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The Identification and Analysis of *C. bifermentans* DPH, an Anaerobic Bacterium that can Dechlorinate by Reductive Dechlorination of Tetrachloroethylene or Other Halogenated Aliphatic Compounds

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PCE 포함한 각종 유기염소화합물 분해능을 보유한 *C. bifermentans* DPH 균주의 동정 및 성질

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국문요약

PCE(tetrachloroethylene)분해능을 보유한 그람 양성, 내생포자 형성의 혐기성균이 일본 기후현(岐阜縣)의 한 전자제품제조공장으로부터 분리되었다. 이 균은 생화학적 특성 및 16S rRNA 분석결과에 의하여 *C. bifermentans*인 것으로 확인되었다. 또한 본 균주는 고농도의 PCE(0.9 mM)를 환원적 탈염소화 반응에 의해 TCE(trichloroethylene)을 거쳐 cDCE(cis-1,2-dichloroethylene)로 전환되었다. 전자공여체로서 효모엑기스는 PCE 분해에 있어 가장 효과적이었으며 효모엑기스를 공급한 조건에서의 PCE탈염소화 속도는 0.41 μmol/h · mg protein이었다. 한편 본 균주는 PCE 뿐만 아니라 각종 유기염소화합물에 대해서도 분해능을 보유하고 있는 신종의 PCE분해균으로서 각종 유기염소화합물에 오염된 지하수 및 토양에서의 *In situ* bioremediation적용에 있어 유용할 것으로 기대된다.

Keywords : Tetrachloroethylene (PCE), Halogenated aliphatic compounds, Reductive dechlorination

I. Introduction

Groundwater pollution by halogenated aliphatic compounds, such as tetrachloroethylene (perchloroethylene; PCE), trichloroethylene (TCE), dichloroethylene (*cis*-1,2-DCE, *trans*-1,2-DCE, and 1,1-DCE), vinyl chloride (VC), dichloromethane (DM), 1,2-dichloroethane (DE), 1,3-dichloropropene (DP), and 1,1,2-trichloroethane (TE), has been a great environmental issue in Japan (1). These chemicals pose serious public health problem and are therefore considered priority pollutants. PCE is an important model for the study of biodegradation of halogenated aliphatic compounds because of its high halogen content and toxicity. PCE is recalcitrant under aerobic conditions because of its oxidized nature (2) but can be reductively dechlorinated by a few microbes; *Dehalobacter restrictus* strain PER-K23 (3), *Dehalospirillium multivorans*(4), *Desulfito bacterium* strain PCE1 (5), and *Dehalococcoides ethenogenes* strain 195 (6), under anaerobic condition. Thus, there is growing interest in anaerobic biological systems for PCE decontamination. Besides, *in situ* contamination is often a mixture of complex chemicals, *i.e.*, halogenated aliphatic compounds containing PCE, and the concentration of such compounds at contaminated sites is very high (7).

Chlorinated alkenes of environmental concern, such as;

TCE, VC, cDCE and *trans*-DCE, and DP were degraded by alkene monooxygenase in *Xanthobacteria* strain, while PCE was not degraded (8). Chloroform, DM, 1,1-dichloroethane, and DE were degraded by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase, but PCE was not converted (9). However, there is paucity of information on anaerobic degradation of a variety of halogenated substances. We recently described a mixed culture that is able to dechlorinate PCE to CO_2 and degrade a number of halogenated aliphatic compounds (10). In the present study, the isolation of the tetrachloroethylene dechlorinating bacterium isolated from the mixed culture was described. The isolate was characterized morphologically, and its phylogenetic position was assigned by comparative sequence analysis of the 16S rRNA. Furthermore, experiment was designed to determine whether the strain DPH was able to dechlorinate high concentration of PCE and other halogenated aliphatic compounds.

II. Materials and Methods

Chemicals All chlorinated chemicals (> 99% pure) were purchased from the Kanto Chemical Co., Inc (Tokyo, Japan). TCE was obtained from the Nacalai Tesque, Inc (Kyoto, Japan). All other chemicals used in this study were of reagent grade.

