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Quantitative PCR Confirms Purity of Strain GT, a Novel Trichloroethene-to-Ethene-Respiring *Dehalococcoides* Isolate

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A novel *Dehalococcoides* isolate capable of metabolic trichloroethene (TCE)-to-ethene reductive dechlorination was obtained from contaminated aquifer material. Growth studies and 16S rRNA gene-targeted analyses suggested culture purity; however, the careful quantitative analysis of *Dehalococcoides* 16S rRNA gene and chloroethene reductive dehalogenase gene (i.e., *vcrA*, *tceA*, and *bvcA*) copy numbers revealed that the culture consisted of multiple, distinct *Dehalococcoides* organisms. Subsequent transfers, along with quantitative PCR monitoring, yielded isolate GT, possessing only *vcrA*. These findings suggest that commonly used qualitative 16S rRNA gene-based procedures are insufficient to verify purity of *Dehalococcoides* cultures. Phylogenetic analysis revealed that strain GT is affiliated with the Pinellas group of the *Dehalococcoides* cluster and shares 100% 16S rRNA gene sequence identity with two other *Dehalococcoides* isolates, strain FL2 and strain CBDB1. The new isolate is distinct, as it respires the priority pollutants TCE, *cis*-1,2-dichloroethene (*cis*-DCE), 1,1-dichloroethene (1,1-DCE), and vinyl chloride (VC), thereby producing innocuous ethene and inorganic chloride. Strain GT dechlorinated TCE, *cis*-DCE, 1,1-DCE, and VC to ethene at rates up to 40, 41, 62, and 127 μmol liter^{-1} day^{-1}, respectively, but failed to dechlorinate PCE. Hydrogen was the required electron donor, which was depleted to a consumption threshold concentration of 0.76 ± 0.13 nM with VC as the electron acceptor. In contrast to the known TCE dechlorinating isolates, strain GT dechlorinated TCE to ethene with very little formation of chlorinated intermediates, suggesting that this type of organism avoids the commonly observed accumulation of *cis*-DCE and VC during TCE-to-ethene dechlorination.

Chlorinated ethenes are pervasive groundwater contaminants resulting from extensive usage, improper disposal, and accidental spills, and the incomplete microbial dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) leads to dichloroethene and vinyl chloride (VG) accumulation. A breakthrough in the anaerobic treatment of chloroethene-contaminated sites was the discovery of bacteria that use chloroorganic compounds as electron acceptors to drive their energy metabolism. This metabolic reductive dechlorination process, also known as (de)chlororespiration, is a focus of current bioremediation approaches to contain or remediate chloroethene plumes.

Numerous bacterial isolates that reductively dechlorinate chloroethenes have been described previously (26); however, no single organism has the ability to couple energy generation with each reductive dechlorination step leading from PCE to ethene. The majority of isolates dechlorinate PCE to *cis*-1,2-dichloroethene (*cis*-DCE), and the ability to respire PCE to *cis*-DCE is distributed among phylogenetic groups and includes members of the classes *Deltaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes* (14). Bacteria with the ability for incomplete dechlorination of PCE to *cis*-DCE are often present at contaminated sites, and this transformation to *cis*-DCE may be achieved by biostimulation (9, 13). In contrast, reductive dechlorination past DCE has been linked exclusively to members of the *Dehalococcoides* cluster, a deeply branching group within the phylum *Chloroflexi* (green non-sulfur bacteria) (20).

Driving the reductive dechlorination process to completion (i.e., formation of ethene) is critical to achieving detoxification, and hence the *Dehalococcoides* group receives considerable attention from the bioremediation community (5, 13, 18). *Dehalococcoides ethenogenes* strain 195 was the first *Dehalococcoides* isolate described to dechlorinate PCE to ethene (20); however, careful investigations demonstrated that strain 195 failed to grow with VC and that the final dechlorination step from VC to ethene was cometabolic and required the presence of a polychlorinated ethene to avoid VC accumulation (19). *Dehalococcoides* sp. strain BAV1 was the first isolate capable of coupling growth to VC reductive dechlorination, which was a relevant observation suggesting that efficient chloroethene dechlorination without VC stall is feasible (6, 7). Strain BAV1 respired all DCE isomers and VC as electron acceptors and cometabolized PCE and TCE in the presence of a growth-supporting DCE isomer or VC (7). Another *Dehalococcoides* isolate, strain VS, also grew with VC (21), and isolate FL2 dechlorinated PCE to ethene, though the PCE-to-TCE and VC-to-ethene steps were cometabolic and required the presence of a growth-supporting electron acceptor (i.e., TCE, *cis*-DCE, or *trans*-1,2-dichloroethene [*trans*-DCE]) (8). The known *Dehalococcoides* strains implicated in chloroethene reductive dechlorination share highly similar 16S rRNA genes (1, 4, 7, 8, 21). In fact, strain BAV1, a VC respirer, strain FL2, an organism that cometabolizes VC, and strain CBDB1, an isolate that cannot grow with chloroethenes (2), share 16S rRNA gene

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sequences with greater than 99.9% identity. Hence, gene targets that provide higher resolution than the 16S rRNA gene are being sought for site assessment and bioremediation monitoring. Three such gene targets have been identified: *tceA*, encoding a TCE reductive dehalogenase (RDase) in strain 195 and strain FL2; *vcrA*, encoding a VC RDase in strain VS; and *bvcA*, encoding a VC RDase in strain BAV1 (12, 17, 21).

