APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2007, p. 4439–4445 Vol. 73, No. 14
0099-2240/07/$08.00+0 doi:10.1128/AEM.00215-07

Expression of Reductive Dehalogenase Genes in *Dehalococcoides ethenogenes* Strain 195 Growing on Tetrachloroethene, Trichloroethene, or 2,3-Dichlorophenol▽†

Jennifer M. Fung, Robert M. Morris,‡ Lorenz Adrian,§ and Stephen H. Zinder*

Department of Microbiology, Wing Hall, Cornell University, Ithaca, New York 14853

Received 26 January 2007/Accepted 15 May 2007

Reductive dehalogenase (RD) gene transcript levels in *Dehalococcoides ethenogenes* strain 195 were investigated using reverse transcriptase quantitative PCR during growth and reductive dechlorination of tetrachloroethene (PCE), trichloroethene (TCE), or 2,3-dichlorophenol (2,3-DCP). Cells grown with PCE or TCE had high transcript levels (greater than that for *rpoB*) for *tceA*, which encodes the TCE RD, *pceA*, which encodes the PCE RD, and DET0162, which contains a predicted stop codon and is considered nonfunctional. In cells grown with 2,3-DCP, *tceA* mRNA was less than 1% of that for *rpoB*, indicating that its transcription was regulated. *pceA* and DET0162 were the only RD genes with high transcript levels in cells grown with 2,3-DCP. Proteomic analysis of PCE-grown cells detected both PceA and TceA with high peptide coverage but not DET0162, and analysis of 2,3-DCP-grown cells detected PceA with high coverage but not TceA, DET0162, or any other potential RD. Cells grown with PCE or 2,3-DCP were tested for the ability to dechlorinate PCE, TCE, or 2,3-DCP with H_2 as the electron donor. 2,3-DCP-grown cells were unable to dechlorinate TCE but dechlorinated PCE to TCE without a lag, and PCE-grown cells dechlorinated 2,3-DCP without a lag. These results show that 2,3-DCP-grown cells do not produce TceA and that DET0162 is transcribed but its translation product is not detectable in cells and are consistent with PceA's being bifunctional, also serving as the 2,3-DCP RD. Chlorophenols naturally occur in soils and are good candidates for the original substrates for PceA.

Dehalococcoides ethenogenes strain 195 reductively dechlorinates tetrachloroethene (PCE) and trichloroethene (TCE) to vinyl chloride (VC) and ethene (21, 32). In addition to chlorinated ethenes, strain 195 has been found to reductively dechlorinate chlorobenzenes and other chloroaromatics (7) and more recently 2,3-dichlorophenol (2,3-DCP) and 2,3,4-trichlorophenol in the *ortho* position to 3-monochlorophenol or 3,4-dichlorophenol, respectively (1). The reduction of halogenated compounds by *Dehalococcoides* is carried out by membrane-bound respiratory reductive dehalogenases (RDs) (12, 19, 26, 27), and although more than 90 RD-homologous genes have been identified in this genus (11, 15, 26, 30), little is known about their specific functions. PCE-RD (PceA) and TCE-RD (TceA) were first characterized in mixed dechlorinating enrichment cultures containing strain 195 and were found to reductively dehalogenate PCE to TCE and TCE to VC and ethene, respectively (19). The gene encoding TCE-RD was subsequently cloned, sequenced, and designated *tceA* (18).

The genome sequence of strain 195 (30) revealed 17 RD-homologous genes in addition to *tceA* (designated DET0079) and *pceA* (designated DET0318; J. Magnuson, personal communication). Common features of RDs include the presence of a putative twin-arginine transport signal sequence used for transport into the periplasm of folded proteins that can contain prosthetic groups, iron-sulfur cluster-binding motifs, and an adjacent "B" RD gene predicted to encode a small hydrophobic protein proposed to serve as a membrane anchor. Two of the RD-homologous genes may not be functional. One (DET0162) contains a verified TGA stop codon that would truncate the predicted gene product from 488 to 59 amino acids and a shorter corresponding "B" gene (DET0163). DET0088 encodes a protein predicted to be 153 amino acids long, corresponding to the C termini of other RDs, and lacks a corresponding "B" gene. Sixteen of the 19 RD genes in *D. ethenogenes* have transcriptional regulator genes in close proximity, including *pceA*, suggesting they are transcriptionally regulated. The genome sequence of *Dehalococcoides* strain CBDB1 revealed 32 potential RD genes (15), 12 of which share up to 95.4% amino acid sequence identity with RDs from strain 195, suggesting a partial overlap of substrates. Multiple RD genes have been identified in *Dehalococcoides* strains FL-2, KB1 (mixed culture), and BAV1 (14, 14, and 10, respectively) (10, 35). Although sequenced *Dehalococcoides* genomes share high sequence similarity and synteny among "housekeeping" genes, isolates harbor different suites of RD genes and exhibit different dehalogenation spectra. Identifying which RD genes are expressed during dehalogenation of different substrates can provide insights into reductive dehalogenase function and their potential activities at contaminated sites.

Here we describe *D. ethenogenes* RD genes expressed during growth and the reductive dehalogenation of PCE, TCE, and 2,3-DCP by comparing RD gene expression and corresponding dehalogenation activities for strain 195. Expression and activity results indicated that *tceA* was under transcriptional control,

* Corresponding author. Mailing address: Department of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14840. Phone: (607) 255-2415. Fax: (607) 255-3904. E-mail: shz1@cornell.edu.

‡ Present address: Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, CA 93106.

