. It remains unknown why (obligate) PCE respirers can be found in pristine river sediments. One explanation is an enzyme system with multiple functions. Another intriguing explanation would be the presence of nonanthropogenic sources of PCE in river sediments. The biotic or abiotic formation of chlorinated ethenes could explain the presence and persistence of obligately chloroethene-respiring populations, although such mechanisms have never been demonstrated in river sediments. There is, however, evidence for the abiotic (7) and biotic (2, 7) formation of PCE and TCE in other environments. Further research is required to understand how apparently obligate chlororespiring populations survive in pristine freshwater sediments.

The nested PCR methodology is useful to detect *Dehalococcoides* and BB1-like populations in environmental samples and to monitor their fate in bioaugmentation approaches. Since the abundance of these populations in environmental samples can be too low for detection, enrichment under appropriate conditions to increase the number of target organisms is recommended to prevent false negative results. This is especially true for samples from oligotrophic environments, such as many aquifers, which generally support a lower biomass than river sediments.

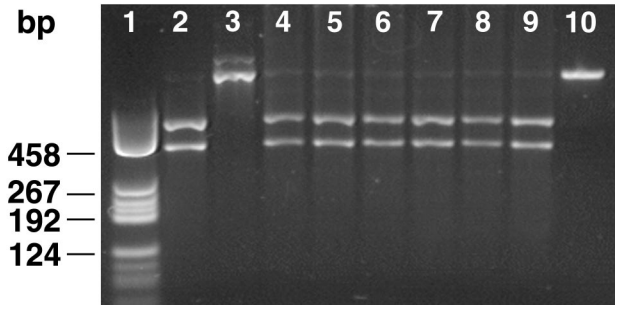

FIG. 4. RFLP analysis of amplicons obtained with the *Desulfuromonas* dechlorinator-targeted primers. Amplicons were digested with the restriction endonuclease *Eco*RI, and fragments were separated on a 2% metaphor agarose gel. Lane 1, DNA marker V; lanes 2 to 9, digested amplicons obtained from *Desulfuromonas* sp. strain BB1, *D. chloroethenica*, the Red Cedar River (collected July 1995, May 1998, and November 1998), the Père Marquette River (locations PM 0 and PM 1, collected September 1998), and the Au Sable River, respectively; lane 10, undigested PCR product obtained from *D. chloroethenica*.

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