Here, we describe a novel *Dehalococcoides* isolate that uses TCE, *cis*-DCE, 1,1-dichloroethene (1,1-DCE), and VC as metabolic electron acceptors and forms negligible amounts of toxic intermediates during TCE dechlorination. These characteristics are desirable in bioremediation applications and expand our understanding of the diversity of metabolic capabilities within the *Dehalococcoides* group. Further, the combined application of qualitative and quantitative 16S rRNA gene- and RDase gene-targeted approaches demonstrated that commonly used 16S rRNA gene-based techniques are insufficient to verify *Dehalococcoides* culture purity.

MATERIALS AND METHODS

Chemicals. PCE and TCE were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other liquid chlorinated organic compounds were obtained from Supelco Co. (Bellefonte, PA). Gaseous VC was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and ethene was purchased from Scott Specialty Gases (Durham, NC). Fluorinated ethenes were purchased from SynQuest Laboratories, Inc. (Alachua, FL). All of the other chemicals used were reagent grade or higher unless otherwise specified. DNA extraction kits were purchased from QIAGEN (Valencia, CA) and Bio-Rad (Hercules, CA). *Taq* DNA polymerase and PCR buffer were from Applied Biosystems (Foster City, CA), and bovine serum albumin and restriction endonucleases were from Promega Biosciences, Inc. (San Luis Obispo, CA). The oligonucleotide primers for PCR were purchased from Integrated DNA Technologies (Coralville, IA).

Microcosms, enrichment, and isolation. Aquifer material from a chloroethene-contaminated site (Hydrite Chemical Co., Cottage Grove, WI) was collected by direct-push technology (Geoprobe, Salina, KS) as described previously (13). The cores were capped immediately to avoid air exposure, shipped to the laboratory on blue ice, and transferred to a glove box (96% N_2-4% H_2, vol/vol) for microcosm setup. The aquifer material was extruded into sterile, 1-liter Mason jars and homogenized. Approximately 2 g (wet weight) of aquifer material was transferred to 24-ml glass vials containing 10 ml of mineral salt medium amended with lactate (5 mM) and received 0.5 μl of neat TCE. Sequential transfers (1 to 2%, vol/vol) from TCE-to-ethene-dechlorinating microcosms to fresh medium yielded a sediment-free, nonmethanogenic, ethene-producing enrichment culture. The dechlorinating culture was maintained and transferred in 160-ml glass serum bottles containing 100 ml mineral salt medium (28) amended with 5 mM acetate plus H_2-CO_2 (80%/20%, vol/vol) headspace and 0.32 mM TCE (5 μl TCE dissolved in 200 μl hexadecane) as the electron acceptor for more than 3 years (approximately 35 transfers). Routinely, L-cysteine (0.2 mM), $Na_2S \cdot 9H_2O$ (0.2 mM), and DL-dithiothreitol (0.5 mM) were used as reductants. Five consecutive transfers received 1 mg/ml of ampicillin before five repeated dilution-to-extinction series in 24-ml vials amended with 0.5 μl of neat TCE were performed. Following this treatment, six additional transfers (0.5%, vol/vol) to VC (0.55 mM aqueous concentration) amended medium occurred, followed by three transfers (0.5%, vol/vol) to TCE (0.32 mM aqueous concentration, diluted in 0.1 ml hexadecane) amended medium in 160-ml serum bottles.

Determination of substrate range. The following compounds were tested as electron acceptors in medium amended with acetate (5 mM) as a carbon source and hydrogen (7.7×10^4 Pa or 0.61 mM) as the electron donor (aqueous concentrations are given in parentheses): PCE (0.33 mM); *cis*-DCE (0.32 mM); *trans*-DCE (0.21 mM); 1,1-DCE (0.19 mM); VC (0.19 to 0.55 mM); monochloroethane (0.1 mM); 1,1-dichloroethane (0.1 mM); 1,2-dichloroethane (0.1 mM); 1,1,1-trichloroethane (0.1 mM); 1,1,2-trichloroethane (0.1 mM); carbon tetrachloride (0.1 mM); 1,2-dichloropropane (0.1 mM); vinyl bromide (0.1 mM); 1,1-dichloro-2,2-difluoroethene (0.1 mM); 1,2-dichloro-1,2-difluoroethene (0.1 mM); 2-chloro-1,1-difluoroethene (0.1 mM); 1,1-difluoroethene (0.1 mM); chlorotrifluoroethene (0.1 mM); trichlorofluoroethene (0.1 mM); sulfate (0.1 to 5 mM); fumarate (1 to 5 mM); nitrate (0.1 to 5 mM); and ferric citrate (5 mM). The inoculum (3%, vol/vol) was transferred from TCE dechlorinating cultures that had consumed all TCE. Liquid chloroethenes (PCE, TCE, *cis*-DCE, *trans*-DCE, and 1,1-DCE) were diluted in 0.1 ml hexadecane. All other halogenated compounds, including gaseous halogenated compounds, were added undiluted by use of gas-tight syringes. Nonhalogenated compounds were added from aqueous, anoxic, neutralized, sterilized stock solutions by syringe. All additions were made prior to inoculation. Growth of strain GT in reduced anaerobic complex media, including full- or half-strength tryptic soy broth and R2A broth, was also explored after addition of 3% (vol/vol) inocula from TCE or VC dechlorinating cultures.

To test the range of electron donors supporting TCE, *cis*-DCE, 1,1-DCE, or VC reductive dechlorination, glucose (2 mM), lactate (5 mM), pyruvate (5 mM), formate (5 mM), or yeast extract (0.01 g/liter, wt/vol) was added to 100 ml of medium with TCE (0.32 mM), *cis*-DCE (0.32 mM), 1,1-DCE (0.19 mM), or VC (0.19 mM) as the electron acceptor. The inoculum (3%, vol/vol) was transferred from TCE dechlorinating cultures that had consumed all hydrogen. The electron donors were added from aqueous, anoxic, neutralized, sterilized stock solutions by syringe before inoculation. Hydrogen consumption threshold concentrations were determined by use of cultures with excess VC. Once the hydrogen concentration was stable over at least 3 weeks, 6.3 μM of hydrogen was added and its consumption to a constant threshold concentration was monitored again (16). Duplicate cultures were established for each substrate. The culture vessels were sealed with black butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK) and incubated upside down at room temperature in the dark without shaking, unless indicated otherwise.

