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Monitoring Abundance and Expression of "*Dehalococcoides*" Species Chloroethene-Reductive Dehalogenases in a Tetrachloroethene-Dechlorinating Flow Column▽†

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We investigated the distribution and activity of chloroethene-degrading microorganisms and associated functional genes during reductive dehalogenation of tetrachloroethene to ethene in a laboratory continuous-flow column. Using real-time PCR, we quantified "*Dehalococcoides*" species 16S rRNA and chloroethene-reductive dehalogenase (RDase) genes (*pceA*, *tceA*, *vcrA*, and *bvcA*) in nucleic acid extracts from different sections of the column. *Dehalococcoides* 16S rRNA gene copies were highest at the inflow port [$(3.6 \pm 0.6) \times 10^6$ (mean ± standard deviation) per gram soil] where the electron donor and acceptor were introduced into the column. The highest transcript numbers for *tceA*, *vcrA*, and *bvcA* were detected 5 to 10 cm from the column inflow. *bvcA* was the most highly expressed of all RDase genes and the only vinyl chloride reductase-encoding transcript detectable close to the column outflow. Interestingly, no expression of *pceA* was detected in the column, despite the presence of the genes in the microbial community throughout the column. By comparing the 16S rRNA gene copy numbers to the sum of all four RDase genes, we found that 50% of the *Dehalococcoides* population in the first part of the column did not contain either one of the known chloroethene RDase genes. Analysis of 16S rRNA gene clone libraries from both ends of the flow column revealed a microbial community dominated by members of *Firmicutes* and *Actinobacteria*. Higher clone sequence diversity was observed near the column outflow. The results presented have implications for our understanding of the ecophysiology of reductively dehalogenating *Dehalococcoides* spp. and their role in bioremediation of chloroethenes.

Tetrachloroethene (PCE) and trichloroethene (TCE) are the most-abundant groundwater contaminants in the United States (32). In situ bioremediation is a promising technology for the removal of these chlorinated solvents from contaminated aquifers (6, 23, 29). Of particular interest for bioremediation are microorganisms of the genus "*Dehalococcoides*" (1, 7, 10, 11, 13, 15, 31). In addition to other recalcitrant chloroorganic pollutants, *Dehalococcoides* spp. reductively dechlorinate PCE, TCE, *cis*-dichloroethene (cDCE), and vinyl chloride (VC) to ethene. While some microbial species other than *Dehalococcoides* spp. degrade chlorinated solvents, reductive dechlorination of PCE past cDCE has been linked exclusively to members of the genus *Dehalococcoides* (11, 31, 36, 45).

The reduction of chloroethenes by *Dehalococcoides* spp. is mediated by reductive dehalogenase (RDase) enzymes. While many RDase genes have been identified, only a few have been characterized for their function. Known RDase genes involved in chloroethene reduction are *pceA*, encoding PCE reductases from *Dehalococcoides ethenogenes* strain 195 (DET0318; GenBank accession no. NC_002936) (28) and *Dehalococcoides* sp. strain CBDB1 (cbdB_A1588; GenBank accession no. NC_007356) (8); *tceA*, encoding TCE reductases from *D. ethenogenes* strain 195 (DET0079; GenBank accession no. NC_002936) (27) and *Dehalococcoides* sp. strain FL2 (GenBank accession no. AY165309) (10); *vcrA*, encoding the VC reductase from *Dehalococcoides* sp. strain VS (GenBank accession no. AY322364) (36); and *bvcA*, encoding the VC reductase from *Dehalococcoides* sp. strain BAV1 (DehaBAV1_0847; GenBank accession no. NC_009455) (18). Little is known about the distribution and activity of *Dehalococcoides* spp. and their RDase genes under PCE-dechlorinating conditions in contaminated aquifers. The detection and quantification of RDase genes can provide information about the abundance, metabolic capabilities, and activity of *Dehalococcoides* spp. in cultures and environmental samples. In previous studies, RDase gene quantification has been used to assess the physiology of *Dehalococcoides* spp. in laboratory cultures, environmental enrichments, and contaminated groundwater (12, 21, 22, 41, 45).

We quantified the known chloroethene RDase genes in DNA and RNA extractions from aquifer solids of a PCE-dechlorinating continuous-flow column. The *Dehalococcoides* species 16S rRNA gene served as the phylogenetic gene marker to determine *Dehalococcoides* species cell numbers. The laboratory-scale flow column contained aquifer solids from the Hanford contaminated-field site (Richland, WA) and

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TABLE 1. Oligonucleotides used for real-time PCR

Name	Sequence (5′→3′)	Position[a]	Product size (bp)	Target organism(s); gene(s)	Reference or source
Eub341F	CCTACGGGAGGCAGCAG	341–357	194	*Bacteria*; 16S rRNA genes	37
Eub534R	ATTACCGCGGCTGCTGGC	534–517		*Bacteria*; 16S rRNA genes	37
Dehalo505F	GGCGTAAAGTGAGCGTAG	505–522	199	*Dehalococcoides* spp.; 16S rRNA genes	This study
Dehalo686R	GACAACCTAGAAAACCGC	703–686		*Dehalococcoides* spp.; 16S rRNA genes	This study
Dre441F	GTTAGGGAAGAACGGCATCTGT	441–461	227	*Dehalobacter* spp.; 16S rRNA genes	44
Dre645R	CCTCTCCTGTCCTCAAGCCATA	645–666		*Dehalobacter* spp.; 16S rRNA genes	44
Dsb406F	GTACGACGAAGGCCTTCGGGT	406–426	225	*Desulfitobacterium* spp.; 16S rRNA genes	44
Dsb619R	CCCAGGGTTGAGCCCTAGGT	610–619		*Desulfitobacterium* spp.; 16S rRNA genes	44
vcrA880F	CCCTCCAGATGCTCCCTTTA	880–899	139	*Dehalococcoides* sp. strain VS; *vcrA*	This study
vcrA1018R	ATCCCCTCTCCCGTGTAACC	999–1018		*Dehalococcoides* sp. strain VS; *vcrA*	This study
bvcA277F	TGGGGACCTGTACCTGAAAA	277–296	247	*Dehalococcoides* sp. strain BAV-1; *bvcA*	This study
bvcA523R	CAAGACGCATTGTGGACATC	504–523		*Dehalococcoides* sp. strain BAV-1; *bvcA*	This study
tceA511F	GCCACGAATGGCTCACATA	511–529	306	*D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain FL2; *tceA*	This study
tceA817R	TAATCGTATACCAAGGCCCG	798–817		*D. ethenogenes* strain 195 and *Dehalococcoides* sp. strain FL2; *tceA*	This study
pceA877F	ACCGAAACCAGTTACGAACG	877–896	100	*D. ethenogenes* strain 195, *Dehalococcoides* sp. strain CBDB1, and *Dehalococcoides* sp. strain VS; *pceA* homologs	This study
pceA976R	GACTATTGTTGCCGGCACTT	957–976		*D. ethenogenes* strain 195, *Dehalococcoides* sp. strain CBDB1, and *Dehalococcoides* sp. strain VS; *pceA* homologs	This study