§ Present address: FG Technische Biochemie, Technical University of Berlin, Berlin, Germany.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 May 2007.

and liquid chromatography-tandem mass spectrometry (LC/MS/MS) proteomic approaches identified TceA protein fragments only in the presence of PCE and TCE but not 2,3-DCP. *pceA* was the only intact RD gene expressed in cells grown with 2,3-DCP and is most likely the 2,3-DCP reductive dehalogenase, which suggests that PceA has broad substrate specificity.

MATERIALS AND METHODS

Chemicals. Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available, and gases were purchased from Airgas East (Elmira, NY).

Growth conditions. *D. ethenogenes* strain 195 was cultured with PCE, TCE, or 2,3-dichlorophenol as previously described (1, 20). Briefly, culture inoculum sizes were 2% (vol/vol) in either 27-ml culture tubes, 120-ml serum vials, or 1,000-ml incubation containers containing 10, 50, or 500 ml of growth medium, respectively. Basal salts medium was amended with 2 mM acetate, a vitamin solution containing 0.05 mg of vitamin B_{12} per liter, 10% (vol/vol) filter-sterilized anaerobic digestor sludge supernatant, and 1% (vol/vol) mixed butyrate-PCE culture extract (20). Doses of PCE, TCE, and H_2 were added as previously described (20), and a filter-sterilized stock solution of 2,3-DCP was added with a syringe at increasing doses of 30, 50, and 75 μM. Culture tubes were sealed with Teflon-coated butyl rubber stoppers and incubated at 35°C. Reductive dechlorination of ethenes was monitored using a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector (8). Chlorophenols were analyzed using a high-pressure liquid chromatograph (Beckman Coulter, Fullerton, CA) equipped with an Alltima C8 3-μM-bead-diameter column (length, 53 mM; inside diameter, 7 mm; Alltech, Deerfield, IL) at ambient temperature. The solvent for isocratic elution was acetonitrile:water:glacial acetic acid (50:50:0.1) at 1.5 ml/min, and chlorophenols were detected by their absorbance at 220 nm. To monitor growth, cells were fixed with 25% formaldehyde, filtered onto 0.2-μm GTBP Isopore membrane filters (Millipore, Billerica, MA), and stained with 5 μg/ml 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride. All resting-cell assays and RNA and protein extractions were performed at near-maximum growth and dechlorination rates.

Nucleic acid extraction. Prior to extraction, 40 ml of PCE-fed or 200 ml of 2,3-DCP-fed cultures were placed on ice for 30 min, centrifuged at 12,000 × *g* for 10 min at 4°C, and resuspended in 500 μl of sterile ultrapure RNase-free water. DNA extractions were performed according to the method of Fennell et al. (6) except for the elimination of a glass bead homogenization step. In short, cell pellets for RNA extractions were processed using the RNeasy minikit according to the manufacturer's instructions (QIAGEN, Valencia, CA). RNA was eluted with 50 μl RNase-free water and quantified by measuring absorbances at 260 and 280 nm with a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). RNA samples were treated twice with RNase-free DNase 1 (Fisher Scientific, Rockville, MD) to eliminate contaminating DNA.

Reverse transcription and quantitative PCR. First-strand cDNA synthesis reactions were performed with random hexamer primers using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were performed in 20-μl solutions containing 10 ng of RNA incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min in a DNA Engine PTC-200 thermocycler (MJ Research, Hercules, CA). Quantitative PCR amplifications were performed on triplicate samples using an ABI 7000 Real Time PCR machine (Applied Biosystems, Foster City, CA). Individual reactions contained iQ SYBR Green Super Mix (Bio-Rad, Hercules, CA) with 1 ng of cDNA template and 200 nM of primer targeting one of the 19 RD genes or the gene encoding the RNA polymerase beta subunit (*rpoB*) (see Table S1 in the supplemental material). Primers were designed to target RD genes and not to amplify any other sequences in the *D. ethenogenes* genome. Specificity of each primer set was tested by PCR amplification and sequencing of amplified DNA. All primers were tested to amplify at a minimum of 97% reaction efficiency for each experiment. PCR amplifications were carried out with the following parameters: 95°C for 10 min and 30 cycles of 95°C for 15 s and 60°C for 1 min. Melting-curve analysis and amplicon sequencing were used to screen for primer dimers, and RNA samples incubated without reverse transcriptase did not lead to a PCR product, showing that no DNA was present. cDNA target amplifications were compared to DNA standards obtained by serial dilution of genomic DNA. RD expression levels were calculated from DNA standard curves generated during each run and with each primer pair and related to corresponding *rpoB* expression levels. In preliminary experiments, we found that using *rpoB* expression as a standard provided the most uniform and reproducible results compared to other potential standards, including *atpA* and the 16S rRNA gene. Rahm et al. (29) found that *rpoB* was highly expressed in mixed cultures containing *D. ethenogenes* that were actively reductively dechlorinating chloroethenes. Values presented and their standard deviations were taken from triplicate samples from at least two different cultures.

Mapping transcriptional start sites. The transcription start sites (TSS) of genes *tceA*, *pceA*, *infA* (DET0497; initiation factor IF-1), and DET1407 were identified using the 5′ rapid amplification of cDNA ends (5′-RACE) system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). A series of two or three nested primers was designed starting 20 bp downstream from the translational start codon (see Table S1 in the supplemental material). The PCR product was sequenced and aligned with the upstream region of the corresponding gene.