Enrichment and isolation Samples were aseptically collected from ditch sludge (mixed with sewage) in Gifu (Gifu Prefecture, Japan), contaminated with PCE and other halogenated aliphatic compounds. Organisms in the samples were enriched and cultivated under anaerobic conditions with mineral medium. The composition of the medium (MMY) was as follows (in grams per liter): K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; sodium citrate, 0.5; yeast extract, 2.0 (pH 7.2). PCE (0.006 mM) was added to MMY medium, and headspaces of the bottles were purged with N_2 gas (> 99.9%), and sealed with Teflon-lined rubber septa, and aluminum crimp caps. Isolation of PCE-dechlorinating bacteria from the enriched culture was performed using a modified roll tube method (11). The procedure was repeated twice. The size, shape and cellular growth habit of an isolated strain were determined by light microscopy (BH-2, Olympus Co., Japan) and scanning probe microscopy (SPM-9500, Shimadzu Co., Japan). Gram staning and spore staining were performed to distinguish between the types of isolated strains. Physiological *characterization was done by the method of Holdeman et al.* (11).

16S rRNA sequence analysis RNA was isolated by well documented procedures (12) from cultures grown to mid-log phase. The sequence was amplified as a polymerase chain reaction product with the use of primers 27f and 1525r under standard conditions. The amplificate was ligated into the pT7Blue(R) vector (Takara, Japan). The ligation product was transformed into competent cell (*E. coli* JM 109) supplied in the pT7Blue(R) vector cloning kit. The presence of correctly sized inserts was checked by restriction analysis with *Eco*RV followed by agarose gel electrophoresis. The 16S rRNA clone was sequenced with ABI PRISM™377 DNA sequencer (PE Applied Biosystem, California, USA). Sequence was added to the 16S rRNA sequence database of BLAST and subsequently screened with homology program of GENETYX-MAC based on the BLAST.

Effect of electron donors on PCE degradation The isolated strain was pregrown on GPY medium (glucose 1.0 g/*l*, Polypepton 10 g/*l*, yeast extract 5.0 g/*l*, and sodium chloride 5.0 g/*l*). Precultures were harvested by centrifugation (10 min at 8,000 × g), washed once with MM medium (based on MMY medium without yeast extract and sodium citrate), and suspended in the MM medium. Two m*l* of cell suspension (0.33 mg protein/m*l*) was transferred to a 26-m*l* serum bottle containing 8.0 m*l* of fresh MM medium and subsequently various compounds (H_2, acetate, glucose, formate, methanol, pyruvate, lactate, and yeast extract) were provided as electron donors for enhancing PCE degradation to a final concentration of 0.61 mM. Yeast extract was added up to a final concentration of 0.1 g/*l*. H_2 was tested at a partial pressure of 0.5×10^5 Pa. The gas phase was N_2, except for the condition when hydrogen was used as an electron donor.

Degradation of high concentrations of PCE The initial concentrations of PCE were 0.06 mM, 0.3 mM, 0.6 mM, or 0.9 mM. Experiments were performed by using 26-m*l* closed bottles. The bottles contained 10 m*l* of MM medium supplement with yeast extract as an electron donor. Cells were added at 0.33 mg of protein/m*l*. Incubation was conducted statically at 30°C for 7 days.

Control, containing no cells, were used in all cases.

Effect of temperature and pH To study the effect of temperature on the biological dechlorination of PCE, cultures in 26-m*l* serum bottles containing 10 m*l* of MM medium with yeast extract as an electron donor and inoculated at 0.33 mg of protein/m*l* were steady and incubated in the dark at different temperatures (10, 20, 30, 37, and 50°C).

To determine the optimum pH range for degradation of PCE, the media were adjusted at different pH values (4, 6, 7.2, 8, 9, 10, 12) using 0.5 M NaOH or 0.5 M HCl before autoclaving.

