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Isolation and characterization of tetrachloroethylene and *cis*-1,2-dichloroethylene-dechlorinating propionibacteria

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Running Title: Isolation and characterization of propionibacteria for dechlorination

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Abstract

Two rapidly growing propionibacteria that could reductively dechlorinate tetrachloroethylene (PCE) and *cis*-1,2-dichloroethylene (*cis*-DCE) to ethylene were isolated from environmental sediments. Metabolic characterization and partial sequence analysis of their 16S rRNA genes showed that the new isolates, designated as strains *Propionibacterium* sp. HK-1 and *Propionibacterium* sp. HK-3, did not match any known PCE or *cis*-DCE-degrading bacteria. Both strains dechlorinated relatively high concentrations of PCE (0.3 mM) and *cis*-DCE (0.52 mM) under anaerobic conditions without accumulating toxic intermediates during incubation. Cell-free extracts of both strains catalyzed PCE and *cis*-DCE dechlorination; degradation was accelerated by the addition of various electron donors. PCE dehalogenase from strain HK-1 was mediated by a corrinoid protein since the dehalogenase was inactivated by propyl iodide only after reduction by titanium citrate. The amounts of chloride ions (0.094 mM and 0.103 mM) released after PCE (0.026 mM) and *cis*-DCE (0.05 mM) dehalogenation using the cell-free enzyme extracts of both strains, HK-1 and HK-3, were stoichiometrically similar (91% and 100%), indicating that PCE and *cis*-DCE were fully dechlorinated. Radiotracer studies with [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE indicated that ethylene was the terminal product: partial conversion to ethylene was observed. Various chlorinated aliphatic compounds (PCE, trichloroethylene, *cis*-DCE, *trans*-1, 2-dichloroethylene, 1, 1-dichloroethylene, 1, 1-dichloroethane, 1, 2-dichloroethane, 1, 2-dichloropropane, 1, 1, 2-trichloroethane, and vinyl chloride) were degraded by cell-free extracts of strain HK-1.

Key words: Tetrachloroethylene, *cis*-1,2-dichloroethylene, chlorinated aliphatic compounds, corrinoid, Propionibacteria, PCE dehalogenase

Introduction

Tetrachloroethylene (PCE), an effective and widely used degreasing solvent and fumigant, is a frequently detected recalcitrant xenobiotic pollutant in soil and groundwater around the world [19]. PCE can be reductively dechlorinated by anaerobic microorganisms to trichloroethylene (TCE) and *cis*-1,2-dichloroethylene (*cis*-DCE) [1, 7, 16, 26, 38, 45, 48, 51]. However, for anaerobic bioremediation to be useful, PCE must be degraded to nonchlorinated, environmentally harmless products. Environmental accumulation of *cis*-DCE and vinyl chloride (VC) is undesirable, because *cis*-DCE is a suspected carcinogen and VC is toxic. Some bacteria have been isolated, including *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. BAV1, FL2, and GT, that can dechlorinate *cis*-DCE via VC to ethane [21, 22, 37, 46, 48]. The strain 195 is the only known microorganism capable of dechlorinating PCE to ethene. Similarly, mixed cultures capable of complete conversion of PCE or TCE to ethene invariably contain organisms closely related to *Dehalococcoides.* Hendrickson *et al.* [24] demonstrated the importance of *Dehalococcoides* in bioremediation by conducting an extensive survey of the presence of *Dehalococcoides* at multiple contaminated sites. The collected evidence from many studies has led to such great interest in the *Dehalococcoides* group of microorganisms.

Hydrocarbon biodegradation can be carried out by a variety microorganisms; activity is generally thought to be ubiquitous. This was also supposed for the biodegradation of PCE and TCE to *cis*-DCE. However, the surprising finding from more than a decade of research is that only one species of bacteria within the genus *Dehalococcoides* has been isolated that can dechlorinate PCE and *cis*-DCE to ethene. However, it would be imprudent to assume that no organisms outside the *Dehalococcoides* group may be capable of the reductive dehalogenation of PCE and *cis*-DCE to innocuous compounds such as ethane.

There is, therefore, interest in the discovery and identification of anaerobes capable of converting PCE and *cis*-DCE without the accumulation of harmful by-products. There is also interest in characterizing the degradation reactions. This work reports the dehalogenation of PCE and *cis*-DCE by cell-free extracts of two isolated microorganisms (*Propionibacterium* sp. HK-1 and *Propionibacterium acnes* HK-3). Data are shown that, for the first time, indicate bacterial degradation of PCE by Propionibacteria may be possible without the accumulation of toxic intermediates.

Materials and Methods

Chemicals

All chemicals were of analytical grade and purchased from Kanto Chemical (Tokyo, Japan). *cis*-DCE was from Tokyo Chemical Industry (Tokyo, Japan) and other chlorinated chemicals (PCE, TCE, *trans*-1,2-dichloroethylene (*trans*-DCE), 1,1-dichloroethylene (1,1-DCE), 1,1-dichloroethane, 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2-trichloroethane, and VC) were purchased from GL

science (Tokyo, Japan). [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Enrichment and Isolation