Proteomic analyses. PCE- and 2,3-DCP-grown cell pellets were prepared from 500 ml of culture by centrifugation at 4°C for 30 min. The supernatant was discarded, and cell pellets were stored at −20°C. Crude pellet fractions were prepared by resuspension in 20 mM Tris, pH 7.4, passing of the cells through a French pressure cell at 55 MPa, and centrifugation for 10 min at 10,000 × *g*. Membrane-enriched material was obtained by transferring 1.5 ml of soluble fraction to new microcentrifuge tubes, and purified membrane material was pelleted at 104,000 × *g* using a tabletop ultracentrifuge. Membrane-enriched proteins were solubilized in 0.1% dodecylmaltoside in 20 mM Tris (pH 7.4), and approximately 5 to 10 μg of protein from PCE-grown cells and <1 μg of protein from 2,3-DCP grown cells was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel lanes in the regions estimated to be between 20 to 120 kDa were then divided into four sections, which were stored at −20°C for subsequent in-gel digestion and manual extraction. In-gel digestion and tryptic peptide extractions were performed following a protocol modified from the method of Shevchenko et al. (31).

Peptide and identification by nano-LC/MS/MS. Samples were reconstituted in 15 μl of 0.1% formic acid with 2% acetonitrile prior to MS analysis. The nano-LC was carried out by using an LC Packings Ultimate integrated capillary high-performance LC system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). The gel-extracted peptides were injected using a Famous autosampler on a C18 μ-precolumn cartridge for on-line desalting and then separated on a PepMap C-18 RP nanocolumn, eluted in a 60-min gradient of 5% to 45% acetonitrile in 0.1% formic acid at 250 nl/min. The nano-LC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap, from ABI/MDS Sciex (Framingham, MA), equipped with a Micro Ion Spray Head ion source.

Data acquisition on the MS was performed using Analyst 1.4.1 software (Applied Biosystems) in the positive-ion mode for information-dependent acquisition (IDA) analysis. In IDA analysis, after each survey scan for *m*/*z* 400 to *m*/*z* 1550 and an enhanced-resolution scan, the three highest-intensity ions with multiple charge states were selected for MS/MS with rolling collision energy applied for detected ions based on different charge states and *m*/*z* values.

MS/MS data generated from nano-LC/electron spray ionization-based IDA analysis were interrogated using the ProID 1.4 search engine (Applied Biosystems) for database searching against the *Dehalococcoides* strain 195 database. One trypsin miscleavage, the carbobamidomethyl modification of cysteine, and a methionine oxidation were used for all searches. Initial protein identification was limited to peptide hits with >95% confidence. Subsequent identification was limited to at least one peptide with a Pro Group confidence score of >95 and at least one additional peptide with a Pro Group confidence score of >20.

Resting-cell assay of reductive dehalogenase activity. Reductive dechlorination resting-cell assays were performed as described by Magnuson et al. and Nijenhuis et al. (18, 27) with the following modifications. Cells grown on PCE or DCP were concentrated 10- or 100-fold relative to their original volumes, respectively, and were prepared with Ti(III) citrate as a reducing agent. Assays were set up in 8-ml crimp-top vials with either 1 ml of cells for PCE and TCE reductive dechlorination assays or 2 ml of cells for the 2,3-DCP reductive dechlorination assay, along with 8.3 mM Ti(III) citrate, 57% hydrogen in the head space, and 0.5 to 1 mM chlorinated ethene or 20 to 50 μM 2,3-DCP. Dechlorination was monitored as described above for chlorinated ethenes and phenols.

RESULTS

RD gene transcript levels. Transcript levels of each of the 19 potential RD genes normalized to *rpoB* (29) in *D. ethenogenes* 195 was examined during exponential-phase growth of cultures on either PCE, TCE, or 2,3-DCP (Fig. 1). In cells growing on PCE or TCE, *tceA* and *pceA* transcript levels were severalfold

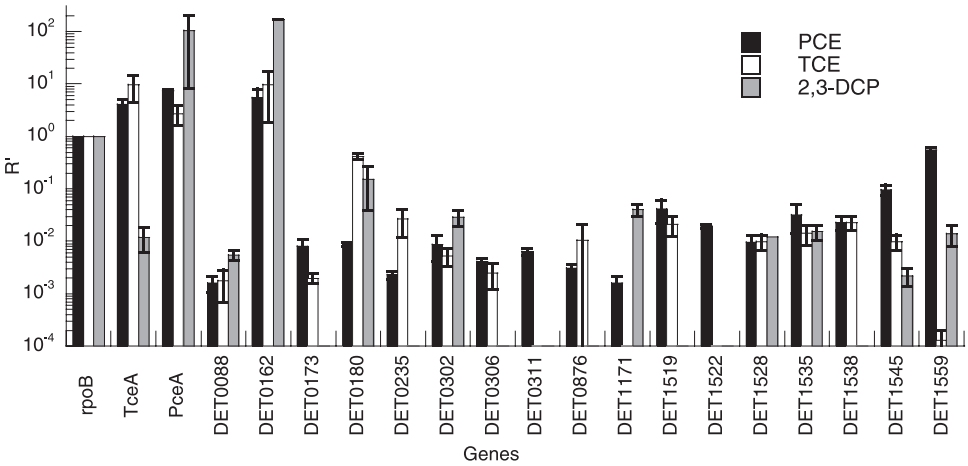

FIG. 1. Transcript levels for genes in the genome of *D. ethenogenes* strain 195 annotated as potential reductive dehalogenases, including *tceA* (DET0079) and *pceA* (DET0318), in cells grown on PCE, TCE, or 2,3-DCP. All transcript levels were normalized to that of *rpoB* (R′), and error bars represent standard deviations.

higher than those for *rpoB*, as were those for DET0162, which is presumably nonfunctional because of a translational stop codon. We confirmed the presence of this stop codon in cultures we were studying by sequencing the PCR product from DET0162 using primers listed in Table S1 in the supplemental material. In PCE-grown cultures, transcript levels of DET1559 approached those of *rpoB*, but not in TCE-grown cells, a finding we confirmed several times. Transcripts from DET0180 in TCE-grown cells and DET1545 in PCE-grown cells were near 10% of the levels of *rpoB* transcripts.