Degradation of PCE and other halogenated aliphatic compounds on growth condition In this study, we observed the conditions that the isolated strain can dechlorinate PCE in growth medium. The growth medium (MY) contained per liter: K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 2.0; $FeSO_4 \cdot 7H_2O$, 0.02, and 0.1 m*l* vitamin solution (containing 1.0 g of p-aminobenzoic acid, 1.0 mg of biotin per liter), and the pH was adjusted to 7.2. The MY medium was purged with N_2 for 30 minutes before autoclaving. Cells were added at 0.007 mg of protein/m*l* in 10 m*l* MY medium for the experiments of degradation of PCE and other halogenated aliphatic compounds. Incubation was conducted statically at 30°C, and samples were taken for analysis after 56 h. After 56 h, the degradation of TCE, *cis*-1,2-dichloroethylene (cDCE), *trans*-1,2-DCE, 1,1-DCE, DE, 1,1-dichloroethane (1,1-DE), DM, DP, 1,2-dichloropropane (1,2-DP), TE, and 1,1,1-trichloroethane (1,1,1-TE) were determined.

Analyses PCE and other aliphatic compounds were analysed by gas chromatography. PCE, TCE, cDCE, *trans*-1,2-DCE, 1,1-DCE, DE, 1,1- 1,1-DE, DM, DP, 1,2-DP, TE, and 1,1,1-TE in a 10-μ*l* 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. 3.2φ×2.1 m ; Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). Temperatures of injection, detection, and column were 200°C, 330°C, and 80°C, respectively. High concentration of PCE was determined gas chromatographically by a model GC-9A gas chromatograph (Shimadzu Co., Japan) equipped with a flame ionization detector (FID) and a glass column (i.d. 3.2φ×2.1 m; Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). VC was determined using a model GC-9A gas chromatograph equipped with FID and a glass column (i.d. 3.2φ×2.2 m; Tricresyl-phosphate (TCP) 20% on Chromosorb WAW DMCS 60/80). Chloride ion was determined using an ion chromatograph (IC; DX-500 system, Dionex Co., California, USA). Protein of whole cells was determined by a modified Lowry method (13). The cells were quantified after washing with phosphate buffer to remove interference from medium components especially yeast extract and ultrasonicated again.After centrifugation (20 min at 8,000×g), the suspension was then used for cell protein.

Results

Taxonomy of isolated organism Strain DPH was a motile, Gram-positive, sporeforming, rod-shaped bacterium. The bacterium was a straight rod to 2.23 to 2.85 μm in lengh as observed under the scanning electron microscopy (Fig. 1). The isolate was characterized on the basis of urease production, liquefaction of gelatin, indole production, and utilization of carbon sources (Table 1).

The 16S rRNA analysis, showed that strain DPH was specifically related to *C. bifermentans* X73437 (96.2% sequence identity) (Table 2) and that *Eubacterium tenue* (95.7%) was a member of the same line of descent. The strain DPH was related to other anaerobic bacteria sush as *C. sordellii* (92%) and *C. difficile* (90.5%) (Table 2).

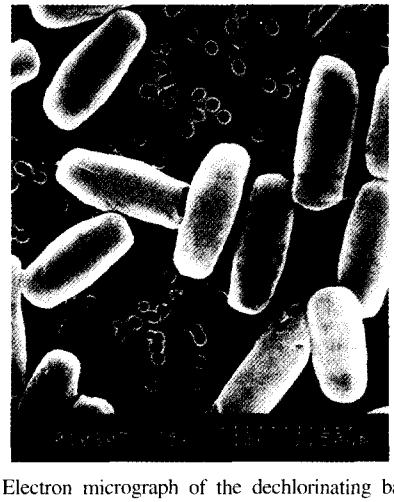

Fig. 1. Electron micrograph of the dechlorinating bacterium DPH.

Table 1. Physiological characteristics of strain DPH

Hydrolyzation of casein	Positive
Urease	Negative
Liquefaction of gelatin	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Lipase	Negative
Lecithinase on egg yolk agar	Positive
Production of toxin	Negative
Production of H_2S	Positive
Utilization of carbon sources	Positive glucose, maltose Negative lactose, salicin

However, strain DPH did not belong to any presently known bacteria that can degrade PCE and other aliphatic compounds. Therefore, we could suggest naming it *Clostridium bifermentans* DPH from the results of physiological characterization and phylogenic classification.

