Phylogenetic and Transcriptional Analyses of a Tetrachloroethene-Dechlorinating “Dehalococcoides” Enrichment Culture TUT2264 and Its Reductive-Dehalogenase Genes

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A dechlorinating microbial enrichment culture designated TUT2264 was cultured with tetrachloroethene and then characterized for tetrachloroethene-dechlorination by culture-dependent and -independent methods. The fourth-transferred TUT2264 culture completely dechlorinated tetrachloroethene and trichloroethene, and accumulated more trans-1,2-dichloroethene than cis-1,2-dichloroethene. A real-time PCR analysis revealed that “Dehalococcoides” cells made up only 0.3% of the total. Eight distinct reductive-dehalogenase-homologous genes (rdh) were detected with degenerate primers. Phylogenetic analyses revealed 5 of the 8 RdhAs to be very similar to RdhAs reported previously but not to share 100% identity. Transcriptional levels were quantified as the number of transcripts per rdhA by combining the reverse transcription real-time PCR and exogenous internal reference mRNA methods. TUT2264 responded to all the chloroethenes tested. rdhA4 was transcribed with all chloroethenes except vinyl chloride, whereas rdhA8 was only transcribed on tetrachloroethene. Furthermore, multiple rdhAs were induced to express by a single chloroethene as a growth-supporting or non-supporting substrate. These results suggested that Rdhs are multi-functional and rdhAs are a powerful tool to evaluate the potential of contaminated sites and isolates to dechlorinate chloroethenes.

Key words: “Dehalococcoides”, enrichment, dechlorination, tetrachloroethene

Chloroethenes, including tetrachloroethene (PCE) and trichloroethene (TCE), have commonly been used as organic solvents and degreasing agents. As a result of past disposal practices and spills, chloroethenes are widely detected as contaminants in soil, groundwater, and subsurface environments around the world (29, 31). Indeed, chloroethenes are considered a potential serious threat to source of drinking water and to health (31).

Complete biological reductive dechlorination from PCE or TCE to ethene is gaining acceptance as a promising method of bioremediation for some chlorinated ethene-contaminated sites (7, 29). At present, however, organisms capable of the complete dechlorination of PCE are restricted to the “Dehalococcoides” bacteria (15). Although “Dehalococcoides” isolates form a tight phylogenetic cluster within the phylum Chloroflexi (>98% similarity to each other) (1, 13, 24, 30), they are functionally diverse with respect to the spectrum of haloorganic compounds they produce (5, 9, 13, 15, 16). This fact suggests that the available information on the 16S rRNA gene is insufficient to definitively evaluate the potential of “Dehalococcoides” isolates for reductive dechlorination. Although over 60 sequences of reductive-dehalogenase (RDase)-homologous genes (rdh) have been reported (24, 25, 32, 36, 40), only a few RDases, PceA, TceA, BvcA and VcrA, have been fully characterized. For effective bioremediation, it is important to precisely evaluate the potential of dechlorinators at contaminated sites by studying the relationships between rdh (genotype) and dechlorinating profiles (phenotype).

Our previous study demonstrated that an enrichment culture designated TUT2264 reductively dechlorinates PCE to TCE and dichloroethenes (10), although the parental culture was enriched with 1,2,3-trichlorobenzene or 4,5,6,7-tetrachloroethylhalide (ethylhalide) from a polychlorinated-dechlorinating microcosm (42). This suggested that TUT2264 has been shifting toward a bacterial community that can respire on chloroethenes. However, no information is available about rdhs of TUT2264, because pceA and tceA were not detected in PCE- and TCE-dechlorinating enrichment cultures (10). The objectives of the present study were to evaluate the chloroethene-dechlorinating activity of fourth-transferred TUT2264 and to analyze the rdhs. We tried to characterize TUT2264 rdhs by comparing deduced amino acid sequences and by measuring transcriptional levels on chloroethenes by real-time reverse transcribed PCR using internal reference mRNA (22, 33).

