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ORIGINAL ARTICLE

Reductive dechlorination of chloroethenes by *Dehalococcoides*-containing cultures enriched from a polychlorinated-dioxin-contaminated microcosm

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The reductive dechlorinating abilities for chloroethenes of seven enrichment cultures from polychlorinated-dioxin-dechlorinating microcosm were investigated using culture-independent and -dependent methods. These cultures were constructed and maintained with 1,2,3-trichlorobenzene (1,2,3-TCB) or fthalide as an electron acceptor and hydrogen as an electron donor. Denaturing gradient gel electrophoresis (DGGE) analysis of the amplified fragments targeting the 16S rRNA gene showed one or two major bands, whose nucleotide sequences were then analyzed and were found to suggest that *Dehalococcoides* was one of the dominant bacteria in all enrichment cultures. The nucleotide sequence data revealed that the identity of the major band was 100% identical to the 16S rRNA gene sequence of the Pinellas subgroup of the *Dehalococcoides* clusters, that is, strains CBDB1 and FL2. Genetic diagnosis targeting the *pceA*, *tceA*, *bvcA*, *vcrA* and reductive dehalogenase homologous (*rdh*) gene was performed to investigate the potential for reductive chloroethene dechlorination of cultures. The required length of PCR-amplified fragments was not observed, suggesting that these cultures are not capable of reductively dechlorinating chloroethenes. However, a culture-dependent test indicated that two cultures, TUT1903 and TUT1952, reductively dechlorinated tetrachloroethene (PCE) to trichloroethene (TCE), although not completely. While, TUT2260 and TUT2264 completely converted PCE to TCE and dichloroethenes, but not further. These results suggest that these TUT cultures might include a novel type of bacteria belonging to the *Dehalococcoides* group and that currently available information on both the 16S rRNA gene and *rdh* gene sequences is insufficient to definitively evaluate the potential abilities for reductive dechlorination.
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Introduction

The contamination of subsurface environments with chlorinated hydrocarbons, especially tetrachloroethene (PCE) and trichloroethene (TCE), is a potentially serious threat to drinking water sources (McCarty, 1997). A number of laboratory studies have demonstrated that TCE is transformed cometabolically by aliphatic and aromatic hydrocarbon-degrading bacteria under aerobic conditions (Ensley, 1991; Semprini, 1997; Futamata *et al.,* 2005), while PCE is not transformed under aerobic conditions. However, PCE is reductively dechlorinated under anaerobic conditions by certain kinds of anaerobic bacteria (Smidt and de Vos, 2004; Futamata and Hiraishi, 2007). At present, it is believed that the organisms that carry out this degradation to completion are restricted to *Dehalococcoides* group bacteria (Hendrickson *et al.,* 2002).

To date, six *Dehalococcoides* strains have been isolated. They are most closely affiliated with the *Chloroflexi*, and these populations share a 16S rRNA gene sequence with more than 99% similarity (Sprott *et al.,* 1994; Maymo-Gatell *et al.,* 1997; Adrian *et al.,* 2000; He *et al.,* 2003, 2005; Krajmalnik-Brown *et al.,* 2004), although it has been suggested that this group is functionally very diverse (Hendrickson *et al.,* 2002; He *et al.,* 2003; Duhamel *et al.,* 2004). The isolate from this group, CBDB1, cannot degrade chlorinated ethenes, but instead dechlorinates tetrachlorobenzenes and trichlorobenzenes to trichlorobenzenes and dichlorobenzenes, respectively, (Adrian *et al.,* 2000) and

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dechlorinates some dioxin congeners (Bunge *et al.,* 2003). Another isolate from this group, strain 195, can only completely dechlorinate PCE to ethene, although degrades vinyl chloride (VC) to ethene cometabolically (Maymo-Gatell *et al.,* 1997). Strains VS and BAV1 isolated from this group can dechlorinate dichloroethenes to ethene, but do not use PCE or TCE as an electron acceptor (Cupples *et al.,* 2003; He *et al.,* 2003; Müller *et al.,* 2004). Strain GT can dechlorinate TCE, *cis*-1,2-dichloroethene and VC to ethene as electron acceptors (Sung *et al.,* 2006). Since little is known about the diversity and function of *Dehalococcoides* group bacteria, it is essential to obtain further knowledge for effective bioremediation.