[a] For the 16S rRNA gene primer, the position numbering corresponds to the relative position in the *E. coli* 16S rRNA gene. The position numbers of the gene-specific primers correspond to positions within the genes of the listed organisms.

was bioaugmented with the Evanite culture, a *Dehalococcoides* species enrichment culture (3). The dechlorinating capacity of the Evanite culture has previously been characterized in batch kinetics studies (49, 50). We analyzed the abundance, distribution, and gene expression of *Dehalococcoides* spp. and their known chloroethene RDase genes in a bioaugmented, aquifer condition-simulating flow column which completely transformed PCE to ethene at the point of sampling. The study addresses the complex interplay of *Dehalococcoides* species population structure and activity under modeled aquifer conditions and is intended to add to our understanding of the ecophysiology and role of *Dehalococcoides* spp. in the environmental cleanup of chlorinated ethenes.

MATERIALS AND METHODS

Column assembly, operation, and sampling. The experimental design, column construction, and performance, as well as chloroethene transformation rates, are described elsewhere (3). The column was bioaugmented with a *Dehalococcoides* species enrichment culture (Evanite) for which transformation rate kinetics for the complete dechlorination of PCE have been studied in detail previously (49, 50). The column was operated for 170 days before aquifer solids were sampled for nucleic acid extraction. Samples for nucleic acid extractions were taken from six sections of the 30-cm-long column under anoxic conditions in a glove box (90% N_2, 10% H_2 atmosphere). The following intervals were collected: 0 to 5 cm, 5 to 10 cm, 10 to 15 cm, 15 to 20 cm, 20 to 25 cm, and 25 to 30 cm. The 0- to 5-cm section was closest to the column influent port. The 25- to 30-cm section at the opposite end of the column was next to the column effluent port. The total aquifer solids of each section were sampled with an autoclaved spatula. The column solids were placed in a separate autoclaved sample container and homogenized before aliquots for storage were prepared. For DNA extraction, 20-g amounts of aquifer solids were placed in 50-ml polypropylene tubes and stored at −20°C. For RNA extraction, aquifer solids (20 g) were mixed with equal volumes of RNA stabilization solution (RNAlater; Ambion, Inc.), immediately frozen in liquid nitrogen, and stored at −80°C.

Primer design for real-time PCR. The program Primer3 (42) was used to design real-time PCR primers that target the 16S rRNA gene of *Dehalococcoides* spp.; the *vcrA* gene of *Dehalococcoides* sp. strain VS (GenBank accession no. AY322364); the *bvcA* gene of *Dehalococcoides* sp. strain BAV1 (GenBank accession no. AY563562); the *tceA* genes of *D. ethenogenes* strain 195 (GenBank accession no. AF228507) and *Dehalococcoides* sp. strain FL2 (GenBank accession no. AY165309); and the *pceA* genes of *D. ethenogenes* strain 195 (DET0318; GeneID 3230306 and GenBank accession no. NC_002936) and strain CBDB1 (cbdB_A1588; GeneID 3623470 and GenBank accession no. NC_007356). The primers for the *pceA* gene in *D. ethenogenes* strain 195 and strain CBDB1 also target a putative gene producing an RDase homologue in *Dehalococcoides* sp. strain VS (locus tag DeVSDRAFT_827; GenBank accession no. ABFQ01000002). The amplicon lengths ranged from 139 to 306 bp. Primer specificity was confirmed with the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast) and by comparison with the genome sequences of *Dehalococcoides* sp. strain VS (draft sequence GenBank accession no. ABFQ00000000), *Dehalococcoides* sp. strain CBDB1 (19), *Dehalococcoides* sp. strain BAV1 (GenBank accession no. NC_009455), and *D. ethenogenes* strain 195 (43). Primer specificity was also confirmed by PCR with genomic DNA of *Dehalococcoides* species cultures, including strains VS, FL2, BAV1, CBDB1, and 195 and the Evanite enrichment culture. The specificity of the *Dehalococcoides* species 16S rRNA gene primers was further evaluated by performing PCR amplification of the cloned 16S rRNA gene sequences from the 16S rRNA gene clone libraries that were constructed from the 0- to 5-cm and 25- to 30-cm sections of the column. The primers failed to amplify all non-*Dehalococcoides* 16S rRNA gene sequences obtained from the column, while they successfully amplified positive *Dehalococcoides* species clones from pure cultures. The universal bacterial 16S rRNA gene primers were those of Muyzer et al. (37). The primers for *Dehalobacter* sp. and *Desulfitobacterium* spp. were those of Smits et al. (44). All primers used in this study are summarized in Table 1.

