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REVIEW

Biochemical and molecular characterization of a tetrachloroethylene (PCE) dechlorinating *Clostridium bifermentans* DPH-1

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Abstract

The tetrachloroethylene (PCE) dehalogenase of *Clostridium bifermentans* DPH-1 (a halorespiring organism) was purified, cloned, and sequenced. This enzyme is a homodimer with a molecular mass of ca. 70 kDa and exhibits dehalogenation of dichloroethylene isomers along with PCE and trichloroethylene (TCE). Broad range of substrate specificity for chlorinated aliphatic compounds (PCE, TCE, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, 1,1-dichloroethylene, 1,2-dichloropropene, and 1,1,2-trichloroethane) for this enzyme was also observed. A mixture of propyl iodide and titanium citrate caused a light-reversible inhibition of enzymatic activity suggesting the involvement of a corrinoid cofactor. A partial sequence (81 bp) of the encoding gene for PCE dehalogenase was amplified and sequenced with degenerateprimers designed from the N-terminal sequence (27 amino acid residues). Southern analysis of *C. bifermentans* genomic DNA using the polymerase chain reaction product as a probe revealed restriction fragment bands. A 5.0 kb *Cla*I fragment, harboring the relevant gene (designated *pce*C) was cloned (pDEHAL5) and the complete nucleotide sequence of *pce*C was determined. The gene showed homology mainly with microbial membrane proteins and no homology with any known dehalogenase, suggesting a distinct PCE dehalogenase. So, *C. bifermentans* could play some important role in the initial breakdown of PCE and other chlorinated aliphatic compounds in sites contaminated with mixtures of halogenated substances.

Key words : tetrachloroethylene, *Clostridium bifermentans* DPH-1, PCE dehalogenase, *pce*C, gene cloning.

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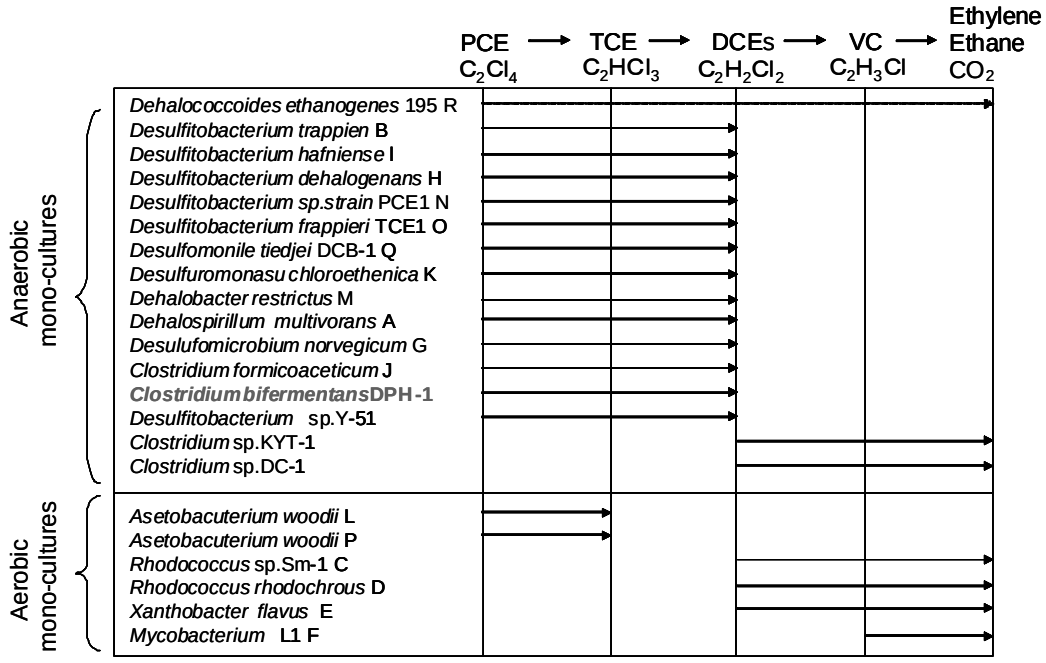
Ⅰ. INTRODUCTION

Chlorinated solvents, such as tetrachloroethylene (also referred to perchloroethylene; PCE) and trichloroethylene (TCE), are among the most prevalent groundwater pollutants. Its frequent occurrence at contaminated sites is due to its widespread use as an industrial solvent. PCE and its incomplete dechlorinationproducts are known or suspected carcinogens. Therefore, the treatment of PCE bearing wastes and the remediation of PCE contaminated soils and aquifers are a global priority on environmental pollution control.

Although aerobic cometabolic dechlorination of PCE by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1 has been recently reported[1)], PCE is recalcitrant under aerobic condition because of its oxidized nature[2)].

Hydrogen is generally considered to be a key electron donor to stimulate the reductive dechlorination of chlorinated ethylene[3-5)]. Our previous work, *Clostridium bifermentans* strain DPH-1 has been found to reductively dechlorinate PCE to cDCE (*cis*-1,2-dichloroethylene) using hydrogen as an electron donor[6)]. Generally, the introduction of halorespiring bacteria is expected to be a cost-effective approach to the remediation of PCE-contaminated site[7,8)]. Such bacteria can grow by anaerobic respiration, a process that has been referred to as halorespiration or dehalorespiration[6,9~16)]. Some pure cultures have been reported to catalyze the reductive dechlorination of PCE to cDCE (Table 1).