Effects of electron donors on PCE degradation

Relatively rapid degradation of PCE was obtained with acetate, lactate, glucose, formate, and yeast extract compared with pyruvate and methanol (Fig. 2). However,

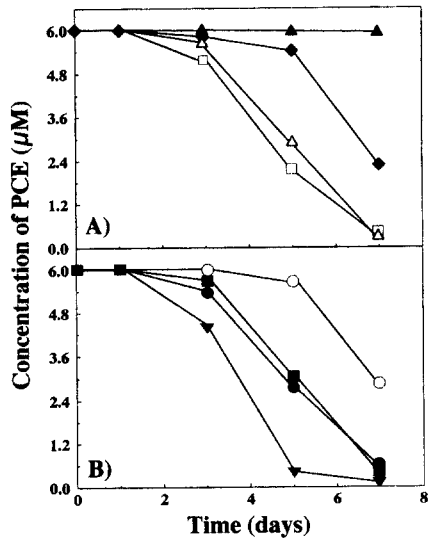

Fig. 2. Effect of electron donors on PCE degradation. MM medium was supplemented with electron donors. The initial concentrations of acetate, lactate, glucose, formate, pyruvate, and methanol were 0.61 mM, respectively, except for yeast extract (1.0 g/*l*). Results are means of duplicate experiments. Symbols: (A) ▲, H_2; ◆, methanol; △, acetate; □, glucose and (B) ○, pyruvate; ■, lactate; ●, formate; ▼, yeast extract.

Table 2. Comparison of homology amont organisms by *K nunc* and sequence similarity

	Sequence similarity(%)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Isolated strain		96.2	95.7	77.0	84.8	92.0	79.6	75.8	73.5	88.1	79.1	79.0	79.0	77.9	77.8	79.3	79.2	82.2	90.5	73.6
2. *C. bifermentans*	0.018		96.8	78.8	86.6	93.0	81.6	78.0	75.6	89.8	81.4	81.4	81.4	80.2	79.5	81.4	81.5	83.8	92.0	76.3
3. *E. tenue*	0.025	0.030		81.9	89.0	92.0	84.4	80.0	78.4	90.7	83.1	83.4	83.1	81.5	81.3	83.0	82.9	85.5	94.0	77.3
4. *C. paradoxum*	0.131	0.134	0.108		89.3	81.6	77.7	80.4	74.0	78.6	68.3	68.3	68.1	74.0	73.4	68.6	68.7	72.9	80.7	66.3
5. *C. thermoalcali-philum*	0.100	0.100	0.078	0.046		91.6	88.2	79.3	83.3	89.2	79.2	79.3	78.8	81.2	78.7	79.4	79.2	83.8	88.8	74.5
6. *C. sordelli*	0.028	0.035	0.013	0.121	0.084		86.5	78.5	80.8	94.3	77.5	77.6	77.8	80.7	79.4	77.8	77.7	88.0	93.5	72.3
7. *C. halophilum*	0.154	0.155	0.128	0.179	0.131	0.139		73.7	83.7	85.4	75.1	75.5	75.1	82.0	79.0	74.4	74.4	79.5	84.0	71.7
8. *Peptostrepto-coccus* sp.	0.129	0.130	0.115	0.192	0.132	0.128	0.196		68.6	78.9	57.8	68.3	68.4	7138	72.7	66.9	66.8	71.5	80.2	63.9
9. *E. thermomarinus*	0.170	0.171	0.148	0.170	0.140	0.154	0149	0.202		79.2	72.6	72.7	72.0	78.3	77.9	71.0	71.2	76.5	78.1	67.3
10. *C. mangenotii*	0.072	0.073	0.062	0.159	0.114	0.066	0.152	0.125	0.175		77.8	78.0	77.7	78.3	76.7	76.9	76.9	87.0	93.3	72.1
11. *M. carouselicus*	0.174	0.172	0.161	0.233	0.178	0.176	0.216	0.219	0.193	0.174		98.8	97.5	71.9	72.1	94.1	94.0	71.5	79.4	84.6
12. *M. equipercicus*	0.177	0.174	0.161	0.235	0.177	0.177	0.213	0.217	0.194	0.174	0.011		98.2	72.0	72.2	94.5	94.4	71.4	79.6	84.9
13. *M. caseolyticus*	0.177	0.174	0.163	0.237	0.183	0.175	0.218	0.215	0.202	0.177	0.025	0.020		72.0	71.8	94.8	94.7	71.4	79.5	84.9
14. *Eubacterium* sp.C2	0.183	0.182	0.175	0.266	0.191	0.178	0.187	0.227	0.186	0.211	0.264	0.264	0.264		81.3	72.3	72.1	74.9	80.0	69.7
15. *C. aminobutyricum*	0.146	0.152	0.136	0.185	0.170	0.144	0.158	0.178	0.184	0.180	0.224	0.227	0.231	0.162		71.9	71.9	74.6	79.2	68.2
16. *S. pisciferm entans*	0.176	0.176	0.167	0.230	0.179	0.178	0.225	0.230	0.212	0.190	0.065	0.062	0.058	0.259	0.228		99.9	71.5	79.2	84.4
17. *S. condimenti*	0.177	0.175	0.168	0.229	0.179	0.178	0.225	0.231	0.210	0.190	0.065	0.063	0.059	0.261	0.228	0.001		71.5	79.1	84.4
18. *C. mayombeii*	0.064	0.070	0.056	0.136	0.102	0.059	0.150	0.120	0.176	0.071	0.071	0.174	0.174	0.192	0.149	0.175	0.175		86.9	65.3
19. *L. difficile*	0.062	0.066	0.046	0.140	0.104	0.055	0.156	0.118	0.176	0.058	0.175	0.175	0.176	0.208	0.168	0.181	0.182	0.060		73.1
20. *L. lindneri*	0.214	0.211	0.209	0.267	0.211	0.214	0.249	0.275	0.248	0.220	0.155	0.151	0.150	0.282	0.266	0.155	0.155	0.218	0.226	