The aim of this study was to characterize the chloroethenes dechlorinating abilities of cultures enriched on 1,2,3-TCB or fthalide and derived from polychlorinated-dechlorinating microcosm (Yoshida *et al.,* 2005). We expected to develop cultures capable of dechlorinating both chlorinated aromatics and alkenes. The chloroethene dechlorinating abilities of seven enrichment cultures were estimated by culture-independent and -dependent methods. Although genetic diagnosis was not sufficient to evaluate the dechlorinating abilities of the cultures, a dechlorinating test revealed that four enrichment cultures exhibited chloroethene dechlorinating activities. We include a discussion of novel *Dehalococcoides* group bacteria with unknown *rdh* genes in enrichment cultures, and consider the reductive dechlorinating ability of enrichment culture TUT2264.

Materials and methods

Chemicals

PCE, TCE, 1,1-dichloroethylene (1,1-DCE), *cis*-1,2-dichloroethylene (*c*DCE), *trans*-1,2-dichloroethylene (*t*DCE) and VC were used in the present study. VC (purity > 99.9%) was purchased from SUPELCO (Bellefonte, PA, USA), and the others chemicals were obtained from Wako Pure Chemicals Industries Ltd. (Chuo-ku, Osaka, Japan).

Construction of enrichment cultures

The parent dioxin-dechlorinating microcosm had been constructed with OAM-1 medium and highly polluted river sediment including polychlorinated dibenzo-*p*-dioxins/dibenzofurans (1.8–3.3 nmol g^{-1} dry weight) (Yoshida *et al.,* 2005). Based on collective information on the anaerobic cultivation of dechlorinating bacteria (Löffler *et al.,* 1996; Adrian *et al.,* 1998; Hölscher *et al.,* 2003), a chemically defined anoxic medium (designated DHE0) was prepared under a N_2/CO_2 (80:20, vol/vol) atmosphere and used in 20 ml anaerobic culture tubes with a culture volume of 10 ml. The medium consisted of (per liter): 1.0 g of NaCl, 0.5 g of $MgCl_2 \cdot 6H_2O$, 0.2 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.3 g of KCl, 0.015 g of $CaCl_2 \cdot 2H_2O$, 1 ml of trace element solution SL8 (3), 1 ml of Se/W solution (Löffler *et al.,* 1996), 1 ml of vitamin mixture PV1 (Hiraishi *et al.,* 2002), 0.048 g of $Na_2S \cdot 9H_2O$, 0.035 g of L-cystein, 2.52 g of $NaHCO_3$, 1 mg of resazurin, 0.75% titanium (III) citrate solution (at a final concentration of 0.075% vol/vol) (Zehnder and Wuhrmann, 1976), 5 mM of acetate, 1 mM of Na_2SO_4, 4 mM of bromoethanesulfonate (BES). The solvent was evaporated using the N_2/CO_2 gas mixture. Hydrogen gas (8% vol/vol in a headspace) and 1,2,3-TCB or fthalide (chloroaromatic compound, 50 μM as an acetone solution) were added as an electron donor and acceptor, respectively. The parent culture was introduced into test tubes (10% vol/vol), and the constructed cultures (TUT2257, TUT2260 and TUT2264) were then further enriched by 10% (vol/vol) transfers into a fresh DHE0 medium approximately every 3–6 months. Fifth transferred cultures were used in this study. TUT1902, TU1903, TUT1951 and TUT1952, which are extremely pure *Dehalococcoides* cultures, have already been reported in the same previous study (Hiraishi *et al.,* 2005b). The total direct counts of microbial cells were measured by fluorescence microscopy after the cells were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA).

DNA isolation

Aliquots of subcultures (4 ml) were centrifuged for 30 min at 4°C and 9000 *g*, and cells were then collected. The cells were suspended in 280 μl of TE buffer and 30 μl of 10% sodium dodecyl sulfate. After mixing gently and completely, freeze–thaw treatment (−80°C and 65°C, respectively) (Sprott *et al.,* 1994) was conducted (three cycles). Ten microliters of proteinase K (20 mg ml^{-1}) was added and the mixture was incubated at 37°C overnight. A total of 50 μl of NaCl solution (5 M) and 40 μl of cetyl trimethyl ammonium bromide (10%)/NaCl (0.7 M) were added and the sample was mixed completely and incubated at 65°C for 10 min. Phenol and chloroform were used for DNA purification, and DNA was precipitated with 3 M acetic sodium solution and 2-propanol. After DNA solution had dried up, the extracted DNA was dissolved in 25 μl of dH_2O.

PCR-DGGE

To analyze the total bacterial community structure, variable region V3 of bacterial 16S rRNA genes (corresponding to positions 341–534 in the *Escherichia coli* sequence) was amplified using primers P2 and P3 (containing a 40-bp GC clump) (Muyzer *et al.,* 1993) and a Takara thermal cycler (Takara Shuzo Co. Ltd, Shiga, Japan) as described previously by Muyzer *et al.* (1993). A DCode Universal Mutation Detection System (Bio-rad, Hercules, CA, USA)

was used for electrophoresis following the manufacturer's instructions. A total of 10 μl of a PCR-amplified mixture was subjected to electrophoresis in a 10% (wt/vol) polyacrylamide gel at 200 V for 3.5 h. The gel gradients used for separation, which were applied in parallel to the electrophoresis direction, were 30–50%. After electrophoresis, the gel was stained with SYBR Green I (Molecular Probes) for 30 min following the manufacturer's instructions. The nucleotide sequences of DGGE bands were determined as described previously (Futamata *et al.,* 2005, Yoshida *et al.,* 2005).

Genetic diagnoses

Five sets of primers were used to evaluate the potential dechlorinating abilities of all enrichment cultures (Table 1). The functional genes targeted in this study were *pceA*, *tceA*, *bvcA*, *vcrA* and the reductive dehalogenase homologous (*rdh*) gene. It has been reported that *rdh* genes are very diverse but include conserved regions, that is, motif of Tat protein export pathway and corrinoid binding motif (Magnuson *et al.*, 2000; Kube *et al.,* 2005). Substrate specificities of some Rdhs were revealed (Magnuson *et al.*, 2000; Suyama *et al.,* 2002). With the exception of a set of primers pceAf and pceAr (targeting *pceA*, hereafter described as a pceAf/Ar), previously reported four sets of specific primers for *tceA*, *bvcA*, *vcrA* and *rdh* genes were used for PCR amplification (Holmes *et al.,* 2006). A set of primers pceAf/Ar was designed by aligning these gene sequences using ClustalW version 1.7 (Thompson *et al.,* 1994), targeting *pceA* (accession number AB070709) from *Desulfitobacterium* sp strain Y51, which reductively dechlorinates PCE to TCE (Suyama *et al.,* 2001, 2002). The validity of specific primers pceAf/Ar was confirmed (shown in Supplementary information). The *tceA* (AF228507) derived from *Dehalococcoides ethenogenes* strain 195 is relevant to reductive dechlorination from TCE to ethene including cometabolism step (Magnuson *et al.*, 2000), and the function of *tceA* (AY165309) from *Dehalococcoides* sp strain FL2 is not yet precisely known. The *bvcA* (AY563562) from *Dehalococcoides* sp strain BAV1 is suggested to reductively dechlorinate VC to ethene (Krajmalnik-Brown *et al.,* 2004), and the *vcrA* (AY322364) from *Dehalococcoides* sp strain VS, which is suggested to reductively dechlorinate TCE to ethene (Cupples *et al.,* 2003, 2004; Sung *et al.,* 2006). Amplification was performed with a Takara thermal cycler (Takara Shuzo) by using a 50 μl mixture containing 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems, Foster City, CA, USA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 100 pmol of each primer and 1.0 μl of extracted DNA solution. The PCR conditions were 10 min to activate the polymerase at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and finally 10 min of extension at 72°C. The availability of pceAf/Ar was confirmed using total DNA of *Dehalococcoides* sp strain Y51 under the PCR conditions as described above. The PCR product with pceAf/Ar was checked by electrophoresis through 1.5% (wt/vol) agarose gel (LO3 agarose; Takara Shuzo) in TBE buffer (Sambrook *et al.,* 1989) and then staining with ethidium bromide. While the PCR products with other specific primers were checked by electrophoresis through 1.2% (wt/vol) polyacrylamide gel and then staining with ethidium bromide, because the purpose length of amplified fragments were less than 100 bp.

Real-time PCR

For measurement of the 16S rRNA gene copy number of the *Dehalococcoides* group, real-time PCR assays were applied to genomic DNA. Template DNAs were prepared as described above. The *Dehalococcoides*-specific primers used for real-time PCR were DHC793f (5′-GGGAGTATCGACCCTCT CTG-3′) and DHC946r (5′-CGTTYCCCTTTCTGTTC ACT-3′) (Hiraishi *et al.,* 2005a; Yoshida *et al.,* 2005),

Table 1 Primers used to diagnose potential dechlorinating abilities for chloroethenes