Table 1. Isolated PCE-dehalorespiring bacteria and dechlorination steps performed.



These organisms belong to species of *Dehalospirillum*, *Desulfomonile*, *Desulfitobacterium*, *Dehalobacter*, and *Clostridium*[6,9~16]. Some strains belonging to *Dehalococcoides* spp. are able to convert PCE to ethylene sequentially (Table 1). He *et al.*[17] recently identified a *Dehalococcoides* strain that uses DCE isomers and vinyl chloride (VC) but not PCE or TCE as metabolic electron acceptors.

PCE dehalogenases have been purified, and their genes cloned, from several bacteria [18~24]. The PCE dehalogenase *pce*A genes were found to be linked with the *pce*B genes coding for small hydrophobic proteins containing two or three transmembrane helices[18,25~27], and *pce*B was assumed to act as a membrane anchor protein to link the dehalogenase to the respiratory chain. The presence of similar *pce* genes among different strains strongly indicates that these genes have a mechanism of transfer among these strictly anaerobic bacteria.

Most anaerobic dehalogenases dechlorinate PCE to principally cDCE; however, a novel PCE dehalogenase from *Dehalococcoides ethenogens* 195[14] can reductively dechlorinate PCE to ethylene, extensively detoxifying it (Table 1). Aerobic degradation of cDCE by *Rhodococcus rhodochrous*[28] and *Nitrosomonas europaea*[29] has been reported. Thus, cDCE accumulation in the anaerobic system can be eliminated by further degradation using such aerobic dehalogenases.

Unlike other dehalogenases from dehalospiring bacteria, the dehalogenase from strain DPH-1, does not have Fe/S clusters, but exhibits a strong dechlorination activity for PCE as well as several other halogenated compounds. Due to this uniqueness and as a representative PCE-dehalorespiring bacterium, we have reviewed the nature of *Clostridium bifermentans* DPH-1, through the special focusing on the biochemical organization and genetic regulation of gene encoding PCE dehalogenase followed by the PCE dechlorination.

II. METHODS

1. Microorganism and culture conditions

An anaerobic bacterium *Clostridium bifermentans* DPH-1 (Photo 1), capable of dechlorinating PCE stoichiometrically to cDCE at concentrations as high as 960 μM and as low as 0.6 μM within 56 h, was originally isolated from PCE-contaminated ditch sludge at a dry-cleaning factory in Gifu, Japan. The characteristics of strain DPH-1 cells were reported previously [6]. The culture was maintained by weekly subculture on MY medium of the following composition (per liter of deionized water): K_2HPO_4, 7.0g; KH_2PO_4, 2.0g; $MgSO_4 \cdot 7H_2O$, 0.1g; $(NH_4)_2SO_4$, 1.0g; yeast extract, 2.0g; resazurin, 0.001g; and pH 7.2. After autoclaving 10 ml of the medium in 26 mL-serum bottles, 0.1 ml of filter sterilized vitamin solution (1 g of *p*-aminobenzoic acid and 1 mg of biotin per liter) and 0.1 ml $FeSO_4 \cdot 7H_2O$ (2 g/l) were added.

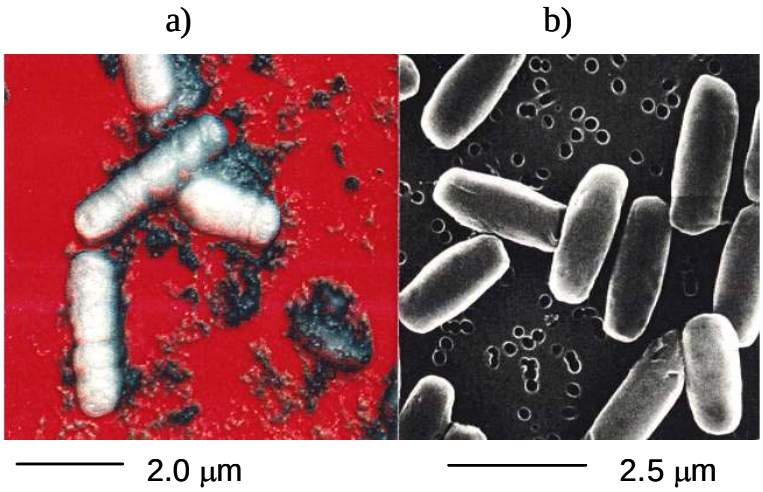


Fig. 1. (A) Time course of PCE dechlorination, cell protein, hydrogen, and chloride ion profile. Symbol: ◇ PCE (without cells); ● cell protein; ■, hydrogen (without PCE); ▲ hydrogen (with PCE); Δ chloride ion. (B) Reductive dechlorination of PCE to TCE and cDCE. Strain DPH-1 was grown in MY medium containing PCE (0.9mM).