PCE was not degraded with H_2. On the basis of these results, we selected yeast extract (0.1 g/*l*) as adequate electron donor.

Degradation of high concentrations of PCE As shown in Fig. 3, the strain DPH had the ability to degrade up to 94% of PCE at an initial concentration of 0.9 mM for 7 days and the rate of PCE degradation was decreased with increasing initial PCE concentration.

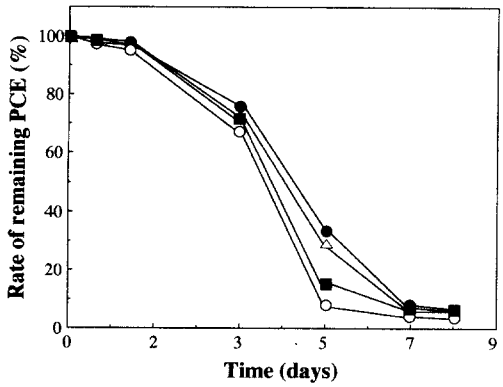

Fig. 3. Degradation of high concentrations of PCE. The culturess were incubated at pH 7.2 and 30°C. Yeast extract (0.1 g/*l*) was added to MM medium as the electron donor for PCE degradation. Results are means of duplicate experiments. Symbols: ○, 0.06 mM; ■, 0.3 mM; △, 0.6 mM; ●, 0.9 mM.

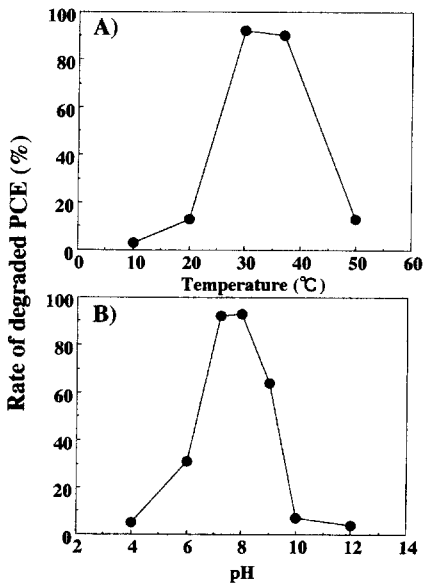

Fig. 4. Effect of temperature (A) and pH (B) on PCE (0.006 mM) degradation. The temperature- and pH-dependence experiments were carried out at pH 7.2 and at 30°C, respectively, for one week in setup. Results are means of duplicate experiments.

As shown in Fig. 4A and 4B, the cultures degraded PCE (0.006 mM) in the temperature range 10 to 50°C, with an optimum between 30°C and 37°C and the pH range of 4.0 to 12.0. and the degradation rates remained constant between pH 7.2 and 8.0.