Primer	*Sequence*	*Positions*	*Target gene*	*Reference(s) or source*
pceAf	5′-ATACTCAACATTTGGGTG-3′	383–400[a]	*pceA* gene	Suyama *et al.* (2002), this study
pceAr	5′-TGAAACCTGCATACTTCTCCC-3′	913–893		
tceAf	5′-ATCCAGATTATGACCCTGGTGAA-3′	446–468[b]	*tceA* gene	Holmes *et al.* (2006)
tceAf	5′-GCGGCATATATTAGGGCATCTT-3′	512–491		
bvcAf	5′-GGTGCCGCGACTTCAGTT-3′	73–90[c]	*bvcA* gene	Holmes *et al.* (2006)
bvcAr	5′-TCGGCACTAGCAGCAGAAATT-3′	140–120		
vcrAf	5′-CTCGGCTACCGAACGGATT-3′	1303–1321[d]	*vcrA* gene	Holmes *et al.* (2006)
vcrAr	5′-GGGCAGGAGGATTGACACAT-3′	1367–1348		
FFR2	5′-SHMGBMGWGATTTYATGAARR-3′	RRXFXK[e]	*rdhA* gene	Krajmalnik-Brown *et al.* (2004)
BIR	5′-CHADHAGCCAYTCRTACCA-3′	WYEW		

[a]Corresponding to the *pceA* sequence of *Desulfitobacterium* sp Y51 (AB070709).
[b]Corresponding to the *tceA* sequence of *Dehalococcoides* ethenogenes strain 195 (AF228507).
[c]Corresponding to the *pceA* sequence of *Dehalococcoides* strain BAVI (AY563562).
[d]Corresponding to the *pceA* sequence of *Dehalococcoides* strain VS (AY322364).
[e]RRXFXK motif is part of the Tat protein export pathway, WYEW is part of the conserved twin arginine motif (Krajmalnik-Brown *et al.*, 2004).

which are capable of detecting diverse *Dehalococcoides* groups (Hiraishi *et al.,* 2005a, b). Standard DNA fragments were produced using a competitive DNA construction kit (Takara Shuzo) according to the manufacturer's recommendations. The PCR profile consisted of preheating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 10 s and extension at 72°C for 20 s. The fluorescence signal was detected at 81°C in each cycle, and a melting curve was obtained by heating the product to 95°C and cooling to 40°C. The reaction was performed using a LightCycler FastStart DNA Master SYBR GREEN I kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The copy number of the amplicons was calculated using LightCycler software version 3.52 (Roche Diagnostics).

Reductive dechlorination of chloroethenes

The enrichment cultures were transferred (5% vol/vol) into fresh DHE2 medium. DHE2, modified DHE0, contains 0.5 g of KH_2PO_4, 0.41 g of $MgCl_2 \cdot 6H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of NH_4Cl, 2.5 g of $NaHCO_3$, 1.0 ml of Se/W solution (Löffler *et al.,* 1996), 1.0 ml of trace elements SL8 solution (Biebl and Pfennig, 1978), 1.0 ml of vitamin solution PV1 (Hiraishi *et al.,* 2002) and 1.0 mg of resazurin. A total of 18 ml of DHE2 medium was put into the reaction bottle (70 ml), and the bottles were sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. The headspace was exchanged with N_2-CO_2 (4:1, vol/vol). Hydrogen gas as an electron donor was added to a level of 8% (vol/vol) in the headspace. The following reagents were filtered: 0.2 mM of L-cystein and Na_2S (pH 7.0), 4 mM of BES, 0.2 mM of acetic acid and butyric acid, and 10 ml of 0.75% titanium (III) citrate solution per liter (pH 7.2). Solutions saturated with PCE or TCE were prepared, and the PCE or TCE solutions were added by microsyringe through the septum at an initial concentration of approximately 10 and 60 μM, respectively. The concentration of chloroethenes used in this study showed liquid concentration after equilibration in liquid and headspace. To investigate reductive dechlorination with cometabolism, enrichment cultures were incubated on all chloroethenes (PCE, TCE, 1,1-DCE, *c*DCE, *t*DCE and VC) under both low (the final concentration of each chloroethene was 5 μM) and high (50 μM) concentrations. The cultures were incubated at 30°C with horizontal shaking for 3 months. The concentrations of ethene, methane and chloroethenes were measured using gas chromatography as described below.

Analytical methods

Methane, ethene and chlorinated ethenes (200 μl of headspace) were analyzed by injecting a 200 μl headspace sample onto a model 353B gas chromatograph equipped with a flame ionization detector (GL Sciences, Shinjuku-ku, Tokyo, Japan) and a GS-Q fused-silica capillary column (length, 30 m; inside diameter, 0.53 mm; J&W Scientific, Agilent Technologies, Santa Clara City, CA, USA). The oven temperature was programmed to maintain a temperature of 50°C for 2 min and then to increase it to 190°C at 50°C/min and finally to maintain the temperature at 190°C for 40 min. The temperature of injection and detection was kept at 220°C. Total mass values were determined using Henry's law constants (Mackay and Shiu, 1981). We used 0.05% and 0.1% gas mixtures of ethene and methane for calibration.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited under DDBJ accession numbers AB288836 to AB288840.

Results

Dominant bacteria in enrichment cultures

PCR-DGGE analysis targeting the 16S rRNA gene was performed to determine what are the dominant bacteria in enrichment cultures (Figure 1). Major bands were excised from DGGE gels, and their nucleotide sequences were determined and used to deduce the relative organisms, although the sequenced DNA lengths were short (only 120–160 bp) for phylogenetic analysis. The sequence of DGGE bands S1, S2 and S3 showed 100% similarity to

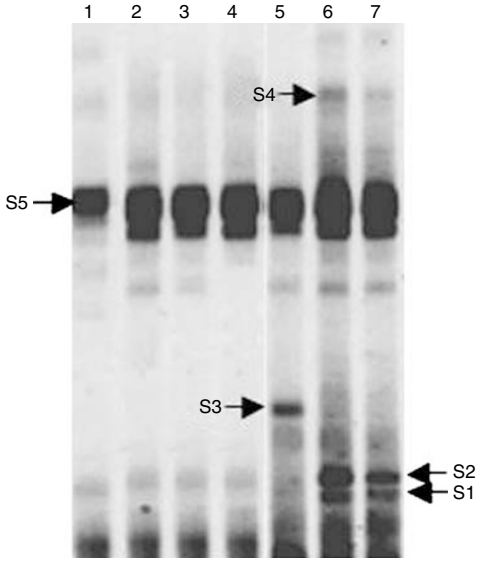

Figure 1 DGGE-profile of PCR-amplified 16S rRNA gene fragments from the cultures enriched on 1,2,3-trichlorobenzene. Lane 1, TUT1902; lane 2, TUT1903; lane 3, TUT1951; lane 4, TUT1952; lane 5, TUT2257; lane 6, TUT2260; lane 7, TUT2264. DGGE, denaturing gradient gel electrophoresis.

uncultured bacteria TSAa22 (accession number AB186816), TSAI15 (AB186755) and TSAF01 (AB186895), which were found in the parent dioxin-dechlorinating microcosm (Yoshida *et al.,* 2005). Major band S4 showed 100% similarity to uncultured *Thermotogales* bacterium clone M79 (AY692052). Major band S5 was observed in all enrichment cultures, showing 100% similarity to the 16S rRNA gene sequence of the Pinellas subgroup of *Dehalococcoides* clusters, that is, strains CBDB1 (AF230641) and FL2 (AF357918). This result is reasonable, because strain CBDB1 cannot degrade chlorinated ethenes but instead dechlorinates some chlorobenzenes (Adrian *et al.,* 2000) and dioxin congeners (Bunge *et al.,* 2003). Strain FL2 dechlorinates TCE and cDCE, but not other chlorinated ethenes (He *et al.,* 2005). It has already confirmed that TUT1902, TUT1903, TUT1951 and TUT1952 include highly enriched *Dehalococcoides* group bacteria as in our previous study (Hiraishi *et al.,* 2005b), while it was shown that three enrichment cultures (TUT2257, TUT2260 and TUT2264) also included the enriched *Dehalococcoides* group bacteria with uncultured bacteria from the parent microcosm.

Dechlorinating abilities of enrichment cultures

To evaluate the potential dechlorinating abilities for chloroethenes, PCR was used as a diagnostic tool targeting functional genes *pceA*, *tceA*, *bvcA*, *vcrA* and *rdhA* (Table 1). Since amplified fragments of the required length were not observed in any samples (Table 2), it was determined that none of enrichment cultures were capable of reductively dechlorinating chloroethenes. However, a culture-dependent technique showed different results: four cultures exhibited reductive dechlorinating activities for some chloroethenes (Table 2). Enrichment cultures TUT1903 and TUT1952 converted PCE to TCE, but not completely (Table 2); culture TUT2260 completely converted PCE to TCE and slightly produced *c*DCE and 1,1-DCE; and TUT2264 completely reductively dechlorinated PCE to TCE and produced *t*DCE and *c*DCE (Figure 2). Not all cultures exhibited reductive dechlorinating activities on all mixed chloroethenes under both low and high concentrations of mixed chloroethenes (Table 2). Methane was observed in TUT2260 and TUT2264 on PCE but not on TCE. Five percent of culture TUT2264 on TCE was enriched in a fresh DHE2 medium on PCE. PCE was completely dechlorinated to TCE, and both *t*DCE and *c*DCE were produced more abundantly than the parent TUT2264 on PCE (Figures 2 and 3). No methane production was observed (Figure 3).

Dechlorinating activities of TUT2260 and TUT2264

PCE-dechlorinating activities of enrichment culture TUT2260 and TUT2264 were evaluated using the method of Holmes *et al.* (2006) (Table 3). Real-time PCR analysis quantified $1.0 \pm 0.1 \times 10^5$ and $1.3 \pm 0.1 \times 10^5$ cells ml^{-1} in TUT2260 and TUT2264, respectively, because the rRNA operons per genome in *Dehalococcoides* is known to be 1 (Kube *et al.*, 2005; Seshadri *et al.*, 2005). The PCE-dechlorinating rate of TUT2260 and TUT2264 was roughly calculated using the initial number of the *Dehalococcoides* 16S rRNA gene copy by applying a protein-to-cell conversion factor of 4.2×10^{-15} g $cell^{-1}$ (Duhamel *et al.,* 2004; Holmes *et al.,* 2006) and 0.5 g of protein/g of cells (He *et al.* 2003, Holmes *et al.,* 2006), resulting in 2.3 ± 2.0 μmol min^{-1} mg^{-1} of protein and 1.5 ± 0.7 μmol min^{-1} mg^{-1} of protein, respectively. These rates were approximately 20-fold less than that of PceA purified from strain 195 (Magnuson *et al.,* 1998). The dechlorinating rate of KB-1 on TCE or *c*DCE, the chloroethenes dechlorinating culture used for bioaugmentation (Major *et al.,* 2002), is approximately 3.2 μmol l^{-1} day^{-1} (Duhamel *et al.,* 2002), and those of TUT2260 and TUT2264 (0.42 ± 0.21 and 0.28 ± 0.01 μmol l^{-1} day^{-1},

Table 2 Reductive dechlorinating abilities for chloroethenes of enrichment cultures

Subculture	*Genetic diagnoses*[a]					*Reductive dechlorinating test*[b]			
	pceA	*tceA*	*bveA*	*vcrA*	*rdhA*	*PCE*	*TCE*	*Mix (low, high)*[c]	
TUT1902	–	–	–	–	–	–	–	–	–
TUT1903	–	–	–	–	–	+(TCE)	–	–	–
TUT1951	–	–	–	–	–	–	–	–	–
TUT1952	–	–	–	–	–	+(TCE)	–	–	–
TUT2257	–	–	–	–	–	–	–	–	–
TUT2260	–	–	–	–	–	+(TCE)	+(1,1-DCE, cDCE)	–	–
TUT2264	–	–	–	–	–	+(TCE)	+(tDCE, cDCE)	–	–

Abbreviations: cDCE, *cis*-1,2-dichloroethylene; 1,1-DCE, 1,1-dichloroethylene; PCE, tetrachloroethene; TCE, trichloroethene; tDCE, *trans*-1,2-dichloroethylene.
[a]Amplified fragments of purpose length were not observed.
[b]No reductive dechlorination occurred; +, reductive dechlorination occurred; the metabolite is given in parentheses.
[c]Reductive dechlorinating activities of subcultures were investigated on all mixed chloroethenes under low (each 5 μM) or high (50 μM) concentrations.