2. Enzyme preparation

Clos*tridium bifermentans* cells (approximately 1.6 g), resuspended in 6 ml of 20 mM Tris-chloride buffer pH 8.2, 2 mM dithiothreitol (DTT) and 5 % glycerol, were lysed in an ice bath, using a TOMY (Tokyo, Japan) ultrasonic disrupter UD-201 at 30 s flash for 5 min. Unbroken cells and debris were separated by centrifugation (17000×g, 20 min, 4°C) and the extraction procedure repeated with the unbrokencells and debris. The two supernatant fractions were combined and passed through a 0.22 μm Millipore filter. The filtrate served as the cell-free enzyme extract.

3. N-terminal sequencing

After SDS-PAGE of protein sample, the protein was electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P^{SQ}, Millipore Corp.) using a Bio-Rad electroblotting apparatus (Bio-Rad, Hercules, Calif., U.S.A.). The amino terminal sequence was analyzed by automated Edman degradation performed using an Applied Biosystems 491 protein sequencer (Perkin Elmer Applied Biosystem, Foster city, Calif., U.S.A), equipped with a PROCISE^{TM} 1.1a data analysis software.

4. Recombinant DNA techniques

Genomic DNA was prepared from an overnight culture of *C. bifermentans*. Plasmid DNA was isolated from cell using a High Pure Plasmid Isolation kit (Boehringer Mannheim Corp., Indianapolis, Ind., U.S.A), according to the manufacturer's instructions. Polymerase chain reaction (PCR) product and restriction enzyme digestion fragments were purified from

agarose gels with the QLAEX II gel extraction kit (Qiagen, Hilden, Germany). All ligations were done using a DNA ligation lit ver. 2 (Takara, Kyoto, Japan), according to the manufacturer's instructions. Plasmid DNA transformation was by standard procedure in *E. coli* DH5α, and colonies were selected and screened by growth on plates containing 100 μg/ml ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranose.

5. Probe construction for gene cloning

Based on the N-terminal sequence of the dehalogenase, two degenerate primers; 5'-GCI-GAR-GTI-TAY-AAY-AAR-GA-3' and3'-GTR-ATR-AAR-ISI-TTR-CTR-TG-5' (I, Inosine; R, A or G; Y, C or T; S, G or C) were designed and used for PCR amplification of the predetermined N-terminal region of the encoding gene. PCR was performed using Ready-To-Go PCR beads (Aersham Pharmacia Biotech, Buckinghamshire, U.K). Approximately 100 ng of *C.nifermentans* genomic DNA was used as the template. DNA amplification was then carried out in 35 PCR cycles as follows: initial denaturation (95°C, 5 min), subsequent denaturation (95°C, 1 min), annealing (43°C, 1.5 min) , and elongation (72°C, 2 min). After being visualized on 1.5% agarose, the expected band (81bp) was purified and cloned into the T-cloning site of pT7Blue (Novagen Inc., Madison, Wis., U.S.A.) generating plasmid pATS81. The insert was subjected to DNA sequencing. The sequence was translated and compared to the known amino terminal sequence of the purified protein.

6. Analyses of PCE and other chlorinated aliphatic substances

PCE and TCE were identified and quantified by static-headspace analysis using a gas chromatograph. PCE, TCE and DCE in a 10-μl headspace sample were determined using a model GC-14B gas chromatograph (Shimadzu Co., Japan) equipped with an electron capture detector (ECD) and a glass column (i.d. 3.2 φ×2.1 m; Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). cDCE, VC, ethylene, and ethane in the vial were determined by injecting 250 μl of headspace gas into a gas chromatograph (GC-14B, Shimadzu Co., Japan), equipped with a glass column, Apiezone grease L 60/80 (i.d. 3.0 φ×2.1 m), and a flame ionization detector (FID). Nitrogen was used for the carrier gas. The column temperature was kept at 75°C for 2 min and then raised to 180°C at a rate of 4°C per min, and injector and detector temperatures were kept at 180°C.

III. RESULTS

1. Growth characteristics and PCE dechlorination

Cells increased exponentially within 14 h and reached a steady state after 16 h (Fig. 1(a)). A similar pattern was observed when PCE was not added to the medium. PCE dechlorination started during the mid-stationary phase, after 30 h, and PCE was completely dechlorinated after 56 h (Fig. 1(a)). Most of the PCE biotransformation occurred during the stationary phase, indicating the stability of the dehalogenation activity even in

resting cells[30]. No growth and reductive dechlorination of PCE was observed when yeast extract was eliminated from the medium. The concentration of chloride ions increased from 0.9 mM to 2.7 mM (Fig. 1(a)). PCE was rapidly converted to cDCE without apparent accumulation of TCE (Fig. 1(b)). In this study, 1.8 mM chloride ions were produced from 0.9 mM of PCE (Fig. 1(a)). This result indicates that the reductive dechlorination of PCE to cDCE can be described by the following equation:

$$2H_2 + C_2C1_4 \text{ "} \rightarrow C_2H_2C1_2 + 2Cl^- + 2H^+$$

Hydrogen was produced exponentially within 40 h and reached a steady state after 40 h (39.4 μmol per 50ml of headspace volume) in control cultures without PCE (Fig. 1(a)). In cultures containing PCE, a similar pattern was observed except that the H_2level was lower (30.7 μmol after 40 h) (Fig. 1(a)), indicating that hydrogen was used for the reduction of PCE. Although several electron donors (hydrogen, lactate, acetate, fumarate, glucose, methanol, formate) enhanced effectively PCE dechlorination when supplied together with yeast extract, ethanol (1.22 mM) was the most effective extra electron donor[31].