Dechlorination of PCE and other halogenated aliphatic compounds on growth condition The cells increased exponentially within 14 h and reached a steady state after 16 h (Fig. 5A). PCE dechlorination started in the mid stationary phase, after 30 h, and was completely degraded after 56 h (Fig. 5A). At 48 h, specific PCE dechlorinating rate was 0.41 μmol/h · mg protein. The concentration of chloride ions increased from 0.9 mM to 2.7 mM. PCE was rapidly converted to cDCE without apparent accumulation of TCE (Fig. 5B). Low level of 1,1-DCE (0.5% of the amount of cDCE) was also

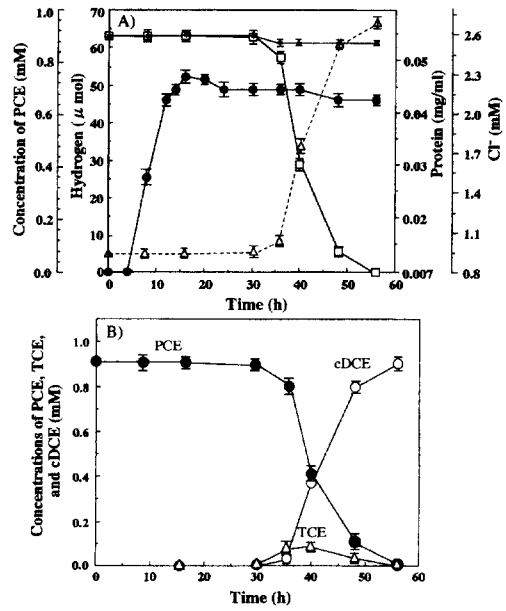

Fig. 5. (A) Time course of PCE degradation, and cell protein and chloride ion concentration profiles in MY medium. Symbols: *, PCE (without cells); □, PCE; ●, cell protein (with PCE); △, chloride ion. (B) Reductive dechlorination of PCE to TCE and cDCE. Strain DPH was grown in MY medium containing PCE (0.9 mM). Results are means of triplicate experiments.

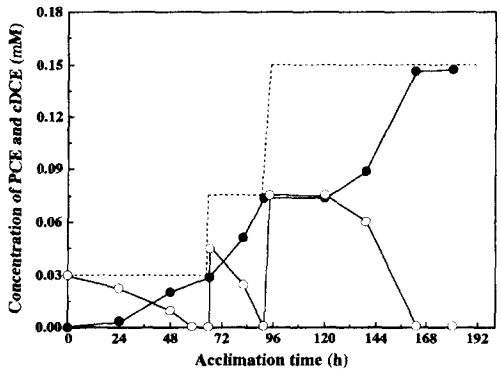

Fig. 6. Changes in the PCE, cDCE concentration after startup of anaerobic culture. MY medium supplemented. PCE was sequentially added following 98% depletion in culture medium. Results are means of duplicate experiments. Symbols: ○, PCE; ●, cDCE; ·····, sum of additional PCE.

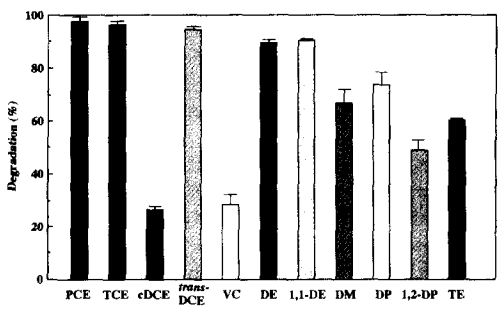

Fig. 7. Degradation of halogenated aliphatic compounds by *Clostridium bifermentans* DPH. Percent degradation compared with controls with no cells. Initial concentration of each compounds was 0.018 mM. Cultivation was performed at 30°C for 56 h. Results are means of triplicate experiments.

detected. Ethene, ethane, or acetylene production were not detected. No growth and reductive dechlorination of PCE was observed when yeast extract was eliminated from the medium.

Figure 6 presents changes in the PCE, cDCE concentration after startup for anaerobic culture with strain DPH. For this sequential dechlorination experiment of PCE, total 0.15 mM of PCE was added in MY medium with strain DPH. It took 56 h to dechlorinate PCE (0.03 mM) to cDCE at the first stage of the pure culture. After sequential acclimation for 65 h, it took 24 h to dechlorinate PCE (0.045 mM), after 90 h, it took 72 h to dechlorinate PCE at as high as 0.075 mM, and cDCE was accumulated.