Materials and Methods

Chemicals

Methane, ethene, PCE, TCE, 1,1-dichloroethene (1,1-DCE), cis-1,2-dichloroethene (cDCE), trans-1,2-dichloroethene (tDCE), vinyl chloride (VC), and methanol were used in the present study. VC (purity >99.9%) was purchased from Supelco (Bellefonte, PA, USA), while chlorinated ethenes and methanol (HPLC grade) were obtained from Wako Pure Chemicals Industries (Osaka, Japan). Methane (purity >99.9%) and ethene (purity >99.5%) were obtained from GL Science (Tokyo, Japan).
Enhancement culture

The dechlorinating culture TUT2264, enriched with 1,2,3-trichlorobenzene or fthalide from a polychlorinated dioxin-dechlorinating microcosm (10, 42), was studied. A chemically defined anoxic medium designated DHE2 was used for cultivation (10). The DHE2 medium contained 0.5 g of NH4Cl, 2.5 g of NaHCO3, 0.5 g of KH2PO4, 0.41 g of MgCl2·6H2O, 0.15 g of CaCl2·2H2O, 1.0 mL of Se/W solution (17), 1.0 mL of trace elements SL8 solution (2), 1.0 mL of vitamin solution PV1 (19), and 1.0 mg of resazurin per liter. The following reagents were filtered: 0.2 mL of L-cysteine and Na2S (pH 7.0), 4 mM of bromoethansulfonate, 0.2 mM each of acetate and butyrate, and 10 mL of 0.75% (v/v) titanium (III) citrate solution (43) per liter (pH 7.5). The headspace was flushed with N2·CO2 (4:1, v/v), and the bottles (70 mL) containing DHE2 medium (15 mL) were sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. Hydrogen gas as an electron donor (8% [v/v]) was added to the headspace. PCE as the initial electron acceptor was purged with a filter-sterilized (0.22 µm) N2·CO2 (80:20, v/v) gas stream until levels of methane, ethene, and chlorinated ethenes were below detectable limits. These limits are approximately 0.01 µM for methane and ethene and 0.2 µM to 0.6 µM for chloroethenes. Following a 5-d starvation period (40), the culture was divided into seven subcultures amended with either PCE, TCE, or DCE, and the topology of the trees was evaluated by bootstrapping with 1,000 resamplings (8).

DNA extraction

Aliquots of the TUT2264 culture (2 mL) were centrifuged for 30 min at 4°C and 13,000 g, and cells were then collected. DNA was extracted according to the conventional method described by Futamata et al. (10).

Real-time PCR

Real-time PCR assays were applied to genomic DNA for measurement of the 16S rRNA gene copy number of “Dehalococcoides”. Template DNAs were prepared as described above. The “Dehalococcoides”-specific primers used for real-time PCR were DHC793f (5’-GGGAGTATCGACCCTCTCTG-3’) and DHC946r (Roche Diagnostics, Mannheim, Germany). One copy of the 16S rRNA gene was counted as 1 cell, because the number of rRNA operons per genome in “Dehalococcoides” is known to be 1 (25, 36).

Clonal analyses

Clones were constructed using the bulk DNA extracted from the culture as described above. The rdhs were amplified with the primers RRF2 and BIR (24). Amplification was performed with AmpliTaq Gold Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and a Takara Thermal Cycler (Takara Bio, Otsu, Japan) as described previously (10). The PCR products were checked by electrophoresis on 1.5% (w/v) agarose gel in TAE buffer (35) and stained with ethidium bromide. The amplicons were purified with a MicroSpin S-HR400 Column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer’s recommendations. Purified PCR products were cloned into the vector pT7Blue-3 and introduced into competent NovaBlue Single™ cells using a Perfectly Blunt Cloning kit according to the manufacturer’s recommendations. Clones were isolated by screening for blue/white phenotypes and incubated in LB medium amended with kanamycin (50 mg L⁻¹). Plasmid DNA was extracted using a Wizard Miniprep DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer’s directions. The DNA was digested with EcoRI and electrophoresed, thereby confirming whether an insert was of expected size. 16S RNA gene fragments were PCR-amplified with a pair of bacterial consensus primers (proR and qepV) corresponding to Escherichia coli 16S RNA positions 8 to 27 and 1,525 to 1,542 (41). FpDHC1 and 1386r were also used for specific amplification of the 16S rRNA gene of the “Dehalococcoides” group (5, 15).