DNA and RNA extraction. Total DNA from the *Dehalococcoides* species cultures and the column soil was prepared as follows: 0.5 ml of culture or 0.5 g of soil were mixed with 0.25 ml of 1× TE buffer (100 mM Tris-HCl, 100 mM EDTA, pH 8.0). A spatula tip of acid-washed glass beads (0.1 to 0.15 μm in diameter; Sigma-Aldrich, St. Louis, MO) and sodium dodecyl sulfate to a final concentration of 2% were added to the sample. The samples were vortexed briefly and incubated in boiling water for 2 min. Subsequently, the samples were frozen in liquid nitrogen and kept on ice until they were completely thawed. The thawed samples were amended with 50 μl of a 10% bovine serum albumin solution and vortexed for 10 min. After centrifugation at 4°C for 3 min at 12,000 × *g*, 0.43 ml extraction buffer (0.8 M NaCl, 500 mM Na acetate, pH 5.5) was added to each sample. The samples were split, and DNA was extracted at least twice with 1.5 volumes of phenol-chloroform-isoamyl alcohol (25:24:1,

vol/vol/vol, pH 8.0; Sigma-Aldrich, St. Louis, MO) in 2-ml phase-lock tubes (heavy gel; Eppendorf, Westbury, NY). After the final centrifugation (5 min at 13,200 × *g*), DNA in the aqueous phase was precipitated with 2.5 volumes of absolute ethanol at −20°C for at least 2 h. The precipitated DNA was collected by centrifugation for 30 min at 13,200 × *g* at 4°C. DNA pellets were washed in 75% ethanol, centrifuged, and dried for 15 min at room temperature. DNA was dissolved overnight at 4°C in 20 μl nuclease-free water. Aliquots of split samples were combined (final volume, 40 μl) and stored at −20°C.

Total RNA was isolated following the hot phenol-chloroform extraction protocol of Oelmüller et al. (38). All solutions were treated with 0.1% (vol/vol) diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO) and subsequently autoclaved prior to use. RNA was dissolved in 20 μl RNase-free water and additionally purified from humic substances by using the RNA cleanup spin columns from a Fast RNA Pro soil-direct kit (MP Biomedicals, Solon, OH). Residual DNA was removed by incubation with 5 U RNase-free DNase (1 U/μl; Roche Diagnostics, Indianapolis, IN) in DNase buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$, pH 8.0) in a total volume of 100 μl. Following DNA digestion, RNA was purified with an RNeasy MinElute cleanup kit (Qiagen, Valencia, CA) by following the manufacturer's instructions. Finally, RNA was eluted in 15 μl RNase-free water, and aliquots were stored at −80°C.

Using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), cDNA synthesis from total RNA was carried out by following the manufacturer's instructions. To test for the absence of residual DNA contamination in the cDNA preparations, we performed reverse transcription control reactions lacking reverse transcriptase enzyme. No PCR amplicons could be obtained from any sample when reverse transcriptase was omitted from reverse transcription reactions.

Construction and application of plasmid standard for real-time PCR. A plasmid (pCR2.1_rdh16S) containing fragments of five different genes was assembled. The plasmid contained fragments of four RDase genes and a fragment of a 16S rRNA gene of *Dehalococcoides* spp., similar to the plasmid standard described by Holmes et al. (12). The 16S rRNA gene and *vcrA* were amplified and cloned from *Dehalococcoides* sp. strain VS genomic DNA (36), *tceA* and *pceA* were amplified and cloned using genomic DNA from *D. ethenogenes* strain 195 (31), and *bvcA* was amplified and cloned from DNA of *Dehalococcoides* sp. strain BAV1 (11). A detailed description of how the plasmid standard was constructed, including a list of primers, is given in the supplemental material. The sequences and orientation of the five gene fragments in plasmid pCR2.1_rdh16S were verified by DNA sequencing. The concentration of pCR2.1_rdh16S was determined by using a Qubit fluorometer and a Quant-iT double-stranded DNA broad-range assay kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Real-time PCR and data analysis. The absolute quantification of genes and transcripts in the aquifer solids from the continuous-flow bioreactor was performed by using iQ Sybr green supermix (Bio-Rad Laboratories, Hercules, CA) and gene-specific primers for different RDases (Table 1). Each sample mixture had a 30-μl reaction volume containing 1× iQ Sybr green supermix, forward and reverse primers at a concentration of 500 nM, and 6 or 2 μl of the prepared DNA or cDNA, respectively. PCR amplification and detection were conducted in an iCycler (Bio-Rad Laboratories, Hercules, CA). Real-time PCR conditions were as follows: 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 45 s at 61.5°C.

For real-time PCR data analysis, the raw data with background subtracted were exported from the iCycler system and analyzed by using Real-Time PCR Miner software (51). The algorithm calculates the efficiency (E) and threshold cycle (C_T) based on the kinetics results of individual real-time PCRs. The start template concentration (N) per reaction mixture was calculated with the equation

$$N = (1 + E)^{CT} \quad (1)$$

Calibration curves (log gene copy number per reaction volume versus log N) were obtained by using serial dilutions of plasmid pCR2.1_rdh16S. Equations 2 and 3 were used to calculate the number of gene copies in a known amount of plasmid DNA.

$$\text{No. of gene copies/}\mu\text{l} = \frac{\text{plasmid concentration (g/}\mu\text{l)}}{\text{no. of plasmid copies (molecules/g)}} \quad (2)$$

$$\text{No. of plasmid copies (molecules/g)} = \frac{\text{molecular weight of plasmid (g/mol)}}{\text{Avogadro's constant (molecules/mol)}} \quad (3)$$

The molecular size of plasmid pCR2.1_rdh16S was determined to be 3.86×10^6 g mol^{-1}. The number of gene copies per reaction volume was calculated by using the corresponding gene's standard curve for pCR2.1_rdh16S. Slopes and y intercepts of standard curves were determined by regression analysis in Excel (Microsoft Office 2003) and used to calculate the number of gene copies per reaction volume based on log N. The number of target genes per volume of sample was determined with equation 4. The value obtained for the number of target gene copies per reaction volume was multiplied by the volume of DNA (40 μl) or RNA (15 μl) extracted from each sample and divided by both the number of μl of DNA (6 μl) and the amount of RNA (2 μl cDNA = 1 μl RNA) used per reaction mixture and the volume of sample from which the DNA was extracted.

$$\text{No. of gene copies per volume of sample} = \frac{\text{(no. of gene copies per reaction volume)} \times \text{(volume of extracted DNA/RNA [}\mu\text{l])}}{\text{(volume of DNA/RNA per reaction mix [}\mu\text{l])} \times \text{(volume of sample used for extraction [ml or g])}} \quad (4)$$

Each real-time PCR (primer/template combination) was performed in triplicate, and triplicate measurements were repeated twice on independent nucleic acid extractions. The error bars shown in the figures represent the averages and standard deviations of the results of six real-time PCRs per sample. To verify the amplification of a single product and correct amplicon size, melting curve analysis was performed. Additionally, aliquots of the amplified product were examined by polyacrylamide gel electrophoresis.