2. Dechlorination of chlorinated compounds

The PCE dechlorination rate increased with increasing initial PCE concentration (6 μM to 900 μM). At 0.9 mM PCE, the maximum specific rate of PCE dechlorination was calculated to be 0.43 μmol/h·mg protein, in relation to protein concentration at 48 h. The dechlorination rate for strain DPH-1 is comparable with those of the previously reported pure cultures, *Desufomonile tiedjei*, 0.02 μmol/h·mg protein[32] *Methanosarcia* sp. 3.5×10^{-5} μmol/h·mg protein[33] and strain MS-1, 0.5 μmol/h·mg protein[34] and lower than those of *S. multivorans*, 4.5 μmol/h·mg protein[21] and *D. restrictus* (strain PER-K23), 1.0 μmol/h·mg protein[9]. The rates of dechlorination of other halogenated aliphatic compounds by strain DPH-1 were determined. After 56 h of cultivation, the dechlorination rates of TCE, cDCE" C*trans*-DCE, VC, 1,2- dichloroethane (DE), 1,1-dichloroethane (1,1-DE), dichloromethane (DM), 1,3-dichloropropene, 1,2-dichloropropene (1,2-DP), and 1,1,2-trichloroethane (TE) were determined to be 96, 27, 94, 30, 90, 90, 70, 62, 50 and 60 %, respectively (Table 2).

Table 2. Dechlorination of halogenated aliphatic compounds by strain DPH-1.

Halogenated aliphatic compounds	Remaining conc. (μM)	Dechlorination (%)[a]
Tetrachloroethylene (PCE)	0.36	98
Trichloroethylene (TCE)	0.72	96
cis-1,2-dichloroethylene (cDCE)	13.14	27
trans-1,2-dichloroethylene	1.08	94
1,1-dichloroethylene (1,1-DCE)	18.00	ND[b]
Vinyl chloride (VC)	12.60	30
1,2-dichloroethane (DE)	1.80	90
1,1-dichloroethane (1,1-DE)	1.80	90
Dichloromethane (DM)	5.40	70
1,3-dichloropropene (DP)	6.84	62
1,2-dichloropropene (1,2-DP)	9.00	50
1,1,2-trichloroethane (TE)	7.20	60
1,1,1-trichloroethane (1,1,1-TE)	18.00	ND

[a]Percent dechlorination compared with controls without cells. [b]ND, Not degraded. Cultivation was performed for 56 h. Initial concentration of each compounds was 18 μM. Results are means of duplicate experiments.

3. Extraction and purification of PCE dehalogenase

The enzyme was easily extracted by

sonication of cells in the Tris-chloride buffer containing dithiothreitol (DTT) and glycerol. Addition of 0.1% Triton X-100 to the extraction buffer did not improve enzyme extraction. Residual cell debris, resuspended in either the same buffer or the extraction buffer containing 0.1% Triton X-100, displayed no PCE degrading activity. This indicates that the enzyme is not an integral membrane protein but a peripheral membrane protein.

Clos*tridium bifermentans* DPH-1 reductive PCE dehalogenase was purified from crude cell extract by monitoring the conversion of PCE to cDCE, via TCE. The initial purification scheme resulted in a 13.2-fold purification of PCE dehalogenase after Superdex pg-75 gel filtration, with approximately 42% recovery of enzyme activity. The specific activity increased from 6.5 to 86.1 U/mg protein. At this stage the enzyme sample contained two apparent impurities (Fig. 2(a)). The enzyme was further completely purified (Fig. 2(b)) from pooled and dialyzed samples by IEX-HPLC and SE-HPLC. This, however, caused a significant decrease in specific activity because of instability under the HPLC purification conditions. Total activity for the IEX-HPLC and SE-HPLC purification steps decreased to 10.9 U (7.1% recovery) and 7.7 U (5.0% recovery), respectively. Afterwards, the IEX-HPLC and SE-HPLC specific activities decreased to 37.7 U/mg protein and increased to 59.5 U/mg protein, respectively. Fractions containing the protein impurities in the semipurified enzyme showed no PCE dehalogenation activity.

The molecular mass of the native PCE dehalogenase, determined by molecular SE -HPLC, was approximately 70 kDa. SDS-PAGE analysis revealed a monomer molecular mass of approximately 35 kDa (Fig. 2(b)), indicating a dimeric protein structure. The precise molecular mass of the monomer determined by MALDI-TOF/MS was 35.7 kDa. The dehalogenase catalyzed PCE dechlorination at an optimum temperature of 35°C. The enzyme was relatively stable in the temperature range 20-35°C. Maximal activity was recorded at pH 7.5 and the enzyme was most stable at pH 7.5-8.0. The optimum temperature (35°C) for *C. bifermentans* dehalogenase is near the 37°C reported for *Desulfitobacterium* sp. Y-51[24], but differs significantiy from 50°C reported for *Desulfitobacterium* sp. PCE-S [35]. The optimum pH (7.5) for *C. bifermentans* dehalogenase activity is similar to that of *Sulfurospirillum multivorans*, *Desulfitobacterium* sp. Y-51, and *Desulfitobacterium* PCE-S, which were maximally active at pH 8.0, 7.2 and 7.2, respectively.