40 soil samples were collected aseptically in Hokkaido, Japan from near a factory of electrical parts, near a dry-cleaner's and from around a livestock farm. 5 sewage sludge samples were also taken. Organisms in the samples were enriched and cultivated under anaerobic conditions by inoculating 3 g samples into 50-ml serum bottles with 30 ml MY medium of the following composition (per liter of deionized water): K_2HPO_4, 0.2 g; NaCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; $FeCl_3 \cdot 6H_2O$, 0.0083 g; $MnCl_2 \cdot 4H_2O$, 0.014 g; $NaMoO_4 \cdot 2H_2O$, 0.017 g; $ZnCl_2$, 0.001 g; yeast extract, 2.0 g; glucose, 2.0 g; *L*-cystein, 0.5 g; resazurin, 0.001 g; at pH 7.2. After autoclaving 30 ml of the medium, the headspaces of the bottles were purged with N_2 gas (>99.9%) for 15 min, and sealed with Teflon-lined rubber septa and aluminium crimp caps. PCE and *cis*-DCE were injected into the bottles via microsyringe at final concentrations of 0.06 mM and 0.1 mM, respectively. Samples were incubated at 30 °C and 120 rpm for 30 days in the dark. To identify cultures that did not accumulate toxic by-products of PCE and *cis*-DCE, the headspaces of the sample bottles were periodically analyzed by gas chromatography. Only two enrichment cultures, from ditch sludge of a dry cleaning shop (Muroran, Hokkaido, Japan), did not accumulate toxic by-products of PCE and *cis*-DCE from all the samples tested. Whenever PCE and *cis*-DCE were degraded, cultures were used to inoculate second-generation cultures, which were then used subsequently to inoculate third-generation cultures, and so on, up to six successive generations. To isolate colonies, 10-fold dilution of the enrichment culture was spread on petri plates containing MY medium with 1.5% agar. A sterile glass tube (0.4 mm i.d×20 mm), filled with cotton fiber soaked with PCE (0.06 mM) or *cis*-DCE (0.1 mM) solution was attached to the inside lid of the petri plate. The plate was then incubated under anaerobic conditions using Anaerobic Gas Generation Kit (Oxoid Ltd, Hants, UK). The procedure was repeated twice to ensure pure cultures. By the above-mentioned isolation procedure, two representative PCE and *cis*-DCE-degrading bacteria were successfully isolated.

DNA sequencing and phylogenetic analysis

For the phylogenetic identification of the two isolates, 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) with a pair of universal primers, 27f and 1392r under standard conditions. The PCR mixture contained 1 μL 10 pmol each primer, 5 μL 10×Ex Taq buffer, 4μ L 2.5 mM each dNTP, 0.25 μL Takara Ex Taq HS (TAKARA BIO, Shiga, Japan), 2 μL DNA extract in a final volume of 50 μL. After initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s were performed. Primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min followed. A final extension was then carried out at 72 °C for 7 min. The PCR product was purified with an ExoSAP-IT (GE Healthcare) PCR purification kit and sequenced using BigDye Teminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 Genetic Analyzer

(Applied Biosystems, Foster City, CA, USA). The sequences determined in this study were compared with other gene sequences in the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences determined in this study and data retrieved from the GeneBank database were aligned using CLUSTAL W. The alignments were refined by visual inspection. A neighbor-joining tree was constructed using a TreeView software package. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates). Physiological characteristics of the isolates were also determined by commercially available identification systems (API 20 A; bioMérieux, Japan).

Degradation experiments

The kinetics of degradation of PCE (0.06, 0.12, 0.3, and 0.6 mM) and *cis*-DCE (0.1, 0.21, 0.52, and 0.98 mM) were determined at 30 ºC using 20 ml MY medium in 50 mL serum bottles, covered with Teflon-lined caps. Two isolated strains were pre-grown on MY medium for 72 h. Pre-cultures were harvested by centrifugation (8000×g, 4 °C, 10 min), washed twice with 50 mM phosphate buffer (pH 7.2), and suspended in MY medium. 200 µL cell suspension inoculated in the medium had an optical density at 590 nm (OD_{590}) of 0.03. The gas phase was N_2. Samples were incubated at 30 °C and 120 rpm for two weeks.

Preparation of cell extracts

Two isolates were pre-grown on MY medium for 96 h. Pre-cultures were harvested by centrifugation (8000×g, 4 °C, 10 min), and suspended in MY medium. 2 ml cell suspension (0.33 mg protein/ ml) was transferred to a 120 mL serum bottle containing 78 mL fresh medium. The culture medium and headspace of the bottle were aseptically purged with N_2 for 15 min. Samples were incubated at 30 °C and 120 rpm for 72 h. Cells (1.2 g wet weight) were harvested by centrifugation (8000×g, 4 °C, 15 min), resuspended in 6 ml 50 mM Tris-HCl (pH 7.5) containing 2 mM dithiothreitol (DTT), and, 5% glycerol, and lysed in an ice bath, using a BRANSON (Danbury, CT, U.S.A.) ultrasonic disrupter SONIFIER 250 at 30 s flash for 5 min. Unbroken cells and debris were recovered by centrifugation (18800×g) and resonicated. Both supernatants were pooled and filtered through a 0.22 µm filter (ADVENTEC, DISMIC-25AS, Bedford, USA). The filtrate served as the enzyme extract.

Enzyme assays

Enzyme activity was determined through PCE and *cis*-DCE degradation. Assays were carried out in 20 mL serum bottles equipped with Teflon septa. The assay mixture comprised: 1 mL enzyme, 3 mL 50 mM Tris-HCl buffer (pH 7.5), 2 mM DTT and 5 mM glucose. The headspace was purged with N_2 gas. PCE (0.12 mM) or *cis*-DCE (0.21 mM) were then injected and incubated at 30 °C for a maximum of 2 h. The reaction was terminated by adding 0.2 mL 5 M H_2SO_4. Headspace samples were analyzed by gas

chromatography. Protein was quantified by the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, California, U.S.A.).

Effect of electron donors

The effects of electron donors on PCE and *cis*-DCE degradation was studied. 1 mL enzyme (0.79 mg protein ml^{-1}) was transferred to a 20 ml serum bottle containing 3 ml 50 mM Tris-HCl (pH 7.5) and DTT (2 mM). The influences of various potential electron donors (ethanol, methanol, glucose, yeast extract, pyruvate, fumarate, acetate, lactate, methyl viologen and formate) were examined at final concentrations of 5 mM; except for yeast extract, which was used at 2.0 g/L). Hydrogen (0.9 µM) was tested at a partial pressure of 0.5×10^5 Pa. The bottles were purged with N_2 gas and sealed with Teflon-coated stoppers before PCE (0.12 mM) and *cis*-DCE (0.21 mM) were added.