16S rRNA gene analysis and detection of RDase genes. Total genomic DNA was extracted from actively dechlorinating cultures by use of a QIAamp DNA Mini kit (QIAGEN, Valencia, CA) or Insta Gene Matrix (Bio-Rad, Hercules, CA). Nearly complete 16S rRNA gene sequences were amplified with genomic DNA obtained from actively TCE, *cis*-DCE, 1,1-DCE, or VC dechlorinating cultures by using the universal bacterial primer pair (8F and 1541R) and PCR conditions described previously (15). PCR-amplified 16S rRNA gene products generated with DNA from TCE and VC dechlorinating cultures were digested for 3 h with the restriction enzymes HhaI, MspI, and RsaI at 37°C, as described previously (24). Fragments were resolved by electrophoresis for 1 h on 2.5% agarose gels (Invitrogen, Carlsbad, CA). For terminal restriction fragment length polymorphism (T-RFLP) analysis, genomic DNA was obtained from VC dechlorinating cultures, and 16S rRNA genes were amplified with hexachlorofluorescein (HEX)-labeled primer 8F-hex (5′-AGA GTT TGA TCC TGG CTC AG-3′) and unlabeled primer 1541R (24). Fluorescently labeled terminal fragments were obtained by digesting the PCR products with HhaI, MspI, and RsaI and analyzed at the High Throughput Sequencing and Genotyping Unit, University of Illinois, Urbana-Champaign. PCR denaturing gradient gel electrophoresis (DGGE) analyses were performed by Microbial Insights (Rockford, TN) with bacterial primers 27F and 519R (22) and *Dehalococcoides*-specific primers 1F-GC and 259R, as described previously (4). All 16S rRNA gene-based analyses were conducted with genomic DNA from actively dechlorinating cultures.

The presence of chloroethene RDase genes characterized for *Dehalococcoides* (i.e., *tceA*, *bvcA*, and *vcrA*) was tested with gene-specific primers, as described previously (12, 17, 21). For increased detection sensitivity, an initial amplification was performed with degenerate primers RRF2 and B1R (12), followed by a second round of PCR (nested PCR) with *bvcA*- or *tceA*-specific primers. Direct PCR with primers vcrAf and vcrAr (21) was used to amplify a 441-bp *vcrA* fragment from TCE and VC dechlorinating GT cultures. The amplicons were sequenced with primers vcrAf and vcrAr (21). Controls included genomic DNA from the following pure cultures: *Dehalococcoides* sp. strains FL2 and BAV1 (7, 8), *Desulfuromonas michiganensis* strain BB1 (28), *Dehalobacter restrictus* (10), and strain SZ, a PCE–to–*cis*-DCE–dechlorinating *Geobacter* sp. isolate (27).

Dechlorination rate measurements. Triplicate culture vessels with fresh medium were amended (aqueous concentrations are given in parentheses) with TCE (0.32 mM), *cis*-DCE (0.53 mM), 1,1-DCE (0.33 mM), and VC (0.55 mM) and received 3% inocula from a TCE-grown culture. For each electron acceptor, dechlorination rates were estimated from the linear portion of the plotted degradation data.

qPCR. Total numbers of bacterial and *Dehalococcoides* 16S rRNA genes, as well as *tceA*, *bvcA*, and *vcrA* genes, were quantified using quantitative real-time PCR (qPCR), as described previously (6, 7, 23). qPCR was performed with a spectrofluorimetric thermal cycler (ABI Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA). A calibration curve (log DNA concentration versus a set cycle threshold value) was obtained using 10-fold serial dilutions of pure culture genomic DNA or plasmid DNA carrying either a cloned *Dehalococcoides* 16S rRNA gene or *bvcA*, *tceA*, or *vcrA* of *Dehalococcoides* sp. strain

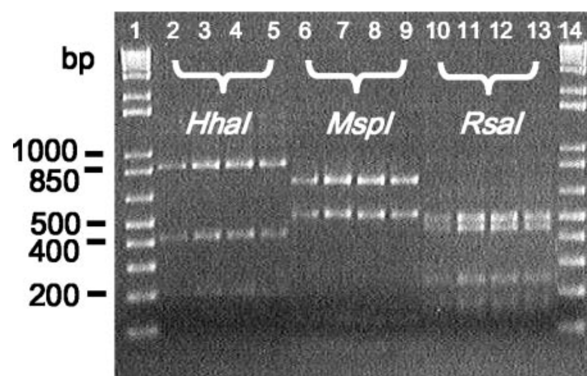

FIG. 1. RFLP analysis of *Dehalococcoides* 16S rRNA gene amplicons generated with a universal bacterial primer pair. Lanes 1 and 14, ladder; lanes 2, 6, and 10, TCE-grown GT culture; lanes 3, 7, and 11, VC-grown GT culture; lanes 4, 8, and 12, VC-grown BAV1 culture; lanes 5, 9, and 13, TCE-grown FL2 culture.