Clone library construction and sequencing. 16S rRNA gene libraries were constructed from column aquifer material sampled close to the inflow (0 to 5 cm) and outflow ports (25 to 30 cm) of the flow column. DNA was extracted as described above. Domain-specific primers were used to amplify almost-full-length 16S rRNA genes from the extracted chromosomal DNAs by PCR; for *Bacteria*, primers GM3F (*Escherichia coli* 16S rRNA position 0008) (37) and Uni1392R (20) were used, and for *Archaea*, primers 20f (30) and Uni1392R or 20f and Arch958R (4) were used. PCRs were performed as follows. Amplifications were carried out in 50-μl volumes containing a final concentration of 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 0.5 U of *Taq* polymerase (Qiagen GmbH, Germany), 200 μg bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 1× Qiagen PCR buffer containing 1.5 mM $MgCl_2$ (pH 8.0). One-microliter amounts of the undiluted and 1:10-, 1:100-, and 1:1,000-diluted environmental DNA were used as template. The PCR amplification parameters included an initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 48°C for 1 min using the *Bacteria* domain-specific primers, and 58°C for 1 min using the *Archaea* domain-specific primers, followed by an elongation step at 72°C for 1 min. The last cycle was followed by a final extension step at 72°C for 9 min. PCR amplifications were performed in a PTC-200 gradient cycler (MJ Research, Inc., Watertown, MA). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen GmbH, Germany) and ligated into the pCR4 TOPO vector (Invitrogen, Carlsbad, CA). *E. coli* XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA) were transformed with the plasmids according to the manufacturer's recommendations.

Sequencing was performed by MCLab (South San Francisco, CA) by using *Taq* cycle sequencing with a model ABI 3730XL sequencer (Applied Biosystems). Sequence assembly was done with the program DNA Baser. The presence of chimeric sequences in the clone libraries was determined with the programs Bellerophon and Mallard version 1.02 (2, 14). Potential chimeras were eliminated before phylogenetic analysis. Sequence data were analyzed with BLAST and the ARB software package using the SILVA database (release date, 18 July 2007) (25, 39).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences from this study have been submitted to EMBL and assigned accession numbers FM178517 to FM178533 (inflow) and FM178802 to FM178834 (outflow).

RESULTS

Column operation, performance, and chloroethene transformation rates. The column operation conditions, performance, and chloroethene transformation rates were reported in a paper by Azizian et al. (3). Here we provide a brief summary of the results of the preceding study. In the flow column experiment, a PCE influent concentration of 0.09 mM was transformed to VC and ethene within a hydraulic residence time of 1.3 days. PCE dechlorination to cDCE was observed after bioaugmentation with the Evanite enrichment culture and following an increase in lactate concentration from

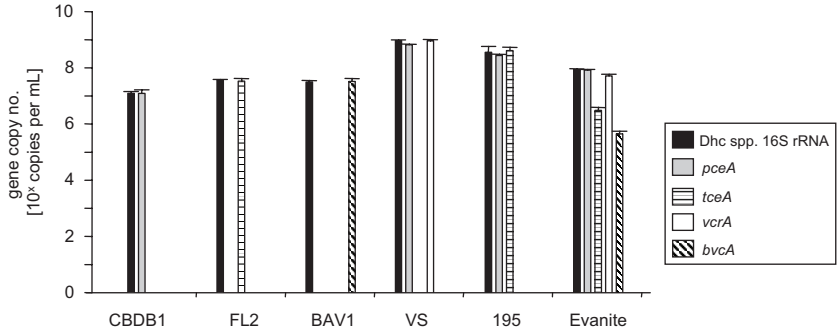

FIG. 1. RDase and 16S rRNA gene copy numbers of pure and enrichment cultures of *Dehalococcoides* spp. determined by real-time PCR. The graph shows the results for five *Dehalococcoides* sp. pure cultures (strains CBDB1, FL2, BAV1, VS, and 195) and one enrichment culture (Evanite). Each bar represents the average of the results of triplicate real-time PCRs performed on two independent DNA extractions ($n = 6$). Dhc, *Dehalococcoides*.

0.35 to 0.67 mM. Further increase in the lactate concentration to 1.34 mM resulted in the complete reduction of cDCE to VC and ethene. PCE and VC transformation rates were determined in batch-incubated microcosms constructed with aquifer soil from the column. In the first 5 cm from the column inlet, the PCE transformation rates were highest, and they decreased toward the column effluent. *Dehalococcoides* spp. accounted for up to 4% of the total *Bacteria* community in the flow column, which was consistent with estimates of electron donor utilization (4%) for dechlorination reactions (3).

Evaluation of the quantitative real-time PCR with axenic and mixed *Dehalococcoides* species cultures. Real-time PCR was used to quantify the copy numbers of *vcrA*, *bvcA*, *tceA*, *pceA*, and *Dehalococcoides* species 16S rRNA genes for *D. ethenogenes* strain 195 and *Dehalococcoides* sp. strains VS, CBDB1, BAV1, and FL2 and the Evanite enrichment culture. There is only a single 16S rRNA gene copy and single copies of multiple genes producing RDase homologues in each of the four sequenced *Dehalococcoides* genomes (*D. ethenogenes* strain 195 [43]; *Dehalococcoides* sp. strain CBDB1 [19]; *Dehalococcoides* sp. strain VS [draft sequence GenBank accession no. ABFQ00000000]; and *Dehalococcoides* sp. strain BAV1 [GenBank sequence accession no. NC_009455]). To accurately reflect this stoichiometry, we constructed and used for quantification a plasmid standard that contains fragments of the four RDase genes and a 16S rRNA gene of *Dehalococcoides* spp. (pCR2.1_rdh16S). Standard curves generated with pCR2.1_rdh16S showed a 1:1 ratio of the RDase gene copy numbers to the 16S rRNA gene copy number ($r^2 = 0.99$) (data not shown). Our real-time PCR method was linear over a dynamic range of 10^1 to 10^7 gene copies per μl of template solution. The detection limit of *Dehalococcoides* species 16S rRNA genes and RDase genes was 1×10^4 copies per ml or gram, respectively.