Degradation of other aliphatic compounds

Enzymes were used at 1.2 mg protein ml^{-1} and compared with controls, containing no enzyme. The initial concentration of each compound was 0.12 mM. After 1 hour, the degradations of PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, 1,1-dichloroethane, 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2-trichloroethane, and VC were determined by gas chromatography.

Effect of propyl iodide on PCE degradation

Cell extract (1 mL; 1.2 mg protein mL^{-1}) was added to 3 ml 0.1 M Tris-HCl (pH 7.5) in a glass bottle wrapped in aluminium foil. Dehalogenase activity was measured in the test system described above except that titanium (III) citrate (2 mM) was used instead of DTT. Propyl iodide, 1-iodopropane (PI; 0.5 mM) was added and the cell extract was illuminated (250 W lamp) for 5 min.

Gas chromatographic analysis of substrates

PCE and TCE were identified and quantified by static-headspace analysis using a gas chromatograph. PCE, TCE and DCE isomers in 250 µL headspace samples were determined using a GC-8A gas chromatograph (Shimadzu Co., Japan) equipped with a flame ionization detector (FID) and a glass column (i.d. 3.2 φ×2.l m; Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). The column was maintained at 60 °C. The injector and detector were kept at 140 °C. The gas samples were analyzed two to three times to verify reproducibility. Ethylene, ethane, and carbon dioxide were analyzed by gas chromatography using a Shimadzu GC-14B, equipped with a glass column, Unibeads C 60/80 (i.d. 3.2 φ×2.l m), and a thermal conductivity detector (TCD). Helium was used as a carrier gas. GCMS analysis was conducted with a Shimadzu GC/MS system (GCMS-QP2010) and an Rxi-5 ms capillary column (30 m, 0.25 mm ID, 1.00 lm *df*; Restek, Pennsylvania, USA). The column temperature program during GC-MS analysis of metabolites was as follows: held at 60 °C for 2 min, increased to 300 °C at 20 °C per

min, and held at 300 °C for 5 min. The injection, interface, and ion-source temperatures were 280, 280, and 250 °C, respectively. Helium (99.995%) was used as a carrier gas at a flow rate of 1.0 ml min^{-1}.

Degradation of [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE

Microcosm preparation, sampling procedures, and methods for verification of ^{14}C-labeled volatile compounds (PCE, TCE, *cis*-DCE, VC, ethylene, and ethane) and $^{14}CO_2$ are well described by Freedman and Gossett [14]. Degradations of [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE by two isolates were performed in 120 ml serum bottles containing 50 ml of MY medium. Pre-cultures were inoculated in the medium. Thereafter, culture medium and the bottles' headspaces were purged with N_2 for 20 min and then spiked with 40,000 dpm [1, 2-^{14}C] PCE (specific activity = 0.6 mCi $mmol^{-1}$) or [1, 2-^{14}C] *cis*-DCE (specific activity = 0.6 mCi $mmol^{-1}$). Labelled PCE and *cis*-DCE were added along with the final addition of unlabeled PCE and *cis*-DCE. ^{14}C-labeled volatile compounds were analyzed with by GC-combustion [14]. After incubation for 10 days, the reaction was stopped by bringing the pH to 2.5 with perchloric acid (3M). Chorinated compounds released by acidification were fractionated by GC and trapped individually in ScientiVerse-E liquid scintillation cocktail (Fisher Scientific). The CO_2 generated was absorbed by CO_2 absorption liquid (Carbo Sorb E, Perkin Elmer, USA). Radioactivity was measured in an LS 6500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). The total disintegrations per minute (dpm) in a bottle of each compound were calculated using the appropriate Henry constants [18].

Other analysis

The concentration of chloride ions released during PCE and *cis*-DCE degradation was measured by an ionic chromatograph Dionex ICS-1000 equipped with a conductivity detector (Dionex Co., CA, USA), using a 4 mm anionic exchanger column, IonPack AS9-HC. The volume of injection was 25 µL. The mobile phase was 9 mM sodium carbonate solution with flow rate of 1 ml min^{-1}.

Data analysis

All results are indicated as mean values with standard deviations (±95% confidence interval) of triplicate experiments, except the experiment of PI effect on PCE degradation. Significant difference was determined by Student's *t* testing with $p < 0.05$.

Nucleotide sequence accession number

The 16S rRNA gene sequences of the isolates (strain HK-1 and strain HK-3) determined in this study were deposited in the DDBJ under accession nos. AB540663 and AB540664, respectively.

Results

Taxonomy of isolated organisms

The isolated strains were named HK-1 and HK-3. The PCE and *cis*-DCE-degrading organisms were anaerobic, Gram-positive, and rod-shaped bacteria. Colonies of each strain appeared white when cultured on MY agar plates with vapors of PCE (0.12 mM) or *cis*-DCE (0.21 mM). The strains showed similar physiological characteristics, they were not able to produce indole, and produced β-glucosidase but not protease (Table 1). The strains displayed positive catalase and oxidase activities but did not produce urease. Based on these characteristics, both strains were considered to belong to the genus *Propionibacterium*. As shown in the phylogenetic tree (Fig. 1), both strains were identified as *Propionibacterium* sp. 16S rRNA gene sequence analysis showed that strain HK-1 was closely related to *Propionibacterium acidipropionici* DH42 AY360222 (98.2% sequence identity). The 16S rRNA gene of the strain HK-3 completely agreed with that of *Propionibacterium acnes* W1392 AY642051 (100% sequence identity).