Enzyme activity was not dependent on metal ions and addition of EDTA-Na, NADH, and cyanocobalamin had no significant effect on PCE dechlorination [22, 31]. The enzyme was oxygen sensitive and lost approximately 50% of its activity during incubation in the presence of air for 20 h. After 48 h, the enzyme completely lost activity. The enzyme lost 90% of its PCE degrading activity upon incubation with a mixture of an alkylating agent (propyl iodide) and a reducing agent (titanium citrate), in the dark. Subsequent exposure to light restored about 95% of the initial activity. Titanium citrate in the absence of propyl iodide did not have any

inhibitory effect and no inhibition was recorded when only propyl iodide was supplied. It was not predicted that the PCE dehalogenase activity would neither be stimulated nor inhibited by the metal ions tested. EDTA, a well known divalent metal ion chelator also had no effect. An explanation for this is that cobalamin (Co^{2+}), a prosthetic group of corrinoid dehalogenases, is not free for chelation by EDTA [36]. Moreover, the cobalt component of corrinoid proteins is enzymatically active in the superreduced state (Co^{2+})[21]. In this state, a divalent metal ion chelator such as EDTA would not immobilize cobalt. Little is known about the exact mechanism of PCE dehalogenation by corrinoid proteins; however, it has been postulated that the superreduced state of the corrinoid (Co^{2+})can bind to the alky1 residue of a halogenated hydrocarbon or an alkylating agent such as propyl iodide, achieving either dechlorination or activity inhibition, respectively[36].

The kinetics of dechlorination of PCE and product formation was studied in time-course experiments. The dehalogenase rapidly converted PCE to cDCE via TCE. The kinetic parameters, V_{max} and K_m"Ccomputed from Lineweaver-Burk double reciprocal plot were 73 nmol/mg protein and 12 μM, respectively. Low concentrations of PCE and other chlorinated aliphatic substances may be unavailable and recalcitrant to microbial degradation in polluted sites. Therefore, low K_m values for microbial dehalogenases could be very important in such sites. The V_{max} (73 nmol/mg protein) of *C. bifermentans*dehalogenase, however, is low compared with 2650 nkat/mg protein and 650 nkat/mg protein reported for *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. PCE-S, respectively. Enzyme activity was generally unstable during purification, possibly because of its oxygen sensitivity and is probably responsible for the low V_{max} of *C. bifermentans* dehalogenase.

Clostri*dium bifermentans* dehalogenase significantly dechlorinated various chlorinated aliphatic compounds. In general, the dechlorination rates observed with PCE and TCE were higher than the rates recorded with other environmentally important chloroaliphatics. Approximately, 25±4.9%, 16±4% , 6±1%, 8±0.8%, 8±4.9%, 25±1.6%, 9±0.4%, 8±0.2% dechlorinations per hour were achieved with PCE, TCE, cDCE, 1,1-DCE, 1,2-dichloroethane, 1,2-DP and 1,1,2-TE, respectively. Vinyl chloride was recalcitrant to dechlorination.

N-terminal amino acid sequencing of the purified PCE dehalogenase revealed the following sequence: AEVYNKDGNKLDLYGKVDGLHYFSNDT. BLAST analysis showed no homology with any known PCE dehalogenase. However, three amino acid residues (KVD) in the sequenced N-terminal region appear to be identical to those of *Desulfitobacterium* sp. PCE-S (Fig. 3).

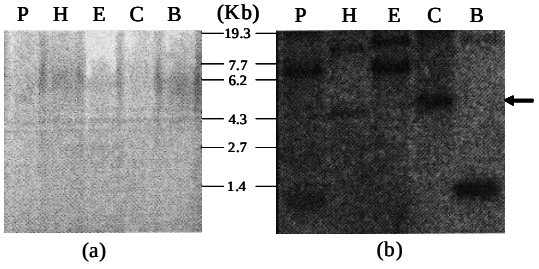


Fig. 3. Alignment of the N-terminal sequences of four PCE dehalogenases.

4. Cloning of PCE dehalogenase and gene sequence

Two degenerate Primers designed from both ends of the N-terminal amino acid sequence successfully amplified an 81 bp putative region of *C. bifermentans* PCE dchalogenase. The translated DNA sequence of the probe (81 bp PCR product) matched the predetermined N-terminal protein sequence.

The PCR product was confirmed by DNA sequencing and was used as a probe for gene cloning. Southern hybridization of *C. bifermentans* genomic DNA cleaved with various restriction enzymes, with probe, with [α-32P] dATP-labelled probe, revealed distinct bands of *Bgl*II, *Cla*I, EcoRI, *Hind*III. After screening 1720 *E. coli* DH5α colonies from a genomic sublibrary (constructed with approximately 4.5-5.5 kb *Cla*I fragments), we isolated a putative clone (pDEHAL5) containing a 5 kb *Cla*I insert. Based on the Southern hybridization analysis data (Fig. 4), it was predicted that a *Bgl*II restriction fragment, less than 1.4 kb could harbor the dehalogenase gene.