Sequencing and phylogenetic analyses

Cloned genes were sequenced with an ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit and analyzed with an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems). Sequence data were compiled with the GENETYX-MAC program (GENETYX Corporation, Tokyo, Japan). 16S rRNA gene sequence data were analyzed for chimeras with the CHIMERA_CHECK program version 2.7, and compared with those retrieved from the Ribosomal Database Project II (4). Sequence data were compared with those deposited in databases using the BLAST homology search system. The multiple alignment of sequences and calculation of the nucleotide substitution rate (Ks) by Kimura’s two-parameter model (23) were performed using the CLUSTAL W program (38). Distance matrix trees were constructed by the neighbor-joining method (34), and the topology of the trees was evaluated by bootstrapping with 1,000 resamplings (8).

Cultivation for transcription experiment

The fourth-transferred TUT2264 culture enriched for 100 d with PCE as the initial electron acceptor was purged with a filter-sterilized (0.22 µm) N2·CO2 (80:20, v/v) gas stream until levels of methane, ethene, and chlorinated ethenes were below detectable limits. These limits are approximately 0.01 µM for methane and ethene and 0.2 µM to 0.6 µM for chloroethenes. Following a 5-d starvation period (40), the culture was divided into seven subcultures amended with either PCE, TCE, cDCE, rDCE, 1,1-DCE, VC, or methanol; 3 mL of the TUT2264 (fourth-transferred) culture was transferred into a glass bottle (70 mL capacity) containing 18 mL of fresh DHE2 medium. Chlorinated ethene was added to the bottle with a syringe to give a final concentration of ca. 50 µM; bottles were prepared in triplicate for each substrate. The control culture contained the same volume of methanol instead of a chloroethene solution, since all chloroethenes dissolved in methanol. The cells in 20 mL of sample solution were collected by centrifugation at 13,000 g for 30 min at 4°C, when the initial dechlorination was observed. The pellets were stored at −80°C prior to use.

Quantification of rdhA mRNA and rdhA

The transcription of rdhA was quantified by real-time reverse

Table 1. Specific primer sequences designed for TUT2264 RDH genes

<table>
<thead>
<tr>
<th>TUT2264 gene target</th>
<th>Primer name*</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>rdhA1</td>
<td>rdhA1_350f</td>
<td>ATTAGGTTCAGTGGTACATTGG</td>
</tr>
<tr>
<td>rdhA1</td>
<td>rdhA1_500r</td>
<td>GAAAGCAGCTATCATTACAGG</td>
</tr>
<tr>
<td>rdhA2</td>
<td>rdhA2_322f</td>
<td>CAGCTTTAAAACGAGTTCTCAGGG</td>
</tr>
<tr>
<td>rdhA3</td>
<td>rdhA3_562f</td>
<td>GTGTTGTTTTCTACACAAC</td>
</tr>
<tr>
<td>rdhA4</td>
<td>rdhA4_1186f</td>
<td>GCAAATCCGCTGACCAAAAG</td>
</tr>
<tr>
<td>rdhA5</td>
<td>rdhA5_1141f</td>
<td>GGTAGGAGCATATCCACCAC</td>
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</tr>
<tr>
<td>rdhA6</td>
<td>rdhA6_1141f</td>
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<td>rdhA6_715f</td>
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<td>rdhA7_1207f</td>
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<tr>
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<td>rdhA8_298f</td>
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<td>rdhA8</td>
<td>rdhA8_456f</td>
<td>TCAGTTGGAATCTACATTGGGC</td>
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</table>

* The position numbers are shown within the names with respect to the first nucleotide of each gene following the RRF2 primer binding site. f, forward; r, reverse.
transcription-PCR (qRT-PCR) as the number of mRNA transcripts per rdhA using the specific primers (Table 1). Total RNA was extracted from frozen cell pellets using the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions. Purified RNA was stored at -80°C prior to use. Although mRNA quantities can be normalized to the quantity of mRNA of an endogenous housekeeping gene, dramatic changes in the expression of housekeeping genes have been observed in prokaryotic cells (33, 39). For the normalization of mRNA losses during sample preparation and inefficiencies in reverse transcription, an exogenous internal reference mRNA (*ref* mRNA) was used along with absolute standard curves (21). The standard for the *ref* mRNA was purchased from Promega (luciferase control RNA [1 mg mL⁻¹]), and added prior to the cell lysis step of the RNA extraction process. Standard fragments for rdhA mRNA and *ref* mRNA were produced using a competitive transcription RNA kit (Takara Bio). Total RNA samples and 10-fold serially diluted rdhA mRNA standards were reverse transcribed in parallel 20-µL reaction mixtures using a LightCycler RNA master SYBR Green I kit (Roche Diagnostics), and mRNA was quantified by one-step RT-PCR according to the manufacturer’s instructions. To quantify contaminating genomic DNA, an additional RT reaction without the reverse transcriptase was performed for each sample. To quantify rdhA s from TUT2264 in experimental samples, real-time PCR (qPCR) assays were applied to genomic DNA in conjunction with the absolute standard curve method. DNA was extracted from frozen cell pellets as described above. Standard fragments for rdhA s were produced using a competitive DNA construction kit (Takara Bio). Sample DNA and 10-fold serially diluted standard DNA were amplified in parallel 20-µL reaction mixtures using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics).

**Analytical methods**

Methane, ethene, and chlorinated ethenes were sampled from the headspace of vials (200 µL samples) and analyzed using Hewlett-Packard model 33B gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA). The oven temperature was programmed to remain at 50°C for 2 min and then to increase to 190°C at 50°C min⁻¹ and then remain at 190°C for 40 min. The temperature for both injection and detection was kept at 220°C. Total mass values were determined with Henry’s law constants (27). Ethene and methane were calibrated with 0.05% (v/v) and 0.1% (v/v) gas mixtures.

**Nucleotide sequence accession numbers**

The nucleotide sequence data reported here have been deposited in the DDBJ under accession numbers AB362892 to AB362926.

**Results**

**PCE-dechlorinating enrichment culture TUT2264**

The PCE-dechlorinating ability of fourth-transferred TUT2264 was investigated (Fig. 1). PCE was almost completely dechlorinated within 35 d at a maximum rate of 0.93±0.06 µM d⁻¹ and then converted to DCE and cDCE via TCE. TCE was also almost completely dechlorinated within 90 d at a maximum rate of 0.27±0.08 µM d⁻¹. DCE and cDCE were accumulated at a 4.1±1.0 (DCE-cDCE) ratio. 1,1-DCE and VC were not detected but ethene and methane were present at very low concentrations (below 0.05 µM).