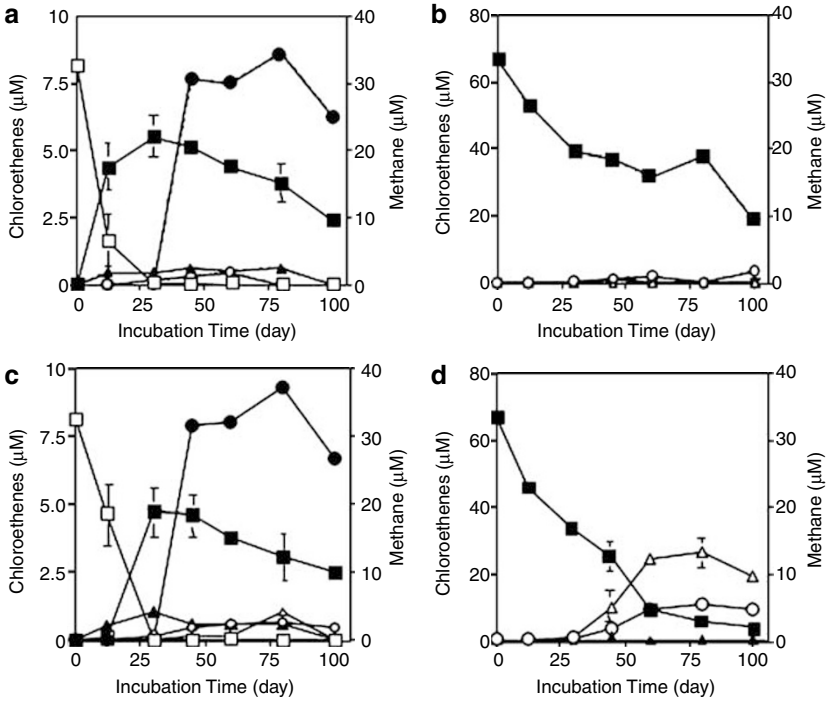

Figure 2 Chloroethene-dechlorination of TUT2260 and TUT2264. TUT2260 on PCE (**a**) and on TCE (**b**), and TUT2264 on PCE (**c**) and on TCE (**d**). Open squares, PCE; filled squares, TCE; open circle, cDCE; filled triangle, 1,1-DCE; open triangle, tDCE; filled circle, methane. Error bars are s.d. of results for three replicate cultures. cDCE, *cis*-1,2-dichloroethylene; 1,1-DCE, 1,1-dichloroethylene; PCE, tetrachloroethene; TCE, trichloroethene; tDCE, *trans*-1,2-dichloroethylene.

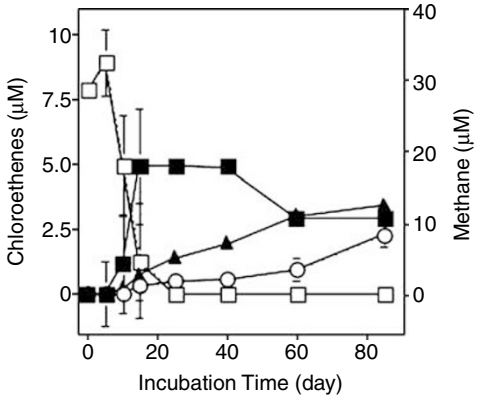

Figure 3 PCE dechlorination of TUT2264 from TUT2264 on TCE. Open squares, PCE; filled squares, TCE; open circle, cDCE; filled triangle, tDCE. Error bars are s.d. of results for three replicate cultures. cDCE, *cis*-1,2-dichloroethylene; PCE, tetrachloroethene; TCE, trichloroethene; tDCE, *trans*-1,2-dichloroethylene.

Table 3 Comparison of PCE reductive dechlorinating rate

Culture/protein	*PCE-dechlorinating rate*	
	$\mu mol\ min^{-1}\ mg^{-1}\ protein$[a]	$\mu mol\ l^{-1}\ day^{-1}$
TUT2260	2.3 ± 2.0[b]	0.42 ± 0.21[b]
TUT2264	1.5 ± 0.7[b]	0.28 ± 0.01[b]
	(3.4 ± 1.3)[c]	(0.78 ± 0.02)[c]
KB-1/TCE	NR[d]	3.2 ± 0.2[e]
PCE-RDase[f]	20	NR[d]

Abbreviation: PCE, tetrachloroethene.
[a]The dechlorinating rate was calculated using cell number of *Dehalococcoides* group bacteria.
[b]PCE reductive dechlorinating rate was measured on medium DHE0.
[c]PCE reductive dechlorinating rate was measured on medium DHE2.
[d]The datum was not reported.
[e]Duhamel *et al.* (2002).
[f]PCE-Rdase was purified from *Dehalococcoides ethenogenes* 195 (Magnuson *et al.*, 1998).

respectively) were 10-fold less than that of KB-1. However, TUT2264 on TCE was transferred into a fresh DHE2 medium with PCE, after which the reductive dechlorinating ability was improved (Figure 3); the PCE-dechlorinating rate and amount of DCEs increased approximately 2.5-fold without methane production, suggesting that TUT2264 rapidly shifts to a bacterial community structure capable of dechlorinating chloroethenes.