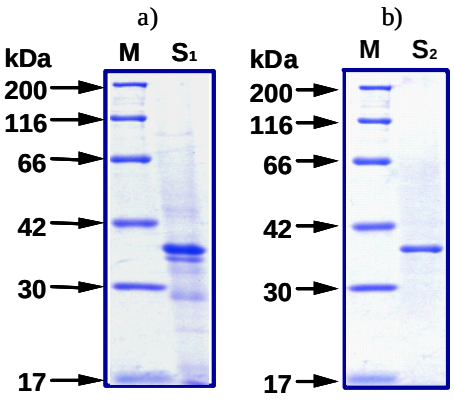


Fig. 4. (A) Agarose gel electrophoresis analysis of restriction fragments of *C. bifermentans* genomic DNA. (B) Southern blot/hybridization analysis. *Bgl*II, B; *Cla*I, C; *Eco*RI, E; *Hind*III, H; and *Pst*I, P. Arrow indicates the approximately 5 kb *Cla*I fragment that were used for the construction of a sublibrary.

The complete nucleotide sequence of the inserts of the two subclones, pDEHAF1 and pDEHAF2 (plasmids used) [22], was determined and the gene was identified using the predetermined N-terminal sequence. The gene was found to contain an internal *Bgl*II site (Fig. 5). From the ATG start codon, a 97 bp of the gene was identified in pDEHAF1 and 1004 bp in pDEHAF2.

AE VYNKDANKLDLY GKVD GL HYFSNDT- *Clostridium bifermentans* DPH-1
ME KKKKPELS RRDF GK L I IG GGAAATIAPF- *Sulfurospirillum multivorans*
A D I VAP ITETSEFP YK VD AY- *Desulfitobacterium* sp. strain PCE-S
A D I VA P I TQT SQF P YK VD AE- *Desulfitobacterium* sp. strain Y-51

Fig. 5. Physical map of pDEHAL5 indicating relevant restriction sites. The region encoding *pce*C is shaded and arrow indicates the direction of transcription.

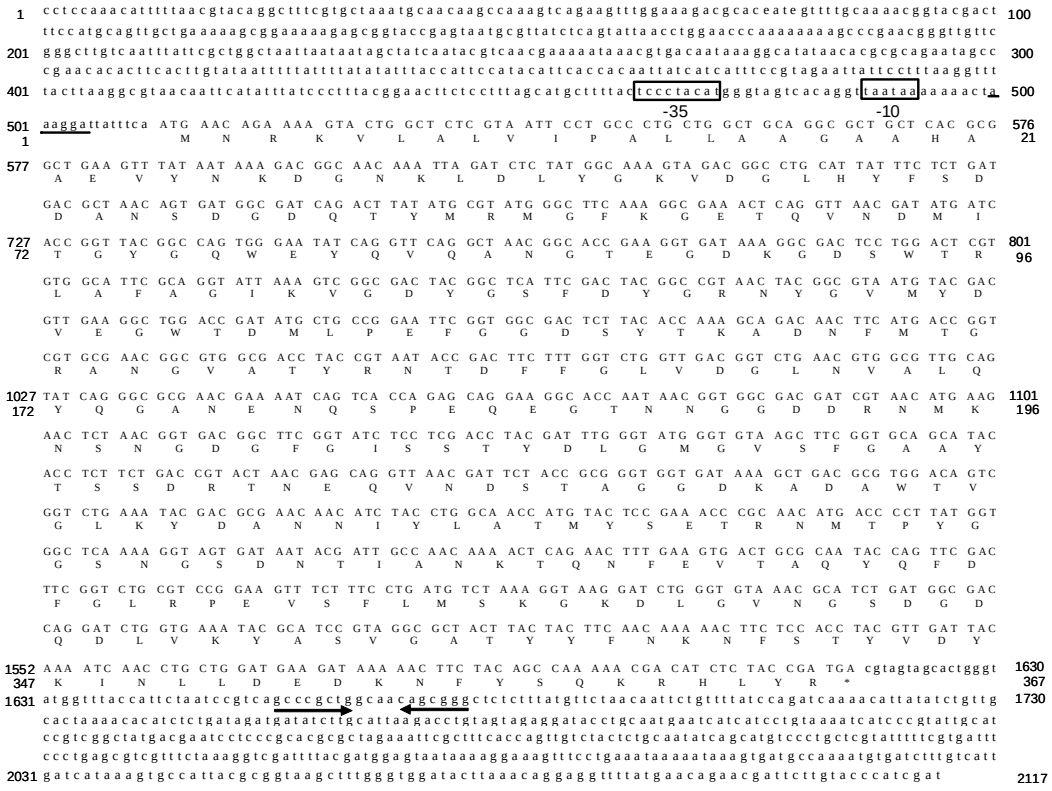


Fig. 6. DNA sequence of *pce*C and its flanking regions. Deduced amino acid sequence (single letters below the DNA sequence), putative transcriptional promoters (boxed), ribosome binding site (underlined), and inverted repeats of possible transcription termination sites (opposite arrows) are shown.