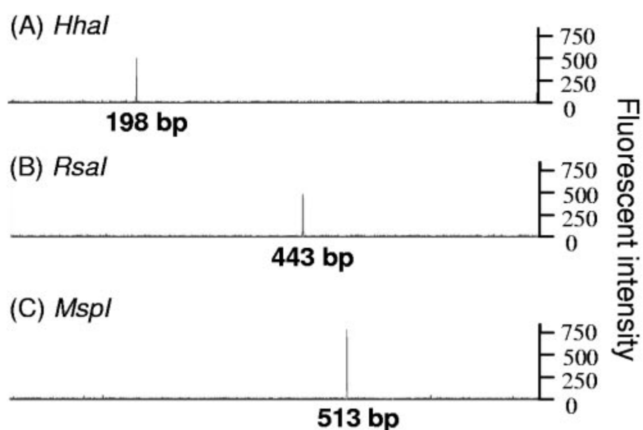

FIG. 2. T-RFLP profiles after digestion of the 16S rRNA gene amplicons obtained with genomic DNA from strain GT grown with VC. HhaI, RsaI, and MspI digests reveal peaks expected for Pinellas group members.

BAV1, strain FL2, or strain GT, respectively. Standard curves spanned a range of 10 to 10^8 gene copies per μl of template DNA.

Growth yield measurements. Growth of isolate GT on TCE, 1,1-DCE, and VC was monitored using qPCR. Total 16S rRNA gene and *vcrA* gene copy numbers were quantified from duplicate TCE and 1,1-DCE and triplicate VC dechlorinating GT cultures. At the time of DNA extraction, ethene was the major dechlorination product (>95%) in all cultures.

Microscopy. Cells grown on TCE were used to obtain scanning electron micrographs by use of procedures and instrumentation previously described (7).

Analytical techniques. Chloroethenes, chloroethanes, and fluorinated ethenes were quantified with a Hewlett-Packard model 6890 gas chromatograph equipped with an HP-624 column (60-m length, 0.32-mm diameter, 1.8-μm film thickness) and a flame ionization detector. Headspace samples (100 μl) were withdrawn using gas-tight, 250-μl glass syringes with gas-tight Teflon valves and Luer Lock adapters (model 1725; Hamilton Co., Reno, NV) and manually injected into a split injector operated at a split ratio of 2:1. To maintain a constant pressure in the culture bottles, 100 μl of sterile N_2 was injected prior to withdrawal of the samples. Chloroethene concentrations are reported as total mass per 160-ml serum bottle, unless indicated otherwise. Chloride release was calculated based on the gas chromatographic chloroethene/ethene concentration measurements, and it was assumed that each reductive dechlorination step liberates one chlorine substituent as chloride. Volatile fatty acids and hydrogen concentrations were quantified by high-performance liquid chromatography and a reduction gas analyzer, respectively, as described previously (16, 28).

Nucleotide sequence accession number. The nearly complete 16S rRNA gene sequence (1,299 bp) of strain GT was submitted to GenBank (accession no. AY914178).

RESULTS

Isolation of *Dehalococcoides* sp. strain GT. A TCE-to-ethene-dechlorinating enrichment culture was obtained from a TCE-fed, ethene-producing microcosm by use of sequential transfers to medium amended with acetate, hydrogen, and TCE. Following repeated transfers in the presence of ampicillin, microscopic analysis revealed a homogeneous culture consisting of small cells (<1 μm in diameter) with a disk-shaped morphology characteristic of *Dehalococcoides*. Amplicons generated with universal bacterial primers and genomic DNA from TCE and VC dechlorinating cultures as the template yielded identical restriction patterns with all restriction enzymes tested (Fig. 1). T-RFLP analysis confirmed the RFLP results and yielded single peaks of 198, 443, and 513 bp (the sizes predicted from in silico analyses) following digestion with HhaI, RsaI, and MspI, respectively (Fig. 2). DGGE analysis with universal primers 27F and 519R yielded a single band, as is expected for a pure culture. Further, DGGE analysis with the *Dehalococcoides*-specific primers 1F-GC and 259R yielded a single band indistinguishable from the band generated with *Dehalococcoides* sp. strain FL2 genomic DNA (Fig. 3). *Dehalococcoides* sp. strain BAV1 DNA, which was included in the analysis with the *Dehalococcoides*-specific primers, yielded a band with different migration properties. The results shown in Fig. 3 suggest that the amplicon contributed by the new *Dehalococcoides* isolate shared an identical 16S rRNA gene sequence with strain FL2 but differed from that of strain BAV1

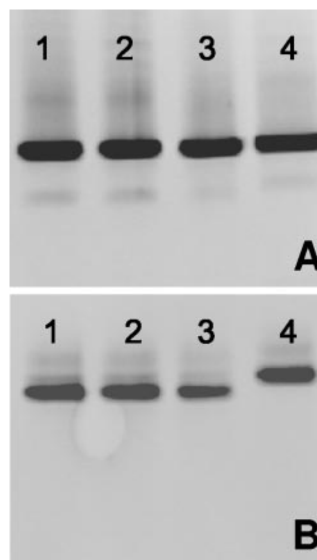

FIG. 3. DGGE. DNA was PCR amplified with a universal bacterial primer pair (A) and a *Dehalococcoides*-specific primer pair (B). Amplified PCR fragments were separated on 8 to 10% acrylamide gels with 30 to 65% (universal bacterial primer) or 45 to 65% (*Dehalococcoides*-specific primers) denaturant. Lane 1, TCE-grown GT culture; lane 2, VC-grown GT culture; lane 3, strain FL2; lane 4, strain BAV1.

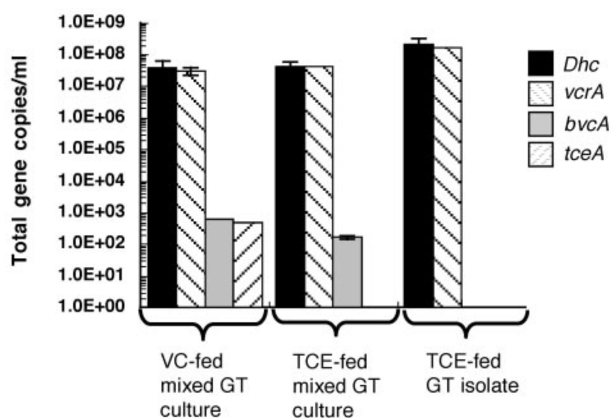

FIG. 4. Monitoring the enrichment process of strain GT under different growth conditions with 16S rRNA gene- and RDase gene-targeted qPCR. Ethene was the major dechlorination product (>90%) in all dechlorinating cultures at the time of DNA extraction. The qPCR data represent at least five independent analyses. Error bars indicate standard deviations and are not shown when they are too small to depict. Dhc, *Dehalococcoides*.

over the 259-bp stretch analyzed with DGGE. The analysis of large 16S rRNA gene fragments (1,299 bp positions analyzed) amplified from TCE-, *cis*-DCE-, 1,1-DCE-, and VC-grown GT cultures yielded identical sequences. Subsequent sequence alignments demonstrated that the sequence of the novel TCE-to-ethene-dechlorinating isolate GT shared an identical 16S rRNA gene sequence with strain FL2 but differed from that of strain BAV1 by 1 bp at position 136 (BAV1 numbering, GenBank accession number AY165308).

Culture-based approaches, microscopic analysis, and 16S rRNA gene-based analyses all suggested culture purity. To further characterize the culture and corroborate culture purity, qPCR analysis using *Bacteria* and *Dehalococcoides* 16S rRNA gene- and RDase gene-targeted primers was performed. The total bacterial cell numbers in TCE- or VC-grown GT cultures (2.26×10^7 to 1.18×10^8 16S rRNA gene copies per ml) almost equaled the total *Dehalococcoides* cell numbers (3.46×10^7 to 1.26×10^8 16S rRNA gene copies per ml), suggesting that all cells in this culture were *Dehalococcoides*. Almost-equal numbers of *vcrA* gene copies were enumerated, indicating that the *Dehalococcoides* cells in the culture carry this gene. Surprisingly, the *bvcA* and *tceA* genes were also quantifiable in this culture, though at much lower numbers, ranging from 4.9×10^2 to 6.1×10^2 gene copies per ml of culture fluid (Fig. 4, left set of columns). The qPCR data suggested that the culture consisted solely of *Dehalococcoides* cells, but the culture was composed of multiple *Dehalococcoides* strains. Apparently, these different strains harbored identical 16S rRNA gene sequences and could not be resolved by 16S rRNA gene-based approaches. The strategy to further purify the dominating *Dehalococcoides* organism bearing the *vcrA* gene involved transfers with VC as the electron acceptor in an attempt to eliminate the strain carrying *tceA*. The middle set of columns in Fig. 4 shows the qPCR results of a culture following six subsequent transfers with VC. *tceA* was no longer detectable, though *bvcA* was still quantifiable. Hence, the culture was fed TCE again, and transfers occurred immediately after the onset of TCE dechlorination. Following three consecutive transfers, qPCR analysis failed to detect *bvcA* and *tceA*, and the total cell numbers inferred from the quantification of bacterial 16S rRNA genes, *Dehalococcoides* 16S rRNA genes, and the *vcrA* gene suggested that a pure culture consisting of a single *Dehalococcoides* organism was obtained (Fig. 4, right set of columns). PCR with RDase gene-targeted primer pair RRF2 and B1R yielded amplicons of the expected sizes (1,500 to 1,700 bp), and nested PCR with *tceA*- and *bvcA*-specific primers did not yield detectable amplicons, whereas *vcrA* was detected by direct PCR with a *vcrA*-targeted primer pair. The combined application of culture-based procedures, qualitative PCR approaches, and qPCR verified culture purity. The isolate was designated *Dehalococcoides* sp. strain GT (for Georgia Tech).

Morphological and physiological characteristics of strain GT. Figure 5 shows scanning electron micrographs of strain GT. Many cells exhibited the disk-shaped morphology observed for other *Dehalococcoides* organisms (7, 20), though cells with a potato-like shape were also seen. The disk-shaped cells had diameters ranging from 0.7 to 1.2 μm and a thickness of about 0.2 to 0.6 μm. Thicker cells, which may represent a predivision stage, were typically spherical or oval (potato-shaped), with diameters of 1.1 to 1.5 μm. Different appendages were observed, including string-like extrusions (Fig. 5C) and short, thick connections between adjacent cells (Fig. 5A and B). Small, round blebs of about 50 to 100 nm in diameter were often observed in proximity to notches or attached to the cell's surface (Fig. 5D). All of the features shown in Fig. 5 were observed repeatedly with replicate samples.

Figure 6 shows the dechlorination of TCE (Fig. 6A), *cis*-DCE (Fig. 6B), and VC (Fig. 6C) to ethene with hydrogen as the electron donor and acetate as the carbon source. Dechlorination started after a lag time of 2 weeks, and differences in lag times with the various electron acceptors were not apparent. Similar lag times of 2 weeks were observed with both TCE-and VC-fed cultures when incubated at 22 or 30°C. Dechlorination occurred at 10°C, but only negligible dechlorination was observed at 35°C over a 3-month incubation period (data not shown). Only small amounts of *cis*-DCE (<19 μM) and VC (<22 μM) were transiently formed in TCE-amended cultures, whereas a considerable buildup of VC (up to 50% of the initial amount of *cis*-DCE added) occurred in *cis*-DCE-fed cultures. Under the conditions tested, TCE, *cis*-DCE, 1,1-DCE, and VC were dechlorinated to ethene at rates of up to 40, 41, 62, and 127 μmol/liter/day, respectively, and hydrogen was consumed to 0.98 ± 0.17 ppm by volume (0.76 ± 0.13 nM; $n = 3$). Replacing hydrogen with glucose, formate, lactate, pyruvate, or yeast extract as the electron donor did not lead to dechlorination of TCE, *cis*-DCE, 1,1-DCE, or VC. No dechlorination occurred with cultures lacking hydrogen or acetate, suggesting that strain GT is strictly hydrogenotrophic, cannot grow autotrophically, and uses acetate as a carbon source. TCE, *cis*-DCE, 1,1-DCE, and VC were the only growth-supporting electron acceptors identified and could not be replaced with PCE; *trans*-DCE; monochloroethane; 1,1-dichloroethane; 1,2-dichloroethane; 1,1,1-trichloroethane; 1,1,2-trichloroethane; carbon tetrachloride; 1,2-dichloropropane; vinyl bromide; 1,1-dichloro-2,2-difluoroethene; 1,2-dichloro-1,2-difluoroethene; 2-chloro-1,1-difluoroethene; 1,1-difluoroethene; chlorotrifluoroethene; trichlorofluoroethene; sulfate; fumarate; nitrate; or ferric citrate. Strain GT failed to dechlorinate PCE,

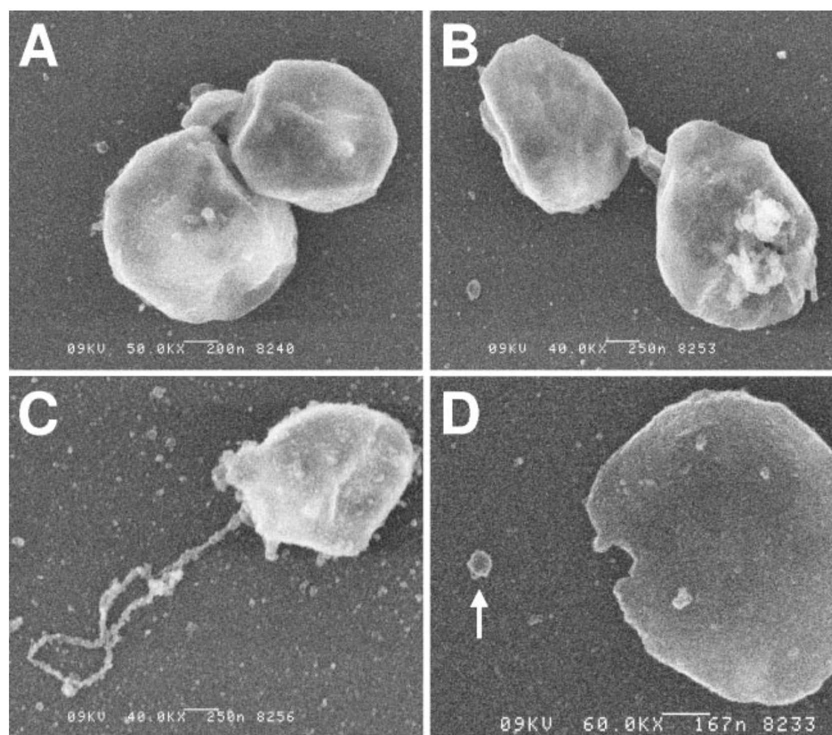

FIG. 5. Scanning electron micrographs of *Dehalococcoides* sp. strain GT. Flattened, potato-shaped cells (A) and two apparently connected cells (B) can be seen. Panel C shows a single cell with an appendage of unknown function. Panel D depicts a bacterial cell with a notch and a bleb (arrow) that may represent a cell extrusion.

even when PCE was added to actively TCE, *cis*-DCE, or VC dechlorinating cultures. The addition of ampicillin to the culture medium did not prohibit the dechlorination of TCE, *cis*-DCE, 1,1-DCE, or VC to ethene. No growth occurred in half- or full-strength complex media over a 6-month incubation period. A doubling time of 2 to 2.5 days was estimated from the linear portion of a semilogarithmic plot of the qPCR growth curve (i.e., during the exponential growth phase).

Detection of *vcrA* in isolate GT. The *vcrA* gene implicated in VC dechlorination in strain VS (21) was detected in strain GT. Amplification of genomic DNA extracted from TCE-, *cis*-DCE-, and VC-grown GT cultures with *vcrA*-specific primers yielded amplicons of the expected size. Sequence analysis confirmed identity to the strain VS *vcrA* gene over the 379-bp stretch analyzed. BioDechlor INOCULUM is a commercially available PCE-to-ethene-dechlorinating consortium that contains multiple *Dehalococcoides* organisms, including strains FL2, BAV1, and GT (23, 25). *tceA*, *bvcA*, and *vcrA* were readily detected in the TCE-grown BDI consortium, indicating that all three RDase genes coexist in the same culture (data not shown).

