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Biodechlorination of Tetrachloroethylene by Anaerobic Bacteria Cell Cultures Isolated from Contaminated and Uncontaminated Soils

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Recently, soil and ground water in various areas were heavily contaminated with tetrachloroethylene (perchloroethylene, PCE), trichloroethylene (TCE) and dichloroethylene (DCE) in Japan. The contamination was due to industrial degreasing solvents, dry cleaning fluids, fumigation and so on. Owing to their widespread not always improper use and disposal, these compounds have become ubiquitous environmental pollutants. In Japan, ground water quality standard of PCE is $0.01 mg.L^{-1}$, and $0.01 - 0.05 mg \cdot L^{-1}$ of PCE is commonly detected in the ground water. PCE is non-mutagenic in bacterial and mammalian cell cultures (Ikeda et al. 1980; Jackson et al. 1993) but was shown to be carcinogenic in experimental animals (Jackson et al. 1993), whereas TCE was mutagenic *in vitro* , and *in vivo* and were carcinogenic in mice and rats (Jackson et al. 1993; Crebelli et al. 1982, 1989; Henschler et al. 1977). It is important to remove PCE, TCE and DCE from the environment.

Some chlorinated ethanes and methanes are biodegraded by methanotrophs (Fathepure et al. 1987, 1988). The biodegradation of PCE has also been reported (Fathepure et al. 1987, 1988; Distefano et al. 1991; Kastner et al. 1991; Egli et al. 1988; Vogel and McCarty 1985). Species of methanogenic bacteria such as *Methanosarcina sp.*, isolated by Fathepure et al. (1987, 1988), that significantly dechlorinate PCE to TCE, have also been discovered. This bio-dechlorination reaction occurred by methanogenesis, which is associated with methane production.

In this study, for isolation of aerobic and anaerobic bacteria, five soil samples that were heavily contaminated with PCE , TCE and DCE were collected from areas around dry cleaning facilities and electronic goods factories, ten uncontaminated soil samples were collected from areas around a paddy field, river subsoil and a pond planted with lotus root, and their ability to dechlorinate PCE in anaerobic culture systems was tested. Fifteen species of bacteria which are capable of dechlorinating PCE to TCE or DCE were isolated from soils around two dry cleaning facilities, an electrical goods factory, and a pond planted with lotus root, and the properties of PCE bio-dechlorination were examined.

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MATERIALS AND METHODS

PCE, TCE and 1,1-, *cis* - 1,2- and *trans* - 1,2- DCEs purchased from Wako Pure Chemical Co. Ltd, were >99% pure, as determined by gas chromatograph - mass spectrometry.

Minimