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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 *brcA*) 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 (*cis*-1,2-DCE) and VC during TCE-to-ethene dechlorination. 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*, *Gammad proteobacteria*, 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 etenes 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% N2-4% H2, 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 TCE-utilizing, nonfluorescent, 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 H2CO3 (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, t-cysteine (0.2 mM), Na2S·9H2O (0.2 mM), and tr-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 × 104 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); 1,1,1-trichloroethane (0.1 mM); 1,1,2-trichloroethane (0.1 mM); carbon tetrachloride (0.1 mM); 1,1,1,2-tetrachloroethane (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); chloro trifluoroethene (0.1 mM); trichlorofluoroethene (0.1 mM); sulfite (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 yeast extract and anoxic soy broth, 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 (0.1% vol/vol) was added from TCE dechlorinating cultures that had consumed all hydrocarbon. 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 (Gilmont Technostat, 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 active TCE, cis-DCE, 1,1-DCE, or VC dechlorinating cultures by using the universal bacterial primer pair (5F and 1514R) 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 Hfhd, MspI, and Rsal 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), 441-bp PCR-amplified 16S rRNA genes were digested with hexachloro-fluorescein (HX)-labeled primer 5F-hex (5'-AGA GTT TGA TCC TGG CTC AG-3') and unlabeled primer 1514R (24). Fluorescently labeled terminal fragments were obtained by digesting the PCR products with Hfhd, MspI, and Rsal 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, IL) using primers D1031R and H11032 (22) and a DGGE Gel System (Bio-Rad) using Taq polymerase and 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 BIR (12), followed by a second round of PCR (nested PCR) with bvcA- or tceA-specific primers. Direct PCR with primers vcrA and vcrA (21) was used to amplify a 441-bp vcrA fragment from TCE and VC dechlorinating GT cultures. The amplicons were sequenced with primers vcrA and vcrA (21). Controls included genomic DNA from the following pure cultures: Dehalococcoides sp. strains FL2 and BAV1 (7, 8), Desulfuromonas michiganensis strain BBI (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 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 spectrophotometric 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 from Dehalococcoides sp. strain.
BAV1, strain FL2, or strain GT, respectively. Standard curves spanned a range of 10 to 10⁸ 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 N₂ 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, Rsal, 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.
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 x 10^7 to 1.18 x 10^8 16S rRNA gene copies per ml) almost equaled the total Dehalococcoides cell numbers (3.46 x 10^7 to 1.26 x 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 x 10^6 to 6.1 x 10^7 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 predifferentiation 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.
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 $4.3 \times 10^6 \pm 1.2 \times 10^6$ (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 ± 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 $3.3 \times 10^8 \pm 0.72 \times 10^8$ ($n = 2$) 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 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
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 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-
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 lab-
oratories 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 grow-
ners, and obtaining isolated colonies is very challenging or im-
possible 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 asserted RDase gene targets is recommended to verify purity of Deha-
lococcoides cultures. As qPCR is becoming standard technol-
ogy 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 puri-
ification procedures.

BioDechlor INOCULUM has been used successfully for bioaugmentation applications (25) and contains multiple De-
halococcoides organisms, including strains FL2, BAV1, and
GT. tceA, vcrA, and bvcA are stably maintained in this consor-
tium, suggesting that multiple Dehalococcoides organisms car-
rying RDase genes with apparently redundant function (i.e.,
vcrA and bvcA) coexist. Similarly, two Dehalococcoides organ-
isms are present in the chloroethene-dechlorinating KB-1 con-
sortium, and both vcrA and bvcA were detected (12, 21). More-detailed studies exploring the dynamics of different Deha-
lococcoides organisms and RDase gene expression under dif-
ferent 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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TABLE 1. Chloroethene utilization by Dehalococcoides isolates

<table>
<thead>
<tr>
<th>Dehalococcoides sp. strain</th>
<th>Metabolic electron acceptors</th>
<th>Chloroethene(s) cometabolized</th>
<th>Major end product(s)</th>
<th>Groupa</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>PCE, TCE, cis-DCE, 1,1-DCE</td>
<td>VC</td>
<td>VC</td>
<td>C</td>
<td>20</td>
</tr>
<tr>
<td>BAV1</td>
<td>cis-DCE, trans-DCE, 1,1-DCE, VC</td>
<td>PCE, TCE</td>
<td>Ethene</td>
<td>P</td>
<td>7</td>
</tr>
<tr>
<td>FL2</td>
<td>TCE, cis-DCE, trans-DCE</td>
<td>PCE, VC</td>
<td>VC, ethene</td>
<td>P</td>
<td>8</td>
</tr>
<tr>
<td>VSb</td>
<td>TCE, cis-DCE, 1,1-DCE, VC</td>
<td>ND</td>
<td>Ethene</td>
<td>V</td>
<td>3, 21; A. Spormann, personal communication</td>
</tr>
<tr>
<td>CBDB1</td>
<td>PCE, TCE</td>
<td>ND</td>
<td>trans-DCE</td>
<td>P</td>
<td>1; L. Adrian, personal communication</td>
</tr>
<tr>
<td>KB-1/VCb</td>
<td>TCE, cis-DCE, VC</td>
<td>ND</td>
<td>Ethene</td>
<td>P</td>
<td>4</td>
</tr>
<tr>
<td>GT</td>
<td>TCE, cis-DCE, 1,1-DCE, VC</td>
<td>None</td>
<td>Ethene</td>
<td>P</td>
<td>This study</td>
</tr>
</tbody>
</table>

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.