The complete nucleotide sequence of *pce*C is presented in Fig. 6. The *pce*C open reading frame spans from base pair 514 to 1614 (367 amino acids; 39.67 kDa for the mature protein). Hydropathy plot revealed the presence of a discernible signal peptideand methionine was absent from the predetermined amino terminal sequence indicating post translational processing. The coding region began with a 21 amino acid signal peptide, followed by the predetermined amino terminal sequence (Fig. 3). Thus, the processed protein (346 amino acids) has a calculated molecular mass of approximately 37.40 kDa, which is identical to that determined by mass-spectroscopic analysis. A putative ribosome binding site (AAAGGA) was found eight bases upstream from the ATG initiation codon. Typical -10 and -35 promoter sequences were found upstream of the coding region. Thirty-nine base pairs downstream of the TGA stop codon is an inverted repeat indicating a rho-independent terminator (Fig. 5). The hydropathy profile of the deduced amino acid sequence indicated a major hydrophobic region at the N-terminus, typical of a traditional leader peptide.

5. PCE dehalogenase: Biochemistry and genetics

PceC is probably a cell-associated extracellular enzyme (peripheral membrane protein) loosely anchored to the cell membrane because it was easily extracted into an aqueous buffer. Moreover, the predetermined N-terminal amino acid sequence started with alanine and the deduced amino acid sequence of *pce*C contained a discernible signal sequence, indicating a processed peripheral membrane protein. After the four purification steps, the completely purified enzyme was not stable and lost about 50% activity at -30°C. Similar instability was observed with preservation of the enzyme on ice at 4°C. The purified enzyme was therefore not suitable for characterization. This is attributed to the many purification steps and the enzyme's oxygen sensitivity. Fractions containing the protein impurities, in the semipurified enzyme, showed no PCE dehalogenation activity, indicating the absence of a second dehalogenase in the semipurifled enzyme used for characterization.

PceC is a homodimer and differs in molecular size from other reported dehalogenases. PCE dehalogenases of *Sulfurospirillum multivorans* [37], *Desulfitobacterium* sp. Y-51 (35), and *Dehalococcoides ethenogenes* 195 [19] were reported to be monomeric proteins with apparent molecular masses of 57, 60, and 51 kDa, respectively. However, the PCE dehalogenase from strain PCE-S ispossibly a homotrimer with an apparent molecular masse of approximately 65 kDa and 200 kDa for monomeric and native forms, respectively.

The most frequently reported dehalogenases consist of a single polypeptide containing one corrinoid cofactor and two iron-sulfur clusters: PCE reductive dehalogenases of *S. multivorans* [37], *Desulfitobacterium* sp. strain PCE-S [35], and *Desulfitobacterium frappieri* TCE-1 [38], *ortho*-chlorophenol reductive dehalogenases of *Desulfitobacterium hafniense* [39], *Desulfitobacterium dehalogenans* [27], and *Desulfitobacterium chlororespirans* [40], and PCE- and TCE-reductive dehalogenases of *Dehalococcoides ethenogenes* [19]. Two reductive dehalogenases with one corrinoid cofactor but without an iron-sulfur cluster have also been reported: the *ortho*-chlorophenol reductive dehalogenase from *Desulfitobacterium frappieri* PCP-1 [41] and the PCE reductive dehalogenase from *C.bifermentans* DPH-1 [22]. These two proteins are different from all the other dehalogenases already described. The third type of dehalogenase is a hemeprotein consisting of two subunits and was isolated only from *Desulfomonile tiedjei* DCB-1 [42]. Abiotic dehalogenation of several halogenated compounds was also observed from the heat-inactivated PCE dehalogenase of *S. multivorans* [43] and from bacterial transition metal coenzymes vitamin B_{12} (Co), coenzyme F_{430} (Ni), and hematin (Fe) [43, 44].

The PCE dehalogenase genes were found to be linked with open reading frames (ORFs) coding for small hydrophobic proteins containing two or three transmembrane helices [18, 23, 25, 26, 27, 45, 46].

Enzymatic cleavage of halogen-carbon bond (dehalogenation) is a critical step in microbial transformation and mineralization of halogenated aliphatic substances. Dehalogenation, generally, decreases toxicity and, consequently, increases susceptibility

of a halogenated molecule to further breakdown. Two strategies have been proposed for the biotreatment of PCE and TCE contamination: (i) complete degradation by reductive dechlorination [14, 47], and (ii) by a combination of an aerobic and aerobic systems, in which PCE or TCE is converted to cDCE by anaerobic reductive dechlorination, followed by complete aerobic metabolism of cDCE [48]. The capacity of *pce*C to effect rapid dechlorination of PCE could be very useful in the proposed two-stage anaerobic and aerobic biotreatment strategy. Mixtures of chlorinated aliphatic substances are often found in polluted environments. However, only a few studies have described the anaerobic transformation of chlorinated hydrocarbons [12, 49]. *C. bifermentans* dehalogenase isunique in that it represents the first characterized anaerobic reductive dehalogenase acting on multiple chlorinated aliphatic molecules. Surprisingly, cDCE was also dechlorinated when added as the initial compound for dechlorination. The product(s) of cDCE dechlorination and the reasons why cDCE as an intermediate product is recalcitrant, are not clear. This aspect requires in depth studies to understand the mechanism and product(s).