Biodegradation of PCE and cis-DCE

Figure 2 shows time courses of the ratios of residual to initial PCE and *cis*-DCE concentrations, and the cell densities (OD_{590}) of liquid cultures of strains HK-1 and HK-3. Tests of PCE degradation by strain HK-1 at high concentrations (0.06, 0.12, 0.3, and 0.6 mM) showed that PCE was not degraded at 0.6 mM. However, when its initial concentration was 0.3 mM, PCE degradation commenced after 24 h and was degraded up to 64% during the incubation period. Similar was observed at 0.06 and 0.12 mM, but PCE degradation was slower than at 0.3 mM PCE. The amount of biomass increased during cultivation. *cis*-DCE was not degraded at 0.98 mM by HK-3, though it was rapidly degraded at all other tested concentrations (0.1, 0.21, and 0.52 mM). PCE and *cis*-DCE were not the sole carbon/energy sources for the growth of these strains (data not shown).

Effect of electron donors and chloride release

The rate of PCE degradation varied with different electron donors (Fig. 3). Methyl viologen, glucose, and yeast extract enhanced PCE degradation. Other electron donors (hydrogen, lactate, and acetate) were slightly more specific than the control for PCE degradation. Although methyl viologen most effectively enhanced PCE degradation, it was not used in subsequent experiments because of concerns for human safety. Similar results were obtained on *cis*-DCE degradation experiment. Based on these results, glucose was chosen as the most effective electron donor for PCE and *cis*-DCE degradation. To determine whether chloride ions were stoichiometrically released during PCE and *cis*-DCE degradation, the chloride ion contents in the enzyme extracts of both strains were determined (Fig. 4). Chloride concentrations generated from PCE after 20, 40 and 60 minutes were 0.022, 0.057, and 0.093, respectively, indicating that *ca*. 91% of stoichiometric chloride ions were generated from PCE (Fig. 4 (A)). Therefore, most of the PCE was dechlorinated by the cell-free extracts of strain HK-1. Chloride concentrations from *cis*-DCE after 20, 40, and 60 minutes were 0.019, 0.084, and 0.096, respectively, consistent with the complete dechlorination of *cis*-DCE (Fig. 4 (B)).

Inactivation of PCE degradation by propyl iodide

The effects of propyl iodide and titanium citrate on PCE degradation by the cell-free enzyme extract are presented in Fig. 5. The extract lost 80% of its initial PCE degradation activity upon incubation with propyl iodide and titanium (III) citrate in the dark within 10 min. Subsequent exposure to light restored 80% of the activity within 20 min. Titanium citrate in the absence of propyl iodide did not have any inhibitory effect and no inhibition was recorded with propyl iodide alone (data not shown).

Degradation of other aliphatic compounds

The degradation of other halogenated aliphatic compounds by crude enzyme was investigated. PCE pregrown enzyme resulted in degradations of PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, 1,1-dichloroethane, 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2-trichloroethane, and VC of 20%, 16% , 25%, 16%, 23%, 23%, 27%, 10%, 25%, and 30%, respectively (Table 2). When *cis*-DCE pregrown enzyme was tested, the degradations of *cis*-DCE, *trans*-DCE, 1, 1-DCE, and VC were as 35%, 18%, 25%, and 40%, respectively. However, PCE, TCE, 1, 1-dichloroethane, 1, 2-dichloroethane, 1, 2-dichloropropane, and 1, 1, 2-trichloroethane showed no degradation after extended incubation (6 h). The degradation of VC was more than that of PCE or *cis*-DCE in reactions with PCE and *cis*-DCE crude enzyme.

Degradation of [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE

Radioisotope experiments were performed with [1, 2-^{14}C] PCE. Radioactivities in 10-day cultures were recovered in ethylene (7%), and cells (8%) in the radioisotope experiments. Although only 15% of radioactivity was recovered in ethylene, the mechanism was concluded to involve reductive dechlorination since the intermediate (ethylene) of reductive dechlorination was observed. Radioisotope experiments were also performed with [1, 2-^{14}C] *cis*-DCE, where radioactivity was only recovered in ethylene (4%). Other metabolites (TCE, *cis*-DCE, VC, and ethane) were not detected. Negligible amounts of radioactivity (<1%) was observed in cells.

Discussion

PCE and *cis*-DCE-degrading anaerobic bacteria, *Propionibacterium* sp. HK-1 and *Propionibacterium* sp. HK-3 were isolated and characterized. 16S rRNA gene sequencing revealed that strains HK-1 and HK-3 were not similar to any of the following known PCE or *cis*-DCE-degrading bacteria: *Desulfuromonas michiganensis* BB1 (AF357915) [48], *Desulfuromonas michiganensis* BRS1 (AF357915) [48], *Dehalococcoides* sp. H10 (AY914178) [50], *Desulfitobacterium* sp. B31e3 (AB289347) [56], *Geobacter lovleyi* SZ (AY914177) [49], *Geobacter lovleyi* GT (AY914178) [[50], *Sulfurospirillum halorespirans* PCE-M2 (AF218076) [35], *Desulfitobacterium hafniense* Y51 (AB049340) [51], *Desulfitobacterium hafniense* JH1 (EU523374) [13], *Dehalococcoides ethenogens* 195 (AF004928) [37],

Desulfitobacterium sp. PCE-S (AJ512772) [39], *Desulfitobacterium* sp. PCE1 (X81032) [16], *Desulfitobacterium* sp. KBC1 (AB194704) [54], *Desulfitobacterium hafniense* TCE1 (X95742) [17], *Dehalobacter restrictus* (U84497) [27], *Sulfurospirillum multivorans* (X82931) [45], *Desulfuromonas chloroethenica* (U49748) [31], *Desulfitobacterium dehalogenans* (L28946) [55], *Desulfomicrobium norvegicum* (AJ277897) [25], *Gammaproteobacterium* MS-1 (L43508) [47], *Dehalococcoides* sp. FL2 (AF357918) [23], *Dehalococcoides* sp. BAV1 (AY165308) [22], *Desulfitobacterium* sp. Viet-1 (AF357919) [33], *Desulfitobacterium hafniense* PCP-1 (U40078) [10], *Desulfitobacterium chlororespirans* (U68528) [34, 44], *Desulfomonile tiedjei* DCB-1 (M26635) [40], *Desulfitobacterium hafniense* DCB-2 (AY013365) [32], *Clostridium* sp. KYT-1 (AB214911) [30], *Acetobacterium woodii* (DD223101) [52], *Rhodococcus* sp. Sm-1 (DQ834672) [42], *Xanthobacter flavus* (DQ834674) [28]. Although several electron donors effectively enhanced PCE and *cis*-DCE dechlorination, glucose and yeast extract were the most effective. No growth or reductive dechlorination of PCE or *cis*-DCE was observed when glucose or yeast extract were eliminated from the MY medium. This observation suggests that glucose and yeast extract served sources of nutrients and electrons.