**Enumeration of the “Dehalococcoides” population and phylogenetic analysis**

Real-time PCR revealed the cell density of “Dehalo-

**RdhAs in enrichment culture TUT2264**

An analysis of the PCE-grown culture showed that, of the 40 rdhA clones obtained and sequenced (over 1,520 bp),
17 were functional genes, of which 8 had distinct rdhA sequences (bold type shown in Fig. 4). All of these genes were also amplified from the DNA of the TUT2264 culture used in the transcription experiment. The amino acid sequence of the TUT2264 RdhAs deduced from the nucleotide sequence was highly similar (97 to 100% identity at the protein level) to RdhAs identified in the "Dehalococcoides" or the enrichment culture KB-1 capable of completely dechlorinating PCE to ethene (6). However, TUT2264 did not contain RdhAs similar to the VC-RDase (VS VcrA and BAV1 BvcA) (24, 32), TCE-RDase (195 TceA [DET0079] and FL2 TceA) (14), and RDase of strain BAV1. TUT2264 RdhA2, RdhA4, and RdhA7 showed 100% identity to CBDB1 RdhA2 (cbdb1638), CBDB RdhA1 (cbdb1560), and FL2 RdhA3, respectively. However, five other TUT2264 RdhAs were highly similar but different from RdhAs reported previously. TUT2264 RdhA3 and RdhA4, RdhA3 and RdhA5, RdhA4 and RdhA5, and RdhA6 and RdhA7 shared over 97% amino acid identity with each other. The TUT2264 RdhAs were not similar (<25% identity) to RdhAs of other dechlorinating bacteria, such as Sulfurospirillum, Dehalobacter, and Desulfito bacteria (data not shown).

Like other RdhAs, the TUT2264 RdhAs contained two iron-sulfur cluster-binding (ISB) motifs in the C-terminal region (Fig. 5A). The consensus motif DX2HX2G was located between the two ISB motifs, whereas the SXL and twin-glycine motifs were located downstream of the second ISB motif, as is the case in other RdhAs from "Dehalococcoides" and KB-1 (20). RdhAs in cluster I (e.g., VcrA, BvcA, and TceA; see Fig. 4 upper cluster), including the TUT2264 RdhA1, did not contain the cobalamine-binding consensus sequence. On the other hand, TUT2264 RdhA2, RdhA3, RdhA4 and RdhA5 contained the twin- or single-glycine motif but not the other motifs for cobalamine-binding.

Transcriptional level of rdhAs

qRT-PCR was performed to investigate the transcriptional levels and substrate specificity of TUT2246 rdhAs (Fig. 6). The subcultures from the fourth-transferred TUT2246 were incubated with chloroethenes and methanol (control). The number of mRNA transcripts per rdhA was calculated and the transcriptional level relative to that of the control was then evaluated. A value of less than 1.0 was ignored as noise. Only the transcriptional levels of rdhA1, rdhA2 and rdhA8 increased markedly on PCE, approximately 620-fold, 3,000-fold, and 600-fold, respectively. The transcriptional levels of rdhA3 increased on PCE (7-fold), TCE (3-fold) and VC (170-fold). The transcriptional levels of rdhA4 increased on PCE (7-fold), TCE (320-fold), cDCE (3-fold), tDCE (14-fold), and 1,1DCE (4-fold). The transcriptional levels of rdhA5 increased on PCE (7-fold), TCE (2-fold), and tDCE (3-fold). The transcriptional levels of rdhA7 increased on PCE (2-fold) and VC (16-fold). No transcription of rdhA6 was found with any of the chloroethenes, since rdhA6 was strongly transcribed on methanol.
**Discussion**

The enrichment culture TUT2264 is unique in that it exhibits reductive dechlorination of chloroethenes despite its history, i.e., repeated enrichment with chloroaromatics such as 1,2,3-trichlorobenzene and fthalide from a polychlorinated-dechlorinating microcosm (10, 17, 18, 42). In this study, we functionally characterized TUT2264 by measuring its chloroethene-dechlorinating activities, identifying rdhA’s, and analyzing transcriptional levels in the presence of different chloroethenes.