In cells grown with 2,3-DCP, the relative transcript levels of *pceA* were 10-fold higher than those in PCE- or TCE-grown cells, as were those for DET0162. In contrast, those for *tceA* transcripts fell 3 orders of magnitude compared to those in cells grown on PCE or TCE, suggesting transcriptional control. The only other RD gene with transcripts approaching 10% of that for *rpoB* was DET0180.

Transcription start sites for *tceA* and *pceA*. We used 5′-RACE to determine TSSs for *tceA*, *pceA*, and two "housekeeping" genes not involved in reductive dehalogenation that are likely to be highly expressed. DET0497 is annotated as encoding translation initiation factor 1 (*infA*) and is highly expressed in growing cells of *Escherichia coli* (3), whereas DET1407 was found to be one of the most abundant membrane-associated proteins in a proteomic survey of strain 195 and was hypothesized to encode part of the S-layer cell wall (25). The TSSs of these genes were determined to be 86 to 156 bp upstream of the predicted translational start codons (Fig. 2A). Attempts to determine TSSs for DET0162 and *rpoB* were not successful.

Potential promoter regions upstream of these TSSs were examined. The canonical σ^{70} promoter site is TTGACa-(16 to 19 bp)-TAtAaT-(5 to 9 bp)-TSS, in which capitalized bases are present more than 50% of the time, and these two conserved hexamers are called −35 and −10 regions, respectively (16). All four upstream regions had acceptable −10 hexamers, all beginning with TA and ending with T. Moreover, the *infA* and DET1407 upstream regions had TGTG motifs one base upstream of the predicted −10 region, known to obviate the need for a strong −35 region in *Bacillus subtilis* (34), and *tceA* had a TG in the same position, which can play a similar role in *E. coli* (23).

The only gene we examined with a suitable −35 region was *infA*, which had the important TTG as the first three bases. DET1407 had essentially no match near −35 but curiously had a TTGACA sequence located only 10 bp upstream of the −10 region. The potential −35 region for *tceA* had a 4/6-bp match

A

tceA	TTTTTCATTCTTTCgTGgCACGTGCGCTTTCCAAGGTGCTATctTCTACTT-122bp-ATG
pceA	GGGTATTCGTTTATgcGAatGCTTACCTGTCTTCACTTATcATTAAACTGT-142bp-ATG
infA	CCAAGTCTACCGAGATTTGAttACCTAGCTGAACTGTGGTATaTTTTGCCA- 86bp-TTG
DET1407	TAAGTTAAAATGCccGtaAACTATTGACAGGCTGTGTTATTATATAAGATA-156bp-ATG
VS*vcrA*	TGCGTATTTTGTGCAATTGACATTACTATATAAAATGCTAgAATACAAATA-104bp-ATG

B

vcrA	TSS-28bp-GC-TTATGGATATTTGGCGTT-56bp-ATG
tceA	TSS-37bp-ATGTTATGGATACTTGGCTTG-64bp-ATG
	********* ***** *

FIG. 2. (A) Regions upstream of transcription starts determined by 5′-RACE for *tceA*, *pceA*, *infA*, and DET1407 from *D. ethenogenes* strain 195 and the region upstream of *vcrA* from strain VS (VS*vcrA*) (26). Underlined bases represent potential −10 regions, TG motifs upstream of −10, or potential −35 regions. (B) Transcription leader regions upstream of *vcrA* and *tceA* showing regions of high sequence identity.

but lacked a beginning T, and only a 2/6 match could be found for the *pceA* −35 region. Matches to other potential sigma factor binding sites were not apparent.

We also examined the leader regions between the TSS and the translation start sites for homology with other potential leader regions. BLAST analyses revealed the upstream region of *tceA* from strain 195 to be nearly 100% identical with upstream regions of close *tceA* homologues sequenced from diverse *Dehalococcoides* enrichments and from strain FL2 (14) (data not presented). Unfortunately, these sequences began downstream of the predicted −10 and −35 regions, so that these regions could not be compared. Comparison of the upstream regions of *tceA* and the *vcrA* vinyl chloride RD of *Dehalococcoides* strain VS (26) revealed 43% sequence identity overall, including low identity in the predicted promoter region (Fig. 2A), which contains a canonical −35 hexamer. Interestingly, there is a conserved stretch of 15 nucleotides with a single T-to-C transition mutation in approximately the same location within the two leader regions (Fig. 2B). The region upstream of the predicted translation start site of *pceA* is ca. 80% identical over 200 bases with that of CbdbA1588 from *Dehalococcoides* strain CBDB1, with only five nucleotide differences found in the promoter region (data not shown). The nucleotide and predicted amino acid sequences of the two genes are 86.1% and 93.7% identical, respectively.

Detection of RDs by LC/MS/MS proteomics. Proteomic analyses were performed on PCE- and 2,3-DCP-grown cells to detect RD polypeptides (24). Similar to previous results (24, 25), TceA and PceA RDs were detected with high peptide coverage in membrane-enriched fractions from PCE-grown cells and were among the top five polypeptides in terms of peptide coverage, along with the products of three genes annotated as cochaperonin GroEL (DET1428), BNR/Asp box repeat domain protein (DET1407), and formate dehydrogenase (DET0187) (Fig. 3). Twenty-three unique peptides corresponding to TceA (54% coverage) and 25 unique peptides corresponding to PceA (55% coverage) were identified in PCE-grown membrane-enriched protein fractions, and no peptides from other RDs were detected. The same dominant proteins were identified in membrane-enriched fractions from 2,3-DCP-grown cells, with the notable exception of TceA. Although fewer proteins were detected and overall peptide coverage was lower in samples from 2,3-DCP-grown cells, which contained less protein than those from PCE-grown cells because the cultures reached lower cell densities, 19 unique peptides corresponding to PceA (51% coverage) were identified, whereas no peptides from other RDs were detected.