Genes coding for the first type of reductive dehalogenases have been reported, such as *cpr*A from *D. dehalogenans* [26, 27], *pce*A from *S. multivorans* [37] and *Desulfitobacterium* sp. strain Y51 [24], and *tceA* from *Dehalococcoides ethenogenes* [18]. These genes are all closely linked to genes *cpr*B, *pcr*B, and *tce*B, respectively, which encode for hydrophobic proteins potentially acting as membrane anchors for the dehalogenases. Villemur *et al.* [50] isolated genes from *D. frappieri* PCP-1 that are highly related to *cpr*A and *cpr*B. Furthermore, they also observed several genes coding for putative CprA and PceA in the genomes of *D. hafniense* DCB-2 and *Dehalococcoides ethenogenes*. Gene coding for reductive dehalogenases containing corrinoid cofactor but without an iron-sulfur cluster have been reported: *crd*A, coding for an enzyme mediating the *ortho*-chlorophenol reductive dehalogenation in *D. frappieri* PCP-1 [41], and *pce*C, coding the PCE reductive dehalogenase in *C. bifermentans* DPH-1 [22]. Both genes and gene products show no similarity with each other and with the first type of reductive dehalogenases.

IV. CONCLUSION AND PERSPECTIVES

It is now widely accepted that anaerobic halorespiring bacteria are among the key players in biologic dechlorination processes under anoxic environments. In order to meet these demands recently, a few of PCE dehalogenase was purified from some halorespiring strains, and cloned like *Pce*A gene. However, details biochemical organization and genetic regulations of this enzyme remain unclear. Literally, function of gene is subjected to physicochemical nature of subtrate i.e. arrangement of amino acid sequences. Functionally, chloroethylene dehalogenase is classified into four categories e.g. *pce*A, *vcr*A, *tce*A, and *cpr*A on the basis of substrate specificities. The substrate is recognized by chemical

structures or the differences between number and position of the chlorine. The substrate might be able to be expanded by specifying the amino acid at the active center and modifying this might improve reactive efficiency. So, X-ray structural analysis of these enzymes need to be performed. It will be urgent to establish DNA recombination experimental system including the host-vector system of obligatory anaerobe.

Table 3. Properties and location in the genome of the putative reductive dehalogenase genes of *Dehalococcoides ethenogenes* strain 195.

Reductive dehalogenase gene	N-terminal amino acid sequence	Lengh of deduced protein (aa)	Position in genome[1]	Location in original region[2]
Det-tceA	MSEKYHSTVTRR	554	23945	A
*Det-rdhA*1	MSSFHSIVSRRD	482	166788	B
*Det-rdhA*2	MSKFHSMVSRRD	500	178825	_3
*Det-rdhA*3	MNKFHTSLSRRD	492	184414	
*Det-rdhA*4	MKEFHSTLSRRD	494	186975	C
*Det-rdhA*5	MHSFHSTVSRRD	469	191688	
*Det-rdhA*6	MSKLHSTLSRRD	507	196819	
*Det-rdhA*7	MNQFHSTVSRRD	505	199588	
*Det-rdhA*8	MTEVNRRDFLKA	532	503166	D
*Det-rdhA*9	MSNFHSTVSRRD	510	767390	E
*Det-rdhA*10	MLNFHSTLTRKD	495	1266422	F
*Det-rdhA*11	MDKFHSTLSRRD	515	1272322	
*Det-rdhA*12	MSKQHSTVSRRD	505	1276843	
*Det-rdhA*13	MNKFHSIVSRRD	514	1280982	
*Det-rdhA*14	MQNFHSTLSRRD	490	1344813	G
*Det-rdhA*15	MDGKINRRDFVK	455	1397826	_3
*Det-rdhA*16	MSKFHSAVTRRD	510	1403184	H
*Det-rdhA*17	MRDGVIAPQEGY	350	1413295	

[1]The position of the first base of each *rdhA* gene is given in accordance to the genome version used this study. [2]The location of the original regions in the genome is depicted in reference (51). [3]The genes *Det-rdhA*2, *Det-rdhA*3 are located between original regions B and C; *Det-rdhA*15 is located between regions G and H.

Next to the numerous technical difficulties of performing genetics on strictly anaerobic bacteria, the tendency of the dehalospiring bacteria to contain multiple reductive dehalogenase gene clusters (Table 3) may attenuate, if not suppress completely, the effect of single gene inactivation. On the other hand, a number of dehalogenase genes are found from the genome of *Dehalococcoides ethenogenes* 195 [51, 52], *Desulfitobacterium* sp. Y51, and *Desulfitobacterium hafniense* DCB-2 [53]. Whole genomic analysis of the strain DPH-1 has also been conducting. Thus, the electron transfer system of dehalorespiring bacteria would be clarified in the near future. It is extremely important for

considering the origin and the evolution of dehalorespiring bacteria to clarify the function of these genes. The study of dehalorespiring bacteria is able not only to be offered an interesting basic finding but also to be applied to the bioremediation of chlorinated hydrocarbons, and to be greatly expected the progress in the future.

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