Growth-linked chloroethene dechlorination and yields. *Dehalococcoides* 16S rRNA gene- and *vcrA* gene-targeted qPCR verified growth of strain GT with TCE, *cis*-DCE, 1,1-DCE, or VC as the electron acceptor. Figure 7 demonstrates that dechlorination of VC to ethene was coupled to an increase in *vcrA* gene copies. Following the consumption of 40 $\pm$ 1.16 μmol VC, the *vcrA* gene and 16S rRNA gene (not shown) copy numbers increased to $1.0 \times 10^8 \pm 0.13 \times 10^8$ and $9.7 \times 10^7 \pm 0.12 \times 10^7$ per ml of culture fluid, respectively. In cultures not provided with VC as an electron acceptor, the cell numbers increased insignificantly, from $4.3 \times 10^6 \pm 1.2 \times 10^6$ (i.e., cells introduced with the inoculum) to $5.4 \times 10^6 \pm 2.1 \times 10^6$. qPCR with strain GT genomic DNA and plasmid DNA containing single copies of strain GT's *vcrA* gene or 16S rRNA gene suggested that both genes occur as single copies on the genome (23). Based on the *vcrA* gene and 16S rRNA gene copy number increase in VC-amended cultures, cell yields of $2.4 \times 10^8 \pm 0.24 \times 10^8$ (average $\pm$ standard deviation, $n = 3$) and $2.5 \times 10^8 \pm 0.13 \times 10^8$ ($n = 3$) cells per μmol of VC dechlorinated to ethene were calculated. Cultures grown with TCE yielded $9.3 \times 10^8 \pm 0.72 \times 10^8$ ($n = 2$) cells per μmol of TCE dechlorinated to ethene. The cell yield with TCE was over three times greater than the yield with VC, indicating that strain GT captures energy from all three dechlorination steps. No increase in *Dehalococcoides* 16S rRNA gene copy number was observed with cultures grown under the same conditions without TCE.

DISCUSSION

A novel TCE-to-ethene-dechlorinating *Dehalococcoides* species, strain GT, was isolated from chloroethene-impacted aquifer material. Similarly to other *Dehalococcoides* isolates, strain GT has a highly restricted metabolism and requires hydrogen as an electron donor and a chloroorganic compound (i.e., TCE, *cis*-DCE, 1,1-DCE, or VC) as an electron acceptor. Strain GT is affiliated with the Pinellas group of the *Dehalococcoides* cluster but exhibits physiological differences with regard to electron acceptor utilization and dechlorination. Table 1 compiles the

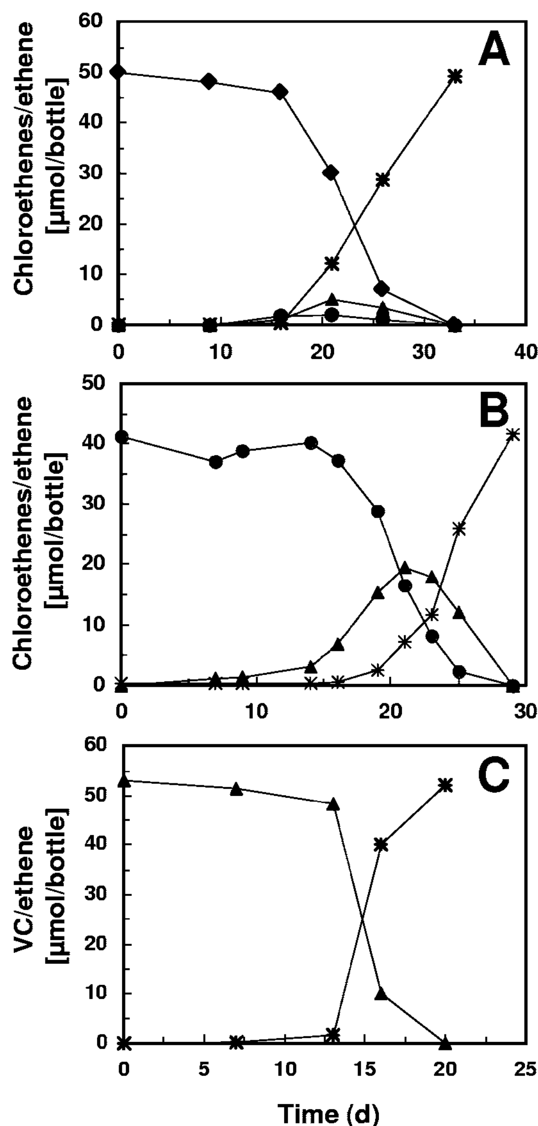

FIG. 6. Reductive dechlorination of TCE (A), *cis*-DCE (B), and VC (C) to ethene by strain GT. Hydrogen was provided as the electron donor and acetate as the carbon source. All data were averaged from triplicate cultures. Symbols: ◆, TCE; ●, *cis*-DCE; ▲, VC; *, ethene. Time is expressed in days (d).

chloroethenes that are metabolically and cometabolically dechlorinated by described *Dehalococcoides* strains. Importantly, strain GT possesses a TCE-to-ethene dechlorination pathway in which each dechlorination step is linked to growth. Strain GT dechlorinated VC at a rate about threefold faster than that for TCE and *cis*-DCE, which led to very little VC accumulation in TCE-grown cultures. Thus, a single organism is capable of efficiently detoxifying the common environmental pollutant TCE to environmentally benign ethene and inorganic chloride. Dechlorination activities similar to that of strain GT have been described for *Dehalococcoides* organisms detected in consortium KB-1 (4) and culture VS (A. Spormann, personal communication), suggesting that this physiology is distributed among the *Dehalococcoides* strains. Of interest is the apparent lack of *tceA* in isolate GT, implying that this strain has a

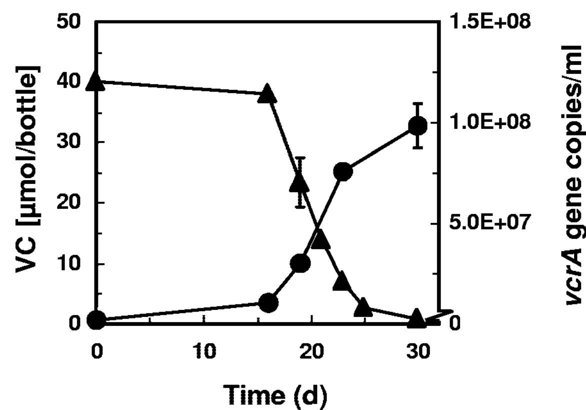

FIG. 7. Increase in *vcrA* gene copies, as determined by PCR during the reductive dechlorination of VC to ethene by culture GT. Data points were averaged from triplicate cultures, and error bars represent standard deviations. Symbols: ●, *vcrA*; ▲, VC. Time is expressed in days (d).

different TCE RDase. Since the sequence diversity of *tceA* genes is currently unknown, it is possible that strain GT possesses a variant *tceA* gene that was not amplified with the PCR primers used in this study. An observation supporting the presence of a novel TCE RDase in strain GT is this organism's inability to cometabolize PCE. Strain FL2, another *Dehalococcoides* isolate that cannot derive energy from PCE dechlorination, dechlorinates PCE in a cometabolic reaction attributed to TceA (8).