RD activity. We examined cells harvested from cultures growing on either PCE or 2,3-DCP for the ability to utilize either substrate independent of growth. PCE-grown cells dechlorinated PCE to TCE and small amounts of dichloroethenes (DCEs) and VC (Fig. 4A), similar to previous results (27), indicating the presence of PceA and TceA activities. PCE-grown cells also converted 2,3-DCP to 3-monochlorophenol with no lag and at rates ca. threefold lower than those of PCE dechlorination (Fig. 4B). 2,3-DCP-grown cells dechlorinated PCE to TCE, but less-chlorinated ethenes were not detected (Fig. 4C). These cells reductively dehalogenated 2,3-DCP ca. fivefold more slowly than PCE (Fig. 4D).

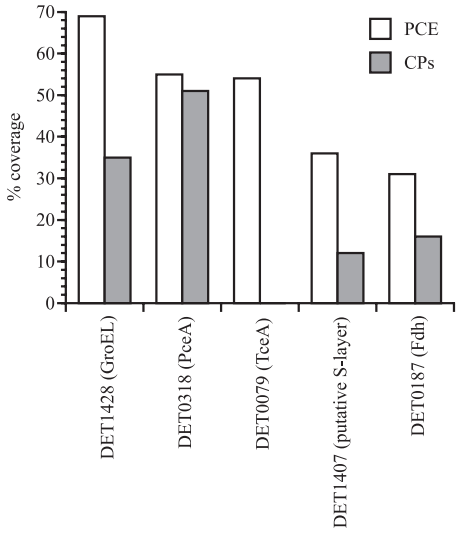

FIG. 3. *D. ethenogenes* protein coverage obtained from PCE-grown cells (PCE; white bars) or 2,3-DCP-grown cells (CPs; gray bars). Peptide fragments identified by electron spray ionization and matrix-assisted laser desorption ionization MS/MS of the five most highly expressed proteins identified in cell membrane-enriched fractions.

DISCUSSION

From previous results (18, 19), it was expected that the genes encoding TceA and PceA would be highly expressed in PCE-grown cells of *D. ethenogenes*, and indeed, the transcript levels of these two genes were higher than that of *rpoB* and orders of magnitude higher than those of genes encoding the other RDs, with the exception of DET0162 (discussed below). Moreover, peptide coverage for TceA and PceA was high in membrane-enriched fractions of PCE-grown cells, in agreement with previous results (25). PceA is not needed for growth on TCE, yet we found high transcript levels in TCE-grown cells; however, Maymó-Gatell et al. (20) found that TCE-grown cells of strain 195 showed high PCE dehalogenation activity, indicating that PceA is present in TCE-grown cells. Transcripts of DET1559 were detected at levels about 10-fold lower than those for *tceA* and *pceA* in PCE-grown cells (Fig. 1), but peptides corresponding to this potential RD were not detected in membrane-enriched cell preparations in this study. However, DET1559 peptides were detected with low coverage in a PCE-grown mixed culture containing *D. ethenogenes* (24), as were peptides from DET1545 in a pure culture preparation different from the one used in these studies. Rahm et al. (29) examined the temporal expression of a select group of RD genes from a mixed culture containing *D. ethenogenes* and found, in similarity to results in this study, that transcript levels of *tceA*, *pceA*, DET0162, and DET1559 increased during PCE reductive dehalogenation. In contrast, DET1545 transcripts were also detected, though they did not reach similar maximum transcript levels to that of *tceA* until after all PCE was reductively dehalogenated to VC. Thus, there appears to be some variability in the detection of RD genes expressed at lower levels than *tceA* and *pceA*.

Transcript levels for *pceA* were higher relative to those for *rpoB* in 2,3-DCP-grown cells than they were in PCE-grown