Reductive dechlorination of PCE has been achieved by many pure cultures belonging to four different metabolic groups: halorespirers, acetogens, methanogens, and facultative anaerobes [9]. To date, only *Dehalococcoides ethenogenes* strain 195 has been known to degrade PCE to nontoxic ethene, while most other natural biotic processes degrade PCE to toxic products [1, 7, 16, 26, 37, 38, 45, 48, 51, 56]. PCE was long thought to be non-biodegradable in the presence of oxygen, but PCE degradation by *Pseudomonas stutzeri* OX1, involving a toluene-o-xylene monooxygenase has been reported [43]. Aerobic PCE degradation has also been reported by the white-rot fungus *Trametes versicolor* [11]. Reductive dehalogenation has been shown to be a major mechanism in the complete degradation of PCE, though other reactions or mechanisms may also facilitate the complete degradation of PCE. An anaerobic oxidative degradation of *cis*-DCE and VC under Fe(III)-reducing and humic acid-reducing conditions has been reported [3, 4, 5]. Under such conditions, no dechlorinated ethenes were detected during the experiments [3, 4, 5].

In this study, no chlorinated ethenes were observed during PCE degradation with cells and cell-free extracts of strain HK-1. *Ca.* 91% of the stoichiometric amount of chloride ions was generated during the incubation of cell-free extracts containing PCE. This indicates that most of the PCE was dechlorinated. Strain HK-3 released chloride ions stoichiometrically with *cis*-DCE dehalogenation, indicating that *cis*-DCE was fully dechlorinated. Similar results were observed in *Clostridium* species. Hata *et al.* [20] and Kim *et al.* [30] reported that *Clostridium* species, such as *Clostridium* sp. DC1, *Clostridium butyricum* NBRC 3315, *Clostridium acetobutylicum* NBRC 13949, and *Clostridium* sp. KYT-1, also showed ability to degrade *cis*-DCE without any accumulation of VC or ethylene. The two isolates were able to dechlorinate PCE and *cis*-DCE in the presence and absence of Fe(III) in the MY medium, respectively. This finding supports that the anaerobic degradation of chlorinated ethenes is not linked to the reduction of Fe (III) to Fe (II) [20]. A similar result was demonstrated by *Clostridium* sp. DC1, which has also been reported to dechlorinate *cis*-DCE in the presence or absence of Fe (III) [20]. The origin of the carbon in the CO_2 gas, whether from PCE or from the materials in the medium, was studied. Radiotracer studies with [1, 2-^{14}C] PCE and [1, 2-^{14}C] *cis*-DCE showed that ethylene was the terminal

product; partial conversion to ethylene was observed. However, the unrecovered reaction products of PCE and *cis*-DCE remain unknown. This requires further study to elucidate the mechanism and products.

Anaerobic halorespiring bacteria (dehalorespirers) are widely accepted as being important to biologic dechlorination in anoxic environments. Compared with dehalorespirers, co-metabolic PCE dechlorination proceeds more slowly. The rates of PCE dechlorination by *Methanosarcia* sp. and *Acetobacterium woodii* were 3.5×10^{-5} and 3.6 nmol h^{-1}·mg $protein^{-1}$, respectively [12]. In comparison, PCE dechlorination by *Sulfurospirillum multivorans*, *Dehalococcoides ethenogenes* strain 195, *Clostridium bifermentans* DPH-1, and *Dehalobacter restrictus* (strain PER-K23) has been reported to occur at 3.0, 4.14, 0.4, and 1.0 μmol h^{-1}·mg $protein^{-1}$ [7, 26, 45]. Dechlorination by strain HK-1 occurred at a rate (18 nmol h^{-1}·mg $protein^{-1}$) close to that of bacteria which degrade PCE co-metabolically but lower than that of dehalorespires. However, the PCE dechlorination rate was higher than that of aerobic white-rot fungus *Trametes versicolor* (2.75 nmol h^{-1}·mg $protein^{-1}$) [11]. In Figure 2, at the highest concentrations of PCE and *cis*-DCE, there was no degradation but substantial growth was observed (cell growth with high PCE), possibly evidence of a co-metabolic process.

Protein-bound cobalamin from a number of anaerobic organisms e.g., *Desulfitobacterium hafniense* Y51, *Dehalococcoides ethenogenes*, *Desulfitobacterium* sp. strain PCE-1, *Clostridium bifermentans* DPH-1, *Desulfitobacterium* sp. PCE-S, *Sulfurospirillum multivorans, Sporomusa ovata* (homoacetogenic strain), *Dehalobacter restrictus*, and *Methanosarcina thermophila* have been reported to degrade PCE [3, 8, 29, 41 52]. It has been postulated that enzyme-bound cobalamin is in a superreduced state [cob(I)alamin], in which the alkyl residue of an alkyl halide can bind to the cobalt atom [15]. The same mechanism has been applied to the binding of the propyl chain of propyl iodide to cobalt, thus inactivating the enzyme [6]. This work clearly shows that the mechanism of PCE degradation by PCE dehalogenase from *Propionibacterium* sp. HK-1 is mediated by a corrinoid protein, since the dehalogenase was inactivated by propyl iodide only after reduction by titanium citrate.

The capability to degrade halogenated aliphatic compounds has mainly been observed in the genera *Desulfitobacterium*, *Clostridium,* and the related *Dehalobacter ristrictus* [2, 8, 27, 30, 38, 41]. This work reports the first demonstration of a broad spectrum of chlorinated aliphatics being degraded by cell-free extracts of *Propionibacterium* sp. HK-1. Biodegradation of xenobiotic pollutants by *Propionibacterium* sp. is not known well. However, a *Propinibacterium* capable of degrading *O*-aryl alkyl ethers and various aromatic hydrocarbons has been reported recently [53].

Two PCE and *cis*-DCE-degrading bacteria (strains HK-1 and HK-3) were isolated and characterized. Neither accumulated toxic intermediate compounds such as TCE, *cis*-DCE, or VC. This is the first report of anaerobic organisms capable of degrading PCE without the accumulation of chlorinated ethenes and with partially mineralization to ethylene. This may be useful for the development of biological remediation of chlorinated ethene-contaminated sites. Furthermore, the ability of strain HK-1 to degrade several halogenated aliphatic compounds has potential to aid the amelioration of environments contaminated with mixtures of halogenated substances. Given that *Propionibacterium* sp. is a ubiquitous microorganism worldwide, it is an interesting model for further studies of PCE degradation.

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Figure legends

Fig. 1. Phylogenic tree based on a comparison of 16S rRNA gene sequences. The phylogenic tree was generated using neighbor-joining methods. Bootstrap values are shown from the bootstrap analysis of 100 replications. Scale bar represents an evolutionary distance (Knuc) of 0.01.

Fig. 2. The time courses of the ratios of residual to initial PCE (A) and *cis*-DCE (B) concentrations, and cell density (OD_{590}). Concentrations used for PCE degradation and growth curve: closed diamonds, 0.06 mM; closed triangles, 0.12 mM; closed circles, 0.3 mM; closed squares, 0.6 mM. Concentrations used for *cis*-DCE degradation and growth curve: closed diamonds, 0.1 mM; closed triangles, 0.21 mM; closed circles, 0.52 mM; closed squares, 0.98 mM. Data represent means of triplicate experiments, and error bars indicate 95% confidence intervals.

Fig. 3. Effects of electron donors on PCE and *cis*-DCE degradation by cell-free extracts of strains HK-1 and HK-3. Initial protein concentration of crude enzyme was 0.79 mg ml^{-1}. Results are means of triplicate independent experiments. Control experiment was conducted with enzyme and without electron donors. Data points are means of triplicate observations and error bars represent ±SD.

Fig. 4. Release of chloride ions during degradation of PCE (A) and *cis*-DCE (B) by cell-free extracts of strains HK-1 and HK-3. A) solid circles (PCE with enzyme); solid squares (PCE without enzyme); solid triangles (chloride ion). B) solid circles (*cis*-DCE with enzyme); solid squares (*cis*-DCE without enzyme); solid triangles (chloride ion). Initial protein concentration of crude enzyme was 1.2 mg ml^{-1}. Data points are means of triplicate observations and error bars represent ±SD.

Fig. 5. Inactivation of PCE dehalogenase by propyl iodide and reactivation by light. The enzyme was reduced by the addition of 2 mM titanium (III) citrate (TC; arrow) prior to inactivation with 0.5 mM propyl iodide (PI). At 40 min, the assay mixture was exposed to light (h*v*). Relative activity was calculated compared with a control (without PI). One unit (U) of enzyme activity was defined as nmol of PCE degraded per hour under assay conditions. Results were normalized with respect to a maximal activity (100%) of 30 U. Initial protein concentration of crude enzyme was 1.2 mg ml^{-1}. Results are means of duplicate experiments.

Figure 1

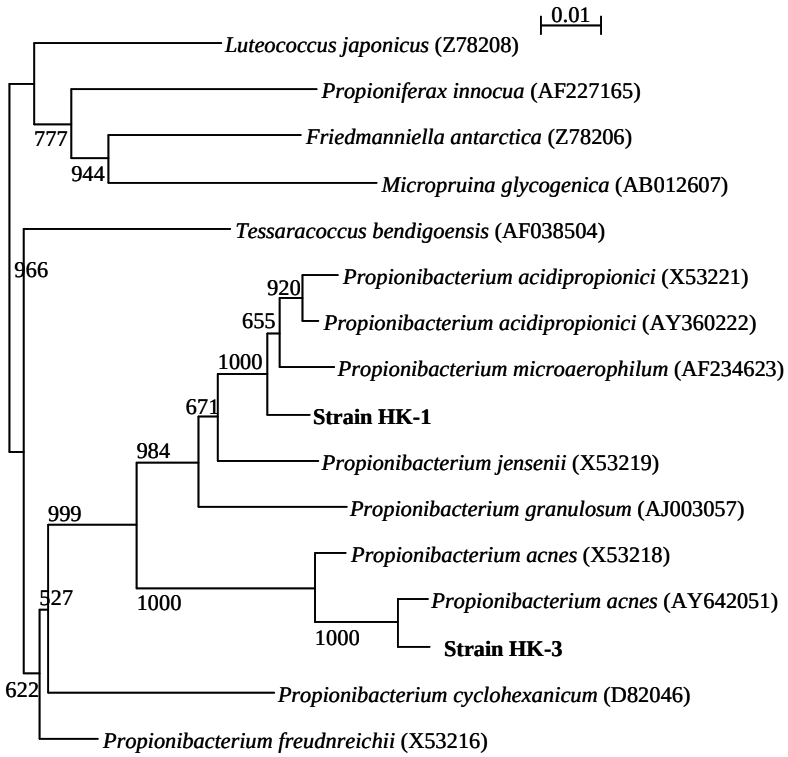

0.01
Luteococcus japonicus (Z78208)
Propioniferax innocua (AF227165)
777
Friedmanniella antarctica (Z78206)
944
Micropruina glycogenica (AB012607)
Tessaracoccus bendigoensis (AF038504)
966
920
Propionibacterium acidipropionici (X53221)
655
Propionibacterium acidipropionici (AY360222)
1000
Propionibacterium microaerophilum (AF234623)
671
Strain HK-1
984
Propionibacterium jensenii (X53219)
999
Propionibacterium granulosum (AJ003057)
Propionibacterium acnes (X53218)
527
1000
Propionibacterium acnes (AY642051)
1000
Strain HK-3
622
Propionibacterium cyclohexanicum (D82046)
Propionibacterium freudnreichii (X53216)

Figure 2.

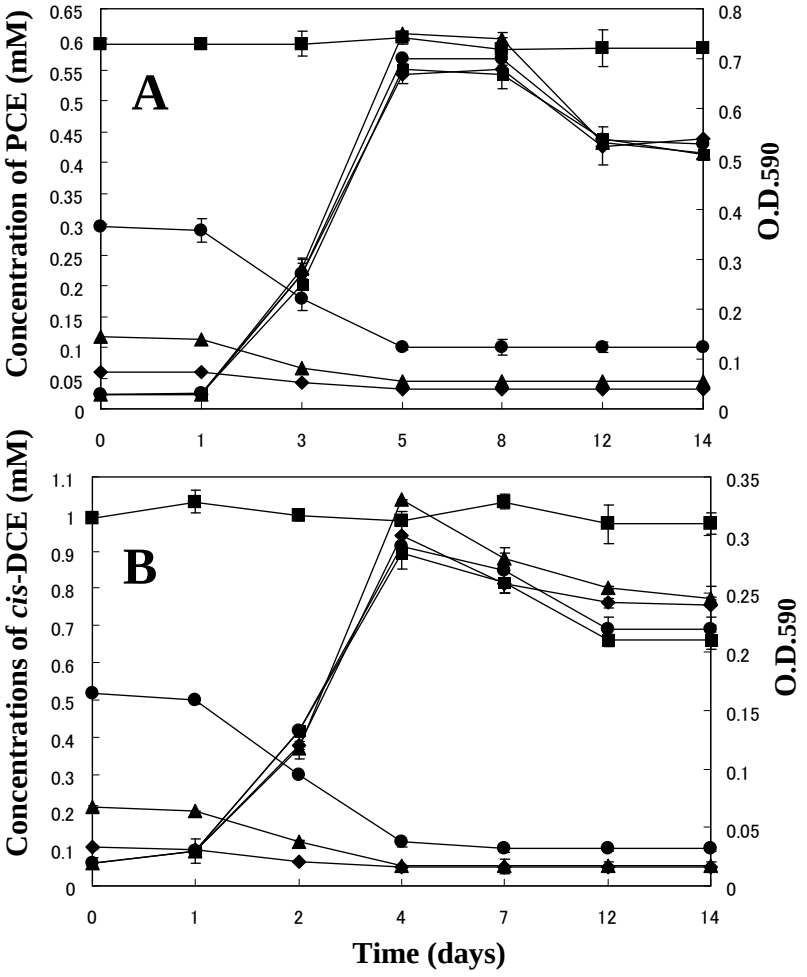

A
B
Concentration of PCE (mM)
Concentrations of cis-DCE (mM)
O.D.590
O.D.590
Time (days)
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 3 5 8 12 14
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 1 2 4 7 12 14

Figure 3.

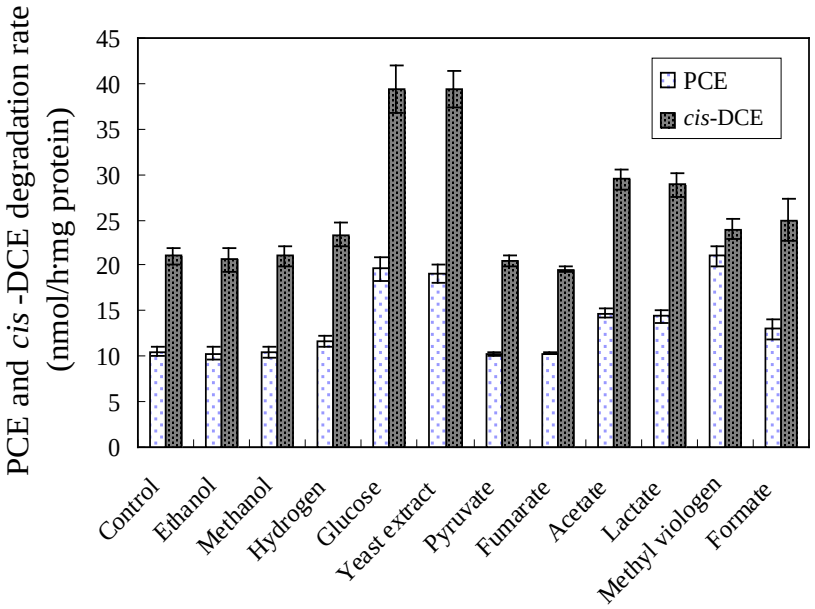

PCE and cis -DCE degradation rate
(nmol/h·mg protein)
45
40
35
30
25
20
15
10
5
0
PCE
cis-DCE
Control
Ethanol
Methanol
Hydrogen
Glucose
Yeast extract
Pyruvate
Fumarate
Acetate
Lactate
Methyl viologen
Formate

Figure 4.

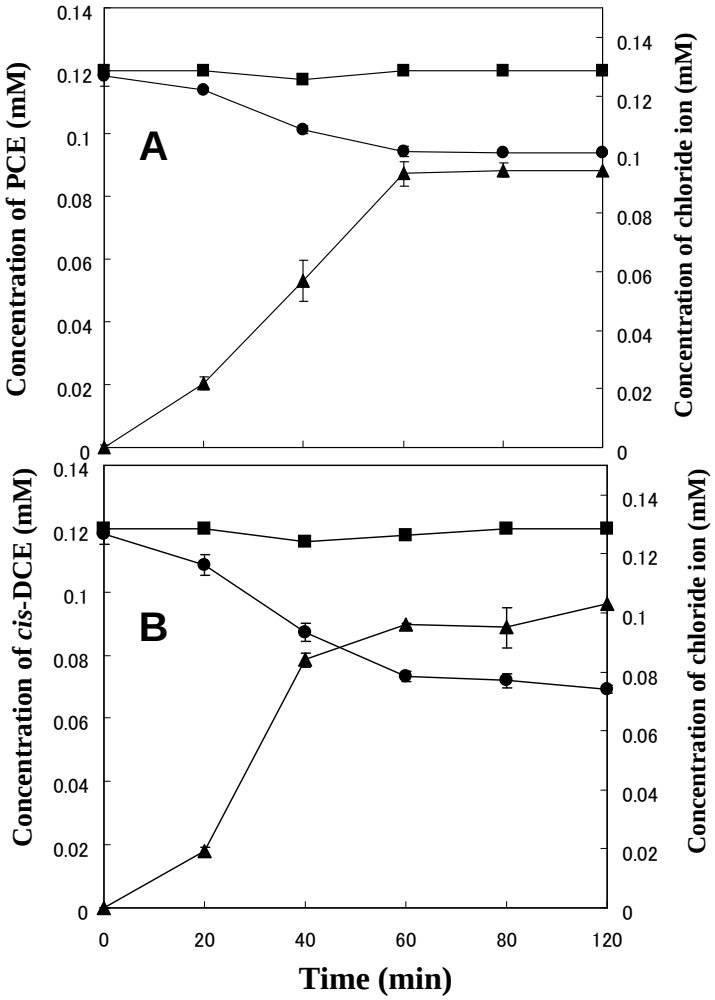

A
B
Concentration of PCE (mM)
Concentration of *cis*-DCE (mM)
Concentration of chloride ion (mM)
Time (min)
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0
20
40
60
80
120

Figure 5.

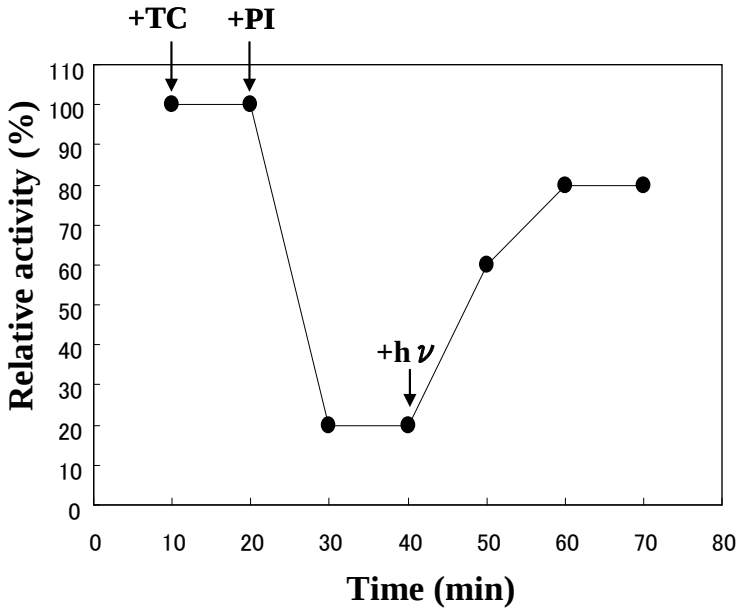
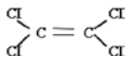
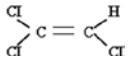
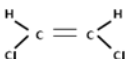
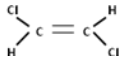
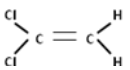
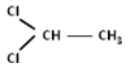
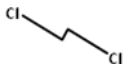
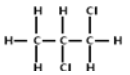
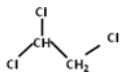
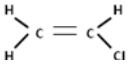

+TC
+PI
+h ν
Relative activity (%)
Time (min)
0
10
20
30
40
50
60
70
80
90
100
110

Table 1. Physiological characteristics of strains HK-1 and HK-3

Gram stain	Positive
Indole production	Negative
Protease hydrolysis	Negative
β-glucosidase hydrolysis	Positive
Urease	Negative
Catalase	Positive
Oxidase	Positive
Utilization of carbon sources (Positive):	glucose, maltose, lactose, saccarose, L-arabinose, glycerol, D-cellobinose, D-mannose, D-melezitose, D-raffinose, D-sorbitol, D-rhamnose, D-trehalose, salicin, D-xylose, D-mannitol

Table 2. Biodegradation of chlorinated aliphatics by cell extracts[a].

Halogenated aliphatic compound	Structure	Strain HK-1		Strain HK-3	
		Residual substrate(μM)	Degradation (%)[b]	Residual substrate(μM)	Degradation (%)
Tetrachloroethylene		96±5.43	20±1.13	120	ND[c]
Trichloroethylene		100.8±7.46	16±1.24	120	ND
cis-1,2-Dichloroethylene		90±8.69	25±2.26	78±5.04	35±2.26
trans-1,2-Dichloroethylene		100.8±6.79	16±1.13	98.4±6.18	18±1.13
1,1-Dichloroethylene		92.4±5.66	23±1.36	90±12.22	25±3.39
1,1-Dichloroethane		92.4±5.10	23±1.22	120	ND
1,2-Dichloroethane		92.4±6.14	23±1.13	120	ND
1,2-Dichloropropane		108±10.86	10.±1.13	120	ND
1,1,2-Trichloroetane		90±9.56	25±2.49	120	ND
Vinyl chloride		84±7.24	30±2.26	72±6.11	40±3.39

[a]Biodegradation experiments were carried out in Tris-HCl (50 mM) with DTT (2 mM) and glucose (5 mM) as the electron donor at 30℃. Concentrations of remaining compounds were measured after 1 h. Initial concentration of protein was 1.2 mg ml^{-1}. [b]Percent degradation compared with controls containing no enzyme. Values are means of triplicate experiments±SD. [c]ND: not degraded.