Quantification of gene copy numbers in genomic DNA obtained from axenic cultures of *Dehalococcoides* sp. strains CBDB1, FL2, BAV1, VS, and 195, respectively, confirmed that the RDase gene copy numbers matched the 16S rRNA gene copy number within 8% (Fig. 1). This result is consistent with *Dehalococcoides* sp. strains CBDB1, FL2, and BAV1 carrying a single copy of either the *pceA*, *tceA*, or *bvcA* gene. *D. ethenogenes* strain 195 contains single copies of *pceA* and *tceA* RDase genes. *Dehalococcoides* sp. strain VS carries a single copy of the *vcrA* gene and also contains a gene producing an RDase that is homologous to the PCE reductase in strain 195, whose function is not yet characterized (Fig. 1).

The Evanite culture was used to inoculate the flow column. It is a mixed microbial enrichment culture that contains *Dehalococcoides* spp. which have not been characterized for their RDase gene composition. Thus, we quantified the *Dehalococcoides* species 16S rRNA and chloroethene RDase gene composition of the Evanite culture prior to column inoculation. The *Dehalococcoides* species 16S rRNA gene copy number of the Evanite culture was $8.5 \times 10^7 \pm 0.7 \times 10^7$ (mean ± standard deviation) per ml. The most-abundant RDase gene was found to be *pceA* ($8.4 \times 10^7 \pm 3.8 \times 10^6$ copies per ml), present in 98% of the *Dehalococcoides* species cells in the culture, as determined by the fraction of RDase gene and 16S rRNA gene copy numbers. The *vcrA* gene was present in 60% ($5.1 \times 10^7 \pm 8.1 \times 10^6$ copies per ml) of all *Dehalococcoides* species cells in the Evanite culture. *tceA*-containing *Dehalococcoides* species cells represented 4% ($3.0 \times 10^6 \pm 0.8 \times 10^6$ copies per ml) and *bvcA*-containing *Dehalococcoides* spp. cells represented 1% ($4.5 \times 10^5 \pm 1.0 \times 10^5$ copies per ml) of the total *Dehalococcoides* species population. This shows that the Evanite enrichment culture contains a diverse population of *Dehalococcoides* spp. which contains all four chloroethene RDase genes. The sum of quantified RDase genes exceeded the *Dehalococcoides* species 16S rRNA gene copy number in the Evanite culture, indicating that the indigenous *Dehalococcoides* spp. carry one or a combination of two or more of the analyzed RDase genes.

Abundance and spatial distribution of RDase genes in the flow column. When complete dechlorination of PCE to ethene was observed at the outflow of the column (after 170 days of column operation), the aquifer solids from the continuous-flow column were sampled and analyzed for the spatial distribution and activity of *Dehalococcoides* species cells and their respective RDase genes. The column was divided into six sections (5 cm each) between the column inflow and outflow ports. We used real-time PCR to monitor the abundance of *Dehalococcoides* species 16S rRNA and *pceA*, *tceA*, *bvcA*, and *vcrA* genes in nucleic acid extracts from aquifer solids of each of the six column sections (Fig. 2). Distinct spatial trends of gene distri-

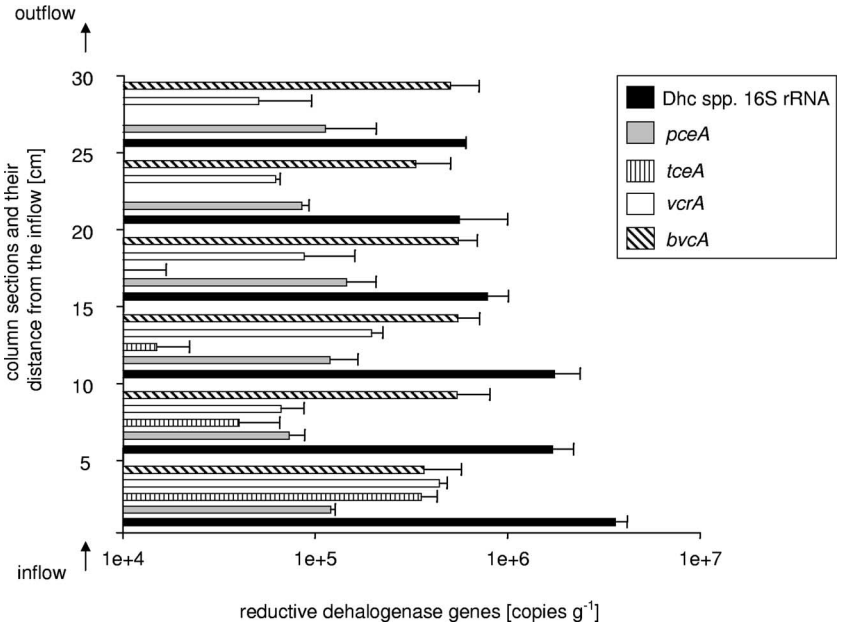

FIG. 2. Chloroethene RDase gene abundance in the flow column. Gene copy numbers were determined by real-time PCR using template DNA extracted from six different column sections. Each bar represents the average of the results of triplicate real-time PCRs performed on two independent DNA extractions. Dhc, *Dehalococcoides*.

bution were observed: the *Dehalococcoides* species 16S rRNA gene was detected at $(3.6 \pm 0.6) \times 10^6$ copies/gram in the first 5 cm from the column inflow, decreasing to $(1.7 \pm 0.5) \times 10^6$ copies/gram between 5 and 15 cm from the column inlet and further dropping to between $(5.6 \pm 0.4) \times 10^5$ and $(7.9 \pm 0.2) \times 10^5$ copies/gram in the second half of the column (15 to 30 cm) (Fig. 2). Thus, the *Dehalococcoides* species population accounted for 1 to 3% of the total *Bacteria* community in the flow column. The relative *Dehalococcoides* abundance estimate has to be considered carefully since the determination of total *Bacteria* 16S rRNA gene numbers by real-time PCR is biased by the various rRNA operon copy numbers in different microbial species (17, 24).