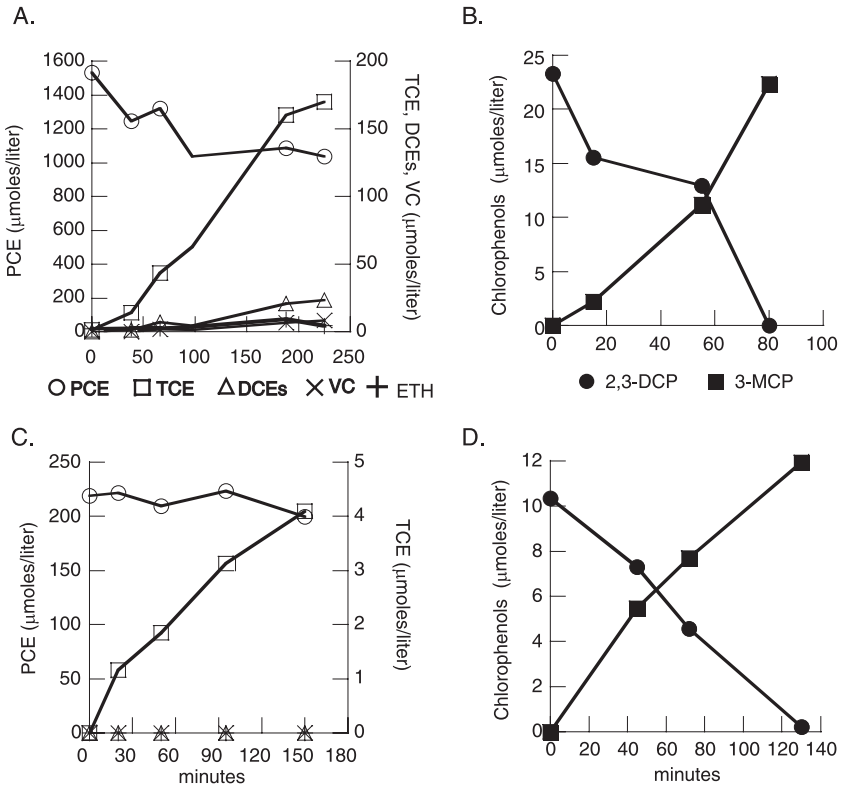

FIG. 4. Reductive dehalogenation activities of resting cells of *D. ethenogenes* strain 195 grown on PCE or TCE. (A) PCE dechlorination by PCE-grown cells. (B) 2,3-DCP dechlorination by PCE-grown cells. (C) PCE dechlorination by 2,3-DCP-grown cells. (D) 2,3-DCP dechlorination by 2,3-DCP-grown cells. Each graph shows results for a representative vial from experiments run in triplicate. 3-MCP, 3-monochlorophenol.

cells. Moreover, PceA was the only RD with significant peptide coverage in proteomic analyses of 2,3-DCP-grown cells. In contrast, *tceA* transcript levels were more than 2 orders of magnitude lower than those of *rpoB*, peptides from TceA were not detected in 2,3-DCP-grown cells, and 2,3-DCP-grown cells did not have detectable TCE RD activity, indicating that *tceA* is not expressed in 2,3-DCP-grown cells.

It is not surprising that evidence for regulation of expression of RD-homologous genes in *Dehalococcoides* spp. is beginning to accumulate, since many are located adjacent to genes predicted to encode transcriptional regulators (15, 30). Johnson et al. (13), in their studies of a TCE-grown enrichment culture from Alameda Naval Air Station that contained *Dehalococcoides* spp., reported increased levels of *tceA* mRNA in starved cells given TCE, *cis*-DCE, *trans*-DCE, or 1,1-DCE but not PCE or VC. These findings suggest that the molecular mechanism of control over *tceA* is finely tuned to recognize specific halogenated compounds. This culture did not use PCE and therefore could not produce TCE potentially needed to induce *tceA*. In strain 195, *tceA* was induced in cells growing on TCE or PCE (which is metabolized to TCE) but not in cells growing on 2,3-DCP. Curiously, unlike most other RD genes, there are no genes with strong resemblances to transcription regulators adjacent to *tceA*. Of the adjacent genes, DET0080 is annotated as having unknown function (www.tigr.org/tdb/mdb) but has similarity to genes encoding the ArsR family of regulators below the noise cutoff in a hidden Markov model search. While this gene product is a possible candidate for regulating *tceA* expression, it is also possible that regulatory circuits encoded elsewhere in the chromosome play a role in regulation.

These results also suggest that the PceA RD is responsible for dechlorination of 2,3-DCP. It has the highest transcript levels in 2,3-DCP-grown cells, and it was detected with high peptide coverage, whereas no other potential RD was detected. Further bolstering this proposition is the finding that PCE-grown cells dechlorinated 2,3-DCP without a lag (Fig. 4), indicating the appropriate reductive dehalogenase was already present. In an analogous experiment, 2,3-DCP-grown cells dechlorinated PCE to TCE without a lag but did not dechlorinate TCE. In *Desulfitobacterium* strain PCE1, a member of the *Firmicutes*, 2-chlorophenol and PCE dechlorination are carried out by distinct RD enzymes with little cross-reactivity towards the other substrate (33). However, the 2-chlorophenol RD from this organism does not require adjacent chlorines, as are present 2,3-DCP and PCE, so that it clearly has different substrate specificity from the 2,3-DCP-dechlorinating RD in strain 195, and its gene sequence is phylogenetically distinct from *pceA*. In light of the large number of potential RD genes present in the *D. ethenogenes* genome (30) with unknown function, it is surprising that PceA rather than one of the other RDs uses 2,3-DCP, but this finding does suggest an evolutionary route for *Dehalococcoides* spp. to take from utilizing chlorophenols, considered to be naturally occurring substrates, especially in soils (4), to utilizing PCE, considered a xenobiotic.

Dehalococcoides strain CBDB1 is adept at dehalogenating chlorinated aromatics (2), including chlorophenols (1). Similar

to strain 195, strain CBDB1 reductively dehalogenates 2,3-DCP from the *ortho* position and reductively dehalogenates PCE to TCE and *trans*-dichloroethene (L. Adrian, personal observation). Strain CBDB1 can also reductively dehalogenate higher chlorinated chlorophenols, such as 2,3,4-trichlorophenol, from the *meta* position. In strain 195, the translated sequence of *pceA* has 93.7% amino acid identity with CbdbA1588 from strain CBDB1 (15). Downstream of cbdbA1588 are predicted histidine kinase (CbdbA1590) and response regulator (CbdbA1589) genes of a two-component regulatory system that have 92.1% and 91.1% amino acid identities to their homologues associated with *pceA* in strain 195. Morris et al. (24) recently found that in cultures of strain CBDB1 grown on 2,3-DCP, CbdbA1588 peptides were detected with 31% coverage, while in contrast to strain 195, CbdbA080, homologous to DET1559, was detected with 13% peptide coverage and CbdbA088, with no homologues in strain 195, was detected with 10% peptide coverage. This result suggests that the Cbdb1588 serves as a 2,3-DCP reductive dehalogenase in strain CBDB1, but with the detection of multiple RDs, the situation may be more complicated in this organism.