The presence of *vcrA* in strain GT suggests that identical genes are shared between members of the Victoria and Pinellas groups. Similarly, the *tceA* gene, which was originally detected in *Dehalococcoides ethenogenes* strain 195 of the Cornell group, was also detected in strain FL2, a member of the Pinellas group (8, 17). On the other hand, isolates that share the 16S rRNA gene signature sequences of the Pinellas group respire different chlorinated substrates. For instance, strains GT and BAV1 respire chlorinated ethenes, whereas strain CBDB1 does not (2, 7). A recent study by Hölscher et al. (11) demonstrated that highly similar RDase genes are shared among the *Dehalococcoides* strains and that unique RDase genes that distinguish different *Dehalococcoides* strains exist. Dividing the *Dehalococcoides* cluster into the Victoria, Pinellas, and Cornell groups was originally suggested by Hendrickson et al. (9) and is based on 16S rRNA gene sequence differences; however, with the accumulated physiological information it becomes apparent that this grouping does not reflect the physiological properties of its members.

Growth yields of *Dehalococcoides* organisms on VC have been determined with qPCR approaches because their fastidious growth, small cell size, and disk-shaped morphology impair traditional procedures (e.g., microscopic counts, protein measurements, determining dry weight, etc.) to estimate biomass. Recently, Duhamel et al. (4) compared growth yields determined with qPCRs of different *Dehalococcoides* organisms grown with VC. The growth yield of strain GT agrees with the values obtained for strain KB-1/VC and strain VS. The application of different DNA extraction procedures from cultures of strain GT demonstrated that the growth yield esti-

TABLE 1. Chloroethene utilization by *Dehalococcoides* isolates

Dehalococcoides sp. strain	Metabolic electron acceptors	Chloroethene(s) cometabolized	Major end product(s)	Group[a]	Reference(s) or source
195	PCE, TCE, *cis*-DCE, 1,1-DCE	VC	VC, ethene	C	20
BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC	PCE, TCE	Ethene	P	7
FL2	TCE, *cis*-DCE, *trans*-DCE	PCE, VC	VC, ethene	P	8
VS[b]	TCE, *cis*-DCE, 1,1-DCE, VC	ND[c]	Ethene	V	3, 21; A. Spormann, personal communication
CBDB1	PCE, TCE	ND	*trans*-DCE	P	1; L. Adrian, personal communication
KB-1/VC[b]	TCE, *cis*-DCE, VC	ND	Ethene	P	4
GT	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	P	This study

[a] Group designations are according to Hendrickson et al. and are based on 16S rRNA gene sequence differences (9). C, Cornell; V, Victoria; P, Pinellas.
[b] Characterized in mixed culture.
[c] ND, not determined.

mates obtained with the qPCR approach can vary by up to 1 order of magnitude. For instance, the DNA extraction protocol applied to estimate the 16S rRNA gene copy numbers of isolate BAV1 (7) consistently yielded approximately 10-fold-lower values than the method used in this study. Hence, comparisons of *Dehalococcoides* growth yield data obtained in different laboratories by use of different DNA extraction protocols must be interpreted cautiously.

A relevant finding from this study is that 16S rRNA gene-based analyses, even when qPCR approaches are used, are not sufficient to prove the purity of a *Dehalococcoides* culture. Unfortunately, *Dehalococcoides* organisms are fastidious growers, and obtaining isolated colonies is very challenging or impossible with the current methodology. Hence, we rely largely on molecular tools to assess and verify culture purity. In our efforts to isolate strain GT, we derived a culture that contained a single Pinellas 16S rRNA gene sequence. Obviously, all 16S rRNA gene-based assays would detect a single sequence, thus suggesting culture purity; however, the quantitative assessment of RDase genes demonstrated that this culture contained three distinct *Dehalococcoides* strains, which obviously could not be distinguished by 16S rRNA gene analyses. Hence, a careful quantitative assessment of 16S rRNA gene copies and assorted RDase gene targets is recommended to verify purity of *Dehalococcoides* cultures. As qPCR is becoming standard technology in the microbiological laboratory, it seems practical to combine 16S rRNA gene- and functional-gene-targeted qPCR approaches to verify purity of cultures that resist clonal purification procedures.

BioDechlor INOCULUM has been used successfully for bioaugmentation applications (25) and contains multiple *Dehalococcoides* organisms, including strains FL2, BAV1, and GT. *tceA*, *vcrA*, and *bvcA* are stably maintained in this consortium, suggesting that multiple *Dehalococcoides* organisms carrying RDase genes with apparently redundant function (i.e., *vcrA* and *bvcA*) coexist. Similarly, two *Dehalococcoides* organisms are present in the chloroethene-dechlorinating KB-1 consortium, and both *vcrA* and *bvcA* were detected (12, 21). More-detailed studies exploring the dynamics of different *Dehalococcoides* organisms and RDase gene expression under different growth conditions are needed to shed light on the strategies of the *Dehalococcoides* community to maintain diversity and metabolic redundancy. Strains GT, FL2, and BAV1 are now available as pure cultures to address these relevant ecological and practical questions.

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