While all four RDase genes (*pceA*, *tceA*, *bvcA*, and *vcrA*) were found to be present in the first four sections (0 to 20 cm from the column inflow), the *tceA* gene could not be detected 20 to 30 cm from the column inlet (Fig. 2). The VC reductase-encoding *bvcA* gene was the numerically dominant RDase gene in all column sections [$(5.6 \pm 1.4) \times 10^5$ to $(3.3 \pm 1.7) \times 10^5$ copies/gram] except for the section closest to the inflow, in which the RDase genes *tceA* and *vcrA* were equally abundant [$(3.6 \pm 0.8) \times 10^5$ and $(4.4 \pm 0.4) \times 10^5$ copies/gram]. The PCE reductase-encoding *pceA* gene was present in all column sections. *pceA* gene copy numbers ranged from $(1.5 \pm 0.6) \times 10^5$ to $(7.4 \pm 1.5) \times 10^5$ copies/gram (Fig. 2).

The sum of all RDase genes accounted for 35 to 50% of all *Dehalococcoides* species 16S rRNA gene copies in the first half of the column (0 to 15 cm). This interesting finding indicates that more than half of the quantified population of *Dehalococcoides* species cells in the first 15 cm of the column did not contain any one of the four known chloroethene RDases. In the second half of the column (15 to 30 cm), 80 to 120% of the 16S rRNA genes could be accounted for by the sum of all RDase genes (Fig. 2). A recovery rate of more than 100% can be explained by the presence of *Dehalococcoides* spp., such as strains 195 and VS, which contain a *pceA*-type RDase gene homolog and, in addition, either a *tceA*- or *vcrA*-type RDase gene.

Expression of RDase genes in the flow column. Total RNA was extracted from aquifer solids sampled from the six 5-cm intervals of the column and served as template in reverse transcription–real-time PCRs to quantify the expression of the four chloroethene RDases. Standard curves generated with plasmid pCR2.1_rdh16S were used to quantify RDase transcript abundance.

Interestingly, despite the presence of the *pceA* gene in all six column sections at copy numbers ranging from $(7.4 \pm 1.5) \times 10^4$ to $(1.5 \pm 0.6) \times 10^5$, no transcripts were detected in any part of the column (Fig. 3). Transcripts of the RDase genes *tceA*, *vcrA*, and *bvcA* could be detected throughout most of the column. The highest copy numbers were observed in the section 5 to 10 cm from the column inflow (Fig. 3). While the *vcrA* and *tceA* transcript levels gradually decreased with increasing distance from the column inflow, *bvcA* transcripts never decreased below $(1.0 \pm 0.1) \times 10^6$ copies/gram. *bvcA* was the only RDase gene expressed in the column section closest to the column outflow (25 to 30 cm) but was not detected in the section 0 to 5 cm from the column inflow (Fig. 3). *tceA* transcripts were not detected in the sections 20 to 30 cm into the column, and expression of the VC reductase-encoding *vcrA* could not be detected in the section 25 to 30 cm from the column inflow (Fig. 3).

Microbial community composition in the column. A comprehensive description of the phylogenetic affiliation and frequencies of the 16S rRNA gene clone sequences obtained is provided in Table S1 in the supplemental material. In total, 123 bacterial clones were obtained. Sixty-nine clones were retrieved from the 0- to 5-cm section close to the column inflow,

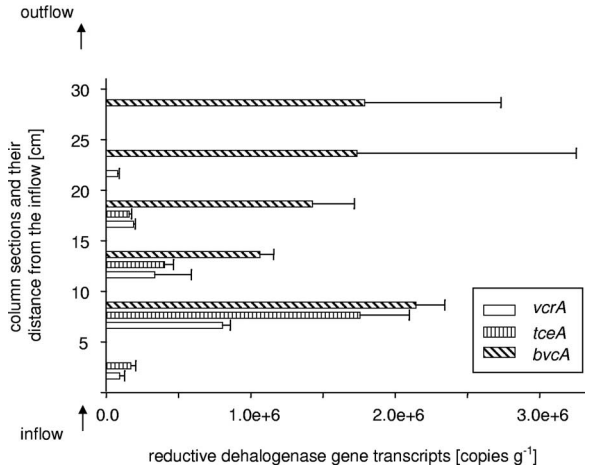

FIG. 3. Transcript copy numbers of chloroethene RDase genes in the flow column. Transcript abundance was determined by real-time PCR using template RNA extracted from six different column sections. Each bar represents the average of the results of triplicate real-time PCRs performed on two independent RNA extractions.

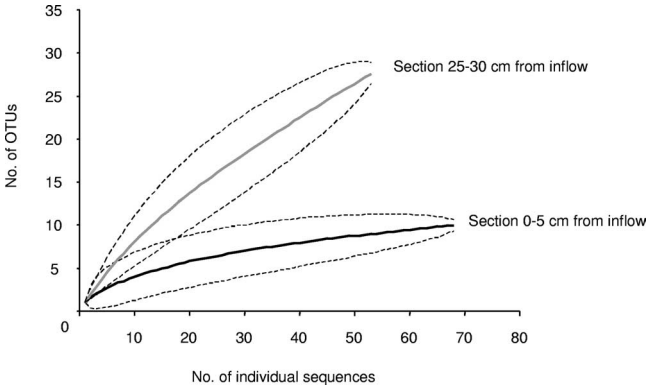

FIG. 4. Rarefaction curves of two 16S rRNA gene libraries from the PCE-dechlorinating continuous-flow column. The clone libraries were constructed from DNA extracted from column aquifer solids sampled 0 to 5 cm from the column inflow and 25 to 30 cm from the column inflow. Dashed lines represent the >95% confidence interval. Sequences were assigned to OTUs if they were less than 3% different from each other.

and 54 clones were obtained from the section closest to the column outflow (25 to 30 cm). No archaeal 16S rRNA gene sequences were amplified from both sections using two different standard primer combinations (see Materials and Methods). The 16S rRNA gene clones obtained from both libraries showed high sequence similarity (>97%) to environmental clones from other chloro-organic-contaminated environments, but the compositions of the two 16S rRNA gene libraries differed remarkably.