Since little is known about transcription initiation and regulation in *Dehalococcoides* spp., we determined the TSSs and examined upstream regions of two expressed RD genes, *tceA* and *pceA*, and two "housekeeping" genes expected to be highly expressed, *infA* and DET1407. The genome of strain 195 contains a gene predicted to encode a σ^{70} homologue (DET0551) (17), as well as two smaller genes (DET0169 and DET1348) predicted to encode σ^{W} and extracellular sigma factor homologues, respectively, which usually regulate accessory functions. The predicted amino acid sequence of DET0551 contains the conserved residues within its region 4.2 that are involved in nucleotide contact with the −10 and −35 hexamers in other σ^{70} homologues (5, 9, 22), suggesting that its recognition sequences should resemble the canonical ones. Thus, it is reasonable to expect some housekeeping genes and other genes to show σ^{70} consensus binding sites. In all four genes, there was a reasonably good match to the consensus −10 σ^{70} binding site, but only *infA* had a close match to the −35 consensus sequence, as does the *vcrA* gene from *Dehalococcoides* strain VS (Fig. 2A). Both *infA* and DET1407 have potential extended −10 regions with a TGTG motif (34), and it is likely that this allows DET1407 to be transcribed in the absence of an acceptable −35 region. The region upstream of the *tceA* TSS has a TG motif extending its −10 region, which may allow transcription despite the moderate match of its −35 region (23), whereas the *pceA* gene has no TG element and has a poor −35 match. For both RD genes, binding by an activator may be needed for transcription, in the case of *pceA*, perhaps the response regulator of the two-component system predicted to be encoded by DET0315 and DET0316 adjacent to it. Finally, the conserved 15-bp sequence in the leader regions of *tceA* from strain 195 and *vcrA* from strain VS may bind homologous regulatory proteins.

Since DET0162 was not considered to encode a functional RD, it was surprising that it showed high transcript levels in cells grown in PCE, TCE, or 2,3-DCP; however, peptides from its translation product were not detected in any cells in this or a previous (25) study, suggesting either that it is not translated or that the translation product is unstable, and it is therefore a nonfunctional pseudogene in the process of degradation. It was recently suggested that pseudogenes are a common feature of microbial genomes (28). While we were unable to identify a transcription start site for DET0162 using 5′-RACE, there is an acceptable −10 region 108 bases upstream of the predicted translation start site, in similarity to other genes (Fig. 2), with TTGACA, a perfect −35 region match, 16 bases upstream of that, making its transcription possible. Why this presumably nonfunctional gene showed such high transcript levels is unclear.

The list of potential RD genes with unknown functions in *Dehalococcoides* spp. has increased rapidly over the past several years, while the list of halogenated substrates has increased at a slower pace. *Dehalococcoides* spp. grow to low densities, making traditional protein purification techniques and genetic analyses of RDs difficult, but PCR-based measurements of transcripts and sensitive proteomic techniques allowed us to identify RD genes expressed in *D. ethenogenes* cells using different electron acceptors, and these techniques were supplemented by measuring RD enzymatic activity in cells. This approach should continue to be useful in identifying RDs involved in use of other substrates by this microbial group important to bioremediation.

ACKNOWLEDGMENTS

This research was supported by NSF Metabolic Biochemistry grant MCB-0236044.

Helpful comments by J. Helmann and help with proteomic analyses from S. Zhang are gratefully acknowledged.

REFERENCES

1. **Adrian, L., S. K. Hansen, J. M. Fung, H. Görisch, and S. H. Zinder.** 2007. Growth of *Dehalococcoides* strains with chlorophenols as electron acceptors. Environ. Sci. Technol. **41:**2318–2323.
2. **Adrian, L., U. Szewzyk, J. Wecke, and H. Görisch.** 2000. Bacterial dehalorespiration with chlorinated benzenes. Nature **408:**580–583.
3. **Allen, T. E., M. J. Herrgard, M. Liu, Y. Qiu, J. D. Glasner, F. R. Blattner, and B. O. Palsson.** 2003. Genome-scale analysis of the uses of the *Escherichia coli* genome: model-driven analysis of heterogeneous data sets. J. Bacteriol. **185:**6392–6399.
4. **de Jong, E., and J. A. Field.** 1997. Sulfur tuft and turkey tail: biosynthesis and biodegradation of organohalogens by basidiomycetes. Annu. Rev. Microbiol. **51:**375–414.
5. **Dove, S. L., S. A. Darst, and A. Hochschild.** 2003. Region 4 of sigma as a target for transcription regulation. Mol. Microbiol. **48:**863–874.
6. **Fennell, D. E., A. B. Carroll, J. M. Gossett, and S. H. Zinder.** 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. **35:**1830–1839.
7. **Fennell, D. E., I. Nijenhuis, S. F. Wilson, S. H. Zinder, and M. M. Häggblom.** 2004. *Dehalococcoides ethenogenes* strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ. Sci. Technol. **38:**2075–2081.
8. **Freedman, D. L., and J. M. Gossett.** 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55:**2144–2151.
9. **Helmann, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
10. **Hölscher, T., H. Görisch, and L. Adrian.** 2003. Reductive dehalogenation of chlorobenzene congeners in cell extracts of *Dehalococcoides* sp. strain CBDB1. Appl. Environ. Microbiol. **69:**2999–3001.
11. **Hölscher, T., R. Krajmalnik-Brown, K. M. Ritalahti, F. Von Wintzingerode, H. Görisch, F. E. Loffler, and L. Adrian.** 2004. Multiple nonidentical reductive-dehalogenase-homologous genes are common in *Dehalococcoides*. Appl. Environ. Microbiol. **70:**5290–5297.
12. **Jayachandran, G., H. Görisch, and L. Adrian.** 2003. Dehalorespiration with hexachlorobenzene and pentachlorobenzene by *Dehalococcoides* sp. strain CBDB1. Arch. Microbiol. **180:**411–416.
13. **Johnson, D. R., P. K. Lee, V. F. Holmes, A. C. Fortin, and L. Alvarez-Cohen.** 2005. Transcriptional expression of the *tceA* gene in a *Dehalococcoides*-containing microbial enrichment. Appl. Environ. Microbiol. **71:**7145–7151.