The rate of dechlorination of halogenated aliphatic compounds by the strain DPH were determined. After 56 h of cultivation, the dechlorination rates of PCE, TCE, cDCE, *trans*-1,2-DCE, VC, DE, 1,1-DE, DM, DP, 1,2-DP, and TE were determined to be 98, 96, 27, 94, 30, 90, 90, 70, 77, 50 and 60%, respectively(Fig. 7). However, dechlorination of 1,1-DCE and 1,1,1-TE were not observed during one week.

Discussion

We isolated and characterised a novel PCE dechlorinating anaerobic bacterium *C. bifermentans* DPH. Results indicate that the newly isolated strain degrades PCE by reductive dechlorination. On the basis of the 16S rRNA analysis, *C. bifermentans* DPH is different from known PCE degrading anaerobic bacteria, such as *Dehalospirillium multivorans* (Gene bank access number X82931), *Dehalobacter restrictus* TEA (Y10164), *Dehalobacter restrictus* PER-K23 (U84497), *Dehalococcoides ethenogens* 195 (AF004928), *Pelobacter* sp. TT4B (U49748), strain MS-1 (L43508).

In this study, although several electron donors effectively enhanced PCE dechlorination, yeast extract was the most effective electron donor. No growth and reductive dechlorination of PCE was observed when yeast extract was eliminated from MY medium. This observation suggest that yeast extract served as a source of nutrients and electrons.

The most widely reported transformation of PCE under anaerobic conditions was reductive dechlorination of lower PCE concentrations (0.003-0.06 mM) (14-16). However, dechlorination of PCE at a higher concentration more than 0.6 mM in contaminated sites, by *Pseudomonas fluorescens* was reported (17). The dechlorinating rate for strain DPH (0.41 μmol/h·mg protein) is comparable with those of the previously reported pure cultures. *Desulfomonile tiedjei*, 0.02 μmol/h · mg protein (18); Methanosarcia sp., 9.8×10^{-5} μmol/h · mg protein (19); and strain MS-1, 0.5 μmol/h·mg dry cell (20); and lower than those of *Dehalospirillium multivorans*, 4.5 μmol/h·mg protein (4); and *Dehalobacter restrictus* (strain PER-K23), 1.0 μmol/h · mg protein (3).

Biotransformation of PCE into innocuous compounds is desirable; unfortunately, cDCE was the main product of PCE degradation in the present study. A bacterium, tentatively named *Dehalococcoides ethenogenes* 195, capable of reducing chloroethenes to ethene, was recently isolated (6) and its PCE-reductive dehalogenase and TCE-reductive dehalogenase have been partially purified (21). Dechlorination by this organism was via a two-component enzyme pathway. The final product of PCE dechlorination by strain DPH is cDCE, but if cDCE was used as the initial chemical for decomposition, 27% of cDCE was dechlorinated (Fig. 7). The product(s) of cDCE dechlorination and the reasons why cDCE as an intermediate product is recalcitrant, are not clear. This aspect requires indepth studies to understand the mechanism and product(s). Probably, some induction and regulation mechanisms of PCE dehalogenase and/or cDCE dehalogenase activity exist in *C. bifermentans*. Complete dechlorination may be realized by a two-step or multistep process (22).

The capability to degrade halogenated aliphatic compounds was mainly observed among the members of the *Pseudomonas* (17, 23), strain GJ10 (24), and strain OB3b (9) under aerobic condition. Only a few studies had described the anaerobic transformation of a number of chlorinated hydrocarbons by members of the genus Desulfitobacterium and the related *Dehalobacter restrictus* (3, 5). In methanogenic cultures, transformation was observed for halogenated methanes, DE, 1,1,2,2-tetrachloroethane, and PCE at low concentrations (0.06-0.18 μM) (25).

Results indicate that strain DPH could play some important role in the initial breakdown of PCE. Furthermore, the ability of strain DPH to degrade several halogenated aliphatic compounds is important for the development of strategies for the biological remediation of environments contaminated with mixtures of halogenated substances.

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