The 0- to 5-cm-section clone library was dominated by two sequence types affiliated with the genus *Eubacterium* of the phylum *Firmicutes*. Of 69 clones obtained from this section, 42 clones had 97 to 98% sequence identity with *Eubacterium* species 16S rRNA sequences. Of the 42 sequences, 23 sequences were most closely related to a sequence of an uncultured bacterium (GenBank accession no. AJ488081) from a chlorobenzene-degrading microbial consortium. The remaining 19 *Eubacterium* species sequences were most similar to clone sequence ROME195Asa (GenBank accession no. AY998135) from a microbial community inhabiting alternating shale-sandstone units of the continental deep subsurface. Only one of the sequences with high sequence identity to clone sequence ROME195Asa was also found in the clone library from the column outflow section (25 to 30 cm). Sequences found in the 0- to 5-cm-section clone library that were also present in comparable numbers in the clone library from the 25- to 30-cm section belonged to the *Propionibacteriaceae* (*Actinobacteria*; 13 clone sequences in the 0- to 5-cm section and 9 clone sequences in the 25- to 30-cm section, with 92 to 99% sequence similarity) and *Desulfovibrionaceae* (*Deltaproteobacteria*; 4 and 6 clone sequences in the respective sections, with 92 to 98% sequence similarity). The most-abundant sequences in the clone library from the 25- to 30-cm section were affiliated with the *Betaproteobacteria* families *Comamonadaceae* and *Oxalobacteraceae* of the *Burkholderiales* (14 out of 54 total clones). The 16S rRNA gene clone sequences unique to the outflow section of the PCE flow column were most similar to uncultured gammaproteobacteria of the *Xanthomonadales* and the deltaproteobacterium *Pelobacter acetylenicus*. No *Dehalococcoides* species sequences were found in both clone libraries. This finding is consistent with the overall low abundance of *Dehalococcoides* spp. in the column (as determined by real-time PCR) and the small size of our two clone libraries. Interestingly, two sequences from the column outflow section were most closely related to the *Anaerolineae*, a subphylum of the *Chloroflexi* with 85% sequence similarity to the *Chloroflexi* subphylum *Dehalococcoides*. Finding another *Chloroflexi* subphylum in the system is interesting because of the possibility of horizontal gene transfer between both related taxa. McMurdie et al. have recently described the alien nature of the *vcrA* and *bvcA* codon usage, supporting the theory of a foreign origin of these genes in *Dehalococcoides* spp. (33).

16S rRNA gene sequences of other chloroethene-dechlorinating bacteria, such as *Desulfitobacterium* spp. or *Dehalobacter* spp., were not found in the clone libraries and also could not be detected by real-time PCR using the primers designed by Smits et al. (44). Rarefaction curves of both clone libraries are shown in Fig. 4. Sequences were assigned to operational taxonomic units (OTUs) if they were less than 3% different from each other. The rarefaction curves of the two 16S rRNA gene libraries from the different column sections show that each section contained significantly different total numbers of bacterial lineages ($P < 0.05$). While the richness of OTUs in the clone library from the 0- to 5-cm section (10 OTUs) seems to plateau for the 69 sequences obtained, the clone library from the 25- to 30-cm section did not contain sufficient sequences to provide a robust estimate of species richness. Twenty-eight OTUs for the 54 sequences from this section did not result in a flattening of the respective rarefaction curve (Fig. 4).

DISCUSSION

In this study, we investigated *Dehalococcoides* species abundance and activity in a modeled aquifer system undergoing reductive dehalogenation of PCE. 16S rRNA gene clone li-

braries were constructed to gain insights into the microbial community composition at the two opposing ends of the column system. Flow column experiments were conducted with Hanford aquifer material bioaugmented with the Evanite culture, which contained an enriched population of *Dehalococcoides* spp. (73.5% of total *Bacteria* 16S rRNA gene copies). The column operation conditions, performance, and chloroethene transformation rates were published previously (3).

Using real-time PCR, we determined the chloroethene RDase gene composition of *Dehalococcoides* spp. in the Evanite culture. We found that *Dehalococcoides* species cell numbers in the culture could be fully accounted for by the sum of the quantified chloroethene RDase genes. In contrast, analysis of the flow column aquifer solids after 170 days of column operation revealed that nearly 50% of the *Dehalococcoides* species population in the first half of the column (0 to 15 cm) contained *Dehalococcoides* spp. which did not contain any of the four known RDases. This indicates the presence of so-far-uncultured *Dehalococcoides* spp. which presumably are indigenous to the Hanford aquifer material. Interestingly, no dechlorinating activity was observed in the flow column before bioaugmentation with the Evanite culture (3), which is in agreement with the presence of *Dehalococcoides* spp. lacking the known chloroethene reductase genes in the Hanford aquifer solids which were used to fill the column. Bioaugmentation of the flow column with the Evanite culture marked the onset of reductive dechlorination, because it introduced *Dehalococcoides* spp. containing known chloroethene RDase genes into the system.

Futamata et al. took a similar PCR-based approach to investigate the chloroethene RDase gene composition in *Dehalococcoides* species-containing enrichment cultures on polychlorinated dioxins (9). They report PCE transformation in the absence of any one of the four chloroethene RDase genes (*vcrA*, *bvcA*, *tceA*, and *pceA*) and concluded that their cultures might include novel *Dehalococcoides* species-type microorganisms.

We further quantified RDase gene transcripts in total RNA extracted from the different column sections and found that a *bvcA*-type VC reductase gene was the most transcriptionally active of the four RDase genes analyzed in this study. Except in the first section, *bvcA* was the most highly expressed RDase gene in all column sections and had the only RDase transcripts detectable closest to the column outflow. The section 5 to 10 cm from the inflow port was the most transcriptionally active column section, with the highest copy numbers for *bvcA*, *vcrA*, and *tceA*. Interestingly, we did not detect any *pceA* transcripts, although we showed that the *pceA* gene was present in every column section. Azizian et al. determined the zero-order PCE transformation rates in microcosms inoculated with column material from the different column sections (3). The PCE transformation rates, per gram of aquifer solids, were highest in the first section, 0 to 5 cm from the column inlet (5.5 nmol h^{-1} g^{-1}) and were decreased by an order of magnitude by the column outlet (0.5 nmol h^{-1} g^{-1}) (3). The fact that we did not detect any *pceA* transcripts despite active PCE reduction in the column might indicate that continuous *pceA* transcription at a detectable level may not be required in *Dehalococcoides* spp. to maintain PCE dechlorination activity under the prevailing conditions in the column. The known PCE reductase of *D. ethenogenes* strain 195 (DET0318) has been shown to be bifunctional, also serving as a 2,3-dichlorophenol reductase (8). Only limited information about its predominant in situ function, regulation, and expression exists to date.