The fourth-transferred TUT2264 culture has adapted better to respire on chloroethenes due to almost complete dechlorination of TCE (Fig. 1), although the second-transferred culture was incapable of complete TCE-dechlorination (10). The cell density of "Dehalococcoides" increased on PCE and TCE (Fig. 2). Interestingly, pceA and tceA, which are used to assess dechlorinating ability, have not been detected in TUT2264 (10). However the transcriptional analyses demonstrated that 7 and 3 of 8 rdhA’s were transcribed on PCE and TCE, respectively (Fig. 6), which suggests that these are novel PCE- and/or TCE-RdhA genes. TUT2264 rdhA1, rdhA2, and rdhA8 were transcribed with only PCE, while rdhA3, rdhA4, rdhA5 and rdhA7 were transcribed with some chloroethenes, including DCEs and VC incapable of dechlorination. Since the expression of tceA is induced by both growth-supporting and non-supporting chloroethenes (21), it is likely that these mechanisms allow for halorespiring bacteria to produce energy. Furthermore, the results suggest that TUT2264 has the potential for dechlorinating these compounds. For a more comprehensive understanding of these findings, it is the important to combine the results of phenotype- and genotype-research, i.e., investigation of nutritional requirements for dechlorination (12) and analysis of transcriptional regulators of these rdh’s.

Of particular interest is that the transcriptional levels of...
Genetic Characterization of TUT2264

rdhA6 and rdhA8 increased to the same extent with PCE, although the similarity of these genes was only 53% (31% similarity in deduced amino acid sequence). In the case of rdhA6 and rdhA7, their level of similarity was 97.6% and their cobalamine-binding consensus (CBC) sequences were identical (Fig. 4), their transcriptional patterns for chloroethenes differed. These results suggest the difficulty of predicting the substrate specificity of RdhAs on the basis of phylogenetic information and CBC sequences, although it has been discussed that different corrinoid cofactors may modulate the substrate specificity of RdhA (40).

One may need to find signature amino acid residues and motifs in relation to substrate specificity and/or to characterize purified RdhA. These results demonstrated that multiple rdhAs are simultaneously transcribed during dechlorination in TUT2264 as well as KB1 (40).

Unexpectedly, 16S rRNA genes closely related to Acidaminobacter hydrogenoformance strain glu65T were cloned from TUT2264 (Fig. 3). Since this strain is reported as a hydrogen-producing bacterium (37), it is presumed that TUT2264 exhibits dechlorinating activity dependent on an interspecies transfer of hydrogen. Another feature of the

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**Fig. 5.** (A) Comparison of iron-sulfur cluster-binding (ISB) motifs. (B) Comparison of cobalamine consensus sequence (CCS) motifs. Numbers above the sequence correspond to the numbering in the CdbdA1598 sequence (accession number: AJ965256, protein ID: CAI83621.1). DET0079 is TceA of “Dehalococcoides ethenogenes” 195. KB1RdhA5 and KB1RdhA6 are RdhAs of the enrichment culture KB-1, which are transcribed by TCE, cDCE and VC (40). (C) The location of ISB motifs and CCS motifs in rdhA.
diagnosing whether polluted sites (11), TUT2264 reported that dechlorination at contaminated sites of complex microbial transcriptional analyses. The findings not only suggested identified and their substrate responses characterized with provide useful information for evaluating the potential for dechlorination of PCE by TUT2264 was that more DCE than cDCE was accumulated (Fig. 1). Since it has been reported that DCE-polluted sites number more than cDCE-polluted sites (11), TUT2264 rdhA/s might be useful for diagnosing whether DCE is produced or not.

In conclusion, 8 distinct rdhA/s in TUT2264 have been identified and their substrate responses characterized with transcriptional analyses. The findings not only suggested novel rdhA for the dechlorination of chloroethenenes but also provide useful information for evaluating the potential for dechlorination at contaminated sites of complex microbial communities as well as of dechlorinating isolates. That TUT2264 contains a bacterium very closely related to the hydrogen-producing Acidaminobacter hydrogenoformans may provide a clue for developing more efficient dechlorination strategies dependent on the interspecies transfer of hydrogen. This interesting subject is currently under investigation.

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References

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