Discussion

In the present study, to obtain more information about reductive dechlorinators, we investigated whether 1,2,3-TCB- or fthalide-dechlorinating enrichment cultures from dioxin-dechlorinating microcosm are capable of dechlorinating chloroethenes reductively. Interestingly, four enrichment cultures reductively dechlorinated PCE or TCE. The diagnostic technique targeting functional genes was not able to correctly estimate the dechlorinating abilities

of the four cultures, although these functional genes have been used to predict the potential for reductive dechlorination, discrimination and monitoring of multiple *Dehalococcoides* strains as well as to analyze the dechlorinating bacterial community structure (Holmes *et al.,* 2006; Lee *et al.,* 2006; Ritalahti *et al.,* 2006; Sung *et al.,* 2006). Full-genome sequence analyses revealed that there are 18 and 32 kinds of *rdh* genes in strains 195 and CBDB1, respectively (Kube *et al.,* 2005; Seshadri *et al.,* 2005). Although the primer set FFR2/B1R amplified 14 of the 32 *rdh* genes in strain CBDB1 (Hölscher *et al.,* 2004), no purpose amplicons were observed in any enrichment cultures in which strain CBDB1-like bacteria were dominant. It has previously been demonstrated that the *Dehalococcoides* bacterial group is functionally very diverse, despite the fact that its members are phylogenetically close to each other with over 99.9% similarity of 16S rRNA gene sequence (Maymo-Gatell *et al.,* 1997; Adrian *et al.,* 2000; Hendrickson *et al.,* 2002; He *et al.,* 2003; Duhamel *et al.,* 2004; Krajmalnik-Brown *et al.,* 2004). Neither of the strains in the Pinellas subgroup of *Dehalococcoides* clusters, strains CBDB1 and FL2, exhibit PCE-dechlorinating abilities. These suggest that novel *Dehalococcoides* group bacteria possessing newly functional genes might be present in the TUT cultures, if the bacteria played a major role for PCE- and TCE-dechlorination.

The proportion of the population density of *Dehalococcoides* group bacteria to total bacteria (P_{deh}) increased to average 3.3% in TUT2260 and TUT2264, although P_{deh} was 0.1% in the parent microcosm (Yoshida *et al.,* 2005). Actually, PCE-dechlorinating activity of TUT2264 increased from 0.28 ± 0.01 to $0.78 \pm 0.02\,\mu mol\,l^{-1}\,day^{-1}$. These results suggest that *Dehalococcoides* group bacteria have adapted to respire on chloroethenes from polychlorinated dioxins or chloroaromatic compounds, resulted in the enrichment of *Dehalococcoides* group bacteria in TUT2264. However, it is unknown that only *Dehalococcoides* group bacteria played a major role of PCE- and TCE-reductive dechlorination in TUT2264, because *Dehalobacter* strains are PCE- and TCE-reductive dechlorinators as well as *Dehalococcoides* group bacteria (Holliger *et al.,* 1998). Therefore, although it is sure that TUT2264 contains *Dehalococcoides* group bacteria, there are three possibilities about what was a major dechlorinator in TUT2264; 1) novel *Dehalococcoides* group bacteria possessing newly functional genes were, 2) other bacteria, that is, *Dehalobacter* or unknown bacteria were, 3) *Dehalococcoides* group bacteria, *Dehalobacter* and others coexisted. This question will be dissolved using species-specific quantitative PCR.

Not all enrichment cultures exhibited reductive dechlorinating activities under low and high concentrations of all mixed chloroethenes (Table 2). It is known that some *Dehalococcoides* strains reductively dechlorinate chloroethenes by cometabolism, that is, strain 195 reductively dechlorinates *t*DCE and VC on PCE (Maymo-Gatell *et al.,* 1997; He *et al.,* 2005). The present results suggest that these cultures might not have a cometabolic system for dechlorination. It remains unclear why the cultures lose their dechlorinating activities on mixed chloroethenes; however, it is possible that there might be a mechanism that suppresses on transcription or translation with chloroethenes or their by-products.

In conclusion, some TUT enrichment cultures exhibited reductive dechlorinating activities for some chloroethenes, although these parent cultures had been enriched on dioxin or chloroaromatic compounds. Therefore, it is conceivable that the chloroethene-dechlorinating TUT cultures, especially enrichment culture TUT2264, have potentially dechlorinating abilities of both chlorodioxins and chlorobenzenes. Furthermore, it is interesting in the evolution of TUT2264 improving chloroethenes-dechlorinating ability. It remains to clarify the functional genes and bacterial community structure in TUT2264. These subjects are currently under investigation in our laboratory.

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