The results of pure-culture studies with *D. ethenogenes* strain 195 revealed that increasing chloroethene respiration rates were not necessarily reflected in an increase of corresponding RDase gene transcript levels (40). The results of microarray and proteomic studies demonstrated that RDase genes other than the designated PCE reductase gene *pceA* (DET0318) are expressed during reductive dechlorination of PCE by *D. ethenogenes* strain 195 and that their induction and expression levels vary with electron acceptor concentration (16, 34, 35). However, in these studies, pure and enrichment cultures were used that have been maintained on chlorinated ethenes in laboratories for decades. It is conceivable that *Dehalococcoides* species RDase genes other than the known *pceA* function as PCE reductase genes under environmental conditions, including the simulated aquifer conditions in our flow column. In order to identify suitable biomarkers for in situ application it is necessary to study their performance under in situ-relevant conditions (22).

To assess the microbial-community diversity present in the aquifer material from the flow column, we constructed 16S rRNA gene clone libraries from the two opposite ends of the flow column. Based on the number of OTUs found in the two clone libraries, the microbial community near the column inflow, where electron donor and acceptor concentrations were highest, was less diverse than the microbial community at the outflow end of the column. The difference in OTUs found at the opposite ends of the flow column also reflects which electron donors are available at the two column ends (i.e., lactate near the inflow and acetate and propionate near the outflow).

In general, the compositions of OTUs found in the column clone libraries were similar to those detected in a TCE-contaminated aquifer undergoing biostimulation with lactate (26). Both 16S rRNA gene clone libraries were dominated by *Clostridia*. In contrast to the results of the field study by Macbeth et al., we could not amplify archaeal 16S rRNA gene sequences. The archaeal library of Macbeth et al. consisted of clones affiliated with acetoclastic methanogens (26). Consistent with this observation, we did not find evidence for methane formation during the operation of our flow column (3).

Although not represented in the 16S rRNA gene clone libraries generated with broad-specificity bacterial primers, *Dehalococcoides* species 16S rRNA genes could be quantified with genus-specific primers in real-time PCR assays. Other known chloroethene-dechlorinating microbial phylotypes, such as *Dehalobacter* spp. and *Desulfitobacterium* spp., were not found in the clone libraries either. The presence of both phylotypes could also not be shown with specific primers. We found one phylotype in both clone libraries which was most similar to the genus *Sporomusa* of the *Acidaminococcaceae*/*Clostridiales*. *Sporomusa ovata* has been shown to dechlorinate PCE to TCE concomitant with homoacetogenesis from methanol and carbon dioxide (46). *Sporomusa* species phylotypes have also previously been found in chlorinated-ethene-dechlorinating mixed cultures (5, 47).

The column inflow clone library was dominated by 16S rRNA gene sequences affiliated with the genus *Eubacterium* of

the phylum *Firmicutes* (61% of the total number of clone sequences obtained). Although clone library abundance does not necessarily reflect natural abundance, the dominance of these sequences in the column inflow section is apparent. The *Eubacterium* species sequences from the column have 97% sequence similarity to *Eubacterium limosum*. Recently, Yim et al. reported reductive dechlorination of methoxychlor and DDT in pure-culture studies with *Eubacterium limosum* (48). Based on the clone library results and the lack of *pceA* expression by *Dehalococcoides* spp., it is conceivable that *Eubacterium* spp. could be responsible for the bulk of the PCE dechlorination near the column inflow. Based on 16S rRNA gene phylogeny, the physiological potential of the column microbial community comprised reductive dechlorination, fermentation, homoacetogenesis, and sulfate reduction. Since phylogenetic affiliation does not necessarily reflect metabolic capabilities, inferring the functional capabilities of a microbial community based on 16S rRNA gene phylogeny alone should be done cautiously.

By quantifying known chloroethene RDase genes and their transcripts in a PCE-dechlorinating flow column, we have shown that *Dehalococcoides* spp. containing functionally different RDase genes occurred spatially separated in the flow column. Spatial separation most likely followed electron acceptor availability. Assuming that *Dehalococcoides* spp. are the dominant dechlorinating microorganisms in the system, the microbial distribution followed gradients of PCE dechlorination products. *Dehalococcoides* spp. capable of PCE and TCE dechlorination were present in the lower half of the column near the PCE inflow (0 to 15 cm) where they compete with other potential PCE dechlorinators in the microbial community for electron acceptor availability. *Dehalococcoides* spp. capable of DCE and VC dechlorination occupied the upper half of the column where most of the PCE and TCE were reduced to DCE and VC (15 to 30 cm). This might explain the higher abundance and activity of *bvcA*-type VC reductase gene-containing *Dehalococcoides* spp. in this column half and supports the exclusive role and importance of these *Dehalococcoides* sp. strains for complete environmental PCE dechlorination. The spatial analysis of *Dehalococcoides* species chloroethene RDase genes in the PCE flow column provided evidence that complete dechlorination to ethene is a cooperative and nonlinear effort of diverse *Dehalococcoides* sp. strains that occupy environmental niches according to their functional RDase gene composition.

The results of the present study and of the complementary column study by Azizian et al. (3) demonstrate that RDase genes can serve as a general indicator of the metabolic potential and function of *Dehalococcoides* spp. by providing useful insights into the organisms' abundance, distribution, and activity. As we continue to study the physiology of *Dehalococcoides* spp. and characterize new functions of putative RDases and other genes associated with reductive dehalogenation, the applicability of the identified target genes as biomarkers for the in situ monitoring of reductive dechlorination needs to be carefully evaluated. We have shown that continuous-flow columns represent assessable model systems to study the complex interplay of transformation rates, organism distribution, and activity under environmentally relevant conditions.

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