14. **Krajmalnik-Brown, R., Y. Sung, K. M. Ritalahti, F. Michael Saunders, and F. E. Loffler.** 2007. Environmental distribution of the trichloroethene reductive dehalogenase gene (*tceA*) suggests lateral gene transfer among *Dehalococcoides*. FEMS Microbiol. Ecol. **59:**206–214.
15. **Kube, M., A. Beck, S. H. Zinder, H. Kuhl, R. Reinhardt, and L. Adrian.** 2005. Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. Nat. Biotechnol. **23:**1269–1273.
16. **Lewin, B.** 1990. Genes IV, 4 ed. Oxford University Press, Oxford, United Kingdom.
17. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. **174:**3843–3849.
18. **Magnuson, J. K., M. F. Romine, D. R. Burris, and M. T. Kingsley.** 2000. Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of *tceA* and substrate range characterization. Appl. Environ. Microbiol. **66:**5141–5147.
19. **Magnuson, J. K., R. V. Stern, J. M. Gossett, S. H. Zinder, and D. R. Burriss.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64:**1270–1275.
20. **Maymó-Gatell, X., T. Anguish, and S. H. Zinder.** 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by "*Dehalococcoides ethenogenes*" strain 195. Appl. Environ. Microbiol. **65:**3108–3113.
21. **Maymó-Gatell, X., Y. T. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science **276:**1568–1571.
22. **Meima, R., H. M. Rothfuss, L. Gewin, and M. E. Lidstrom.** 2001. Promoter cloning in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. **183:**3169–3175.
23. **Mitchell, J. E., D. Zheng, S. J. Busby, and S. D. Minchin.** 2003. Identification and analysis of 'extended -10′ promoters in *Escherichia coli*. Nucleic Acids Res. **31:**4689–4695.
24. **Morris, R. M., J. M. Fung, B. G. Rahm, S. Zhang, D. L. Freedman, S. H. Zinder, and R. E. Richardson.** 2007. Comparative proteomics of *Dehalococcoides* spp. reveals strain-specific peptides associated with activity. Appl. Environ. Microbiol. **73:**320–326.
25. **Morris, R. M., S. Sowell, D. Barofsky, S. Zinder, and R. Richarson.** 2006. Transcription and mass-spectroscopic proteomic studies of electron transport oxidoreductases in *Dehalococcoides ethenogenes*. Environ. Microbiol. **8:**1499–1509.
26. **Muller, J. A., B. M. Rosner, G. Von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann.** 2004. Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. Appl. Environ. Microbiol. **70:**4880–4888.
27. **Nijenhuis, I., and S. H. Zinder.** 2005. Characterization of hydrogenase and reductive dehalogenase activities of *Dehalococcoides ethenogenes* strain 195. Appl. Environ. Microbiol. **71:**1664–1667.
28. **Ochman, H., and L. M. Davalos.** 2006. The nature and dynamics of bacterial genomes. Science **311:**1730–1733.
29. **Rahm, B. G., R. M. Morris, and R. E. Richardson.** 2006. Temporal expression of respiratory genes in an enrichment culture containing *Dehalococcoides ethenogenes*. Appl. Environ. Microbiol. **72:**5486–5491.
30. **Seshadri, R., L. Adrian, D. E. Fouts, J. A. Eisen, A. M. Phillippy, B. A. Methe, N. L. Ward, W. C. Nelson, R. T. Deboy, H. M. Khouri, J. F. Kolonay, R. J. Dodson, S. C. Daugherty, L. M. Brinkac, S. A. Sullivan, R. Madupu, K. E. Nelson, K. H. Kang, M. Impraim, K. Tran, J. M. Robinson, H. A. Forberger, C. M. Fraser, S. H. Zinder, and J. F. Heidelberg.** 2005. Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. Science **307:**105–108.
31. **Shevchenko, A., M. Wilm, O. Vorm, and M. Mann.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68:**850–858.
32. **Smidt, H., and W. M. de Vos.** 2004. Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. **58:**43–73.
33. **van de Pas, B. A., J. Gerritse, W. M. de Vos, G. Schraa, and A. J. Stams.** 2001. Two distinct enzyme systems are responsible for tetrachloroethene and chlorophenol reductive dehalogenation in *Desulfitobacterium* strain PCE1. Arch. Microbiol. **176:**165–169.
34. **Voskuil, M. I., and G. H. Chambliss.** 2002. The TRTGn motif stabilizes the transcription initiation open complex. J. Mol. Biol. **322:**521–532.
35. **Waller, A. S., R. Krajmalnik-Brown, F. E. Loffler, and E. A. Edwards.** 2005. Multiple reductive-dehalogenase-homologous genes are simultaneously transcribed during dechlorination by *Dehalococcoides*-containing cultures. Appl. Environ. Microbiol. **71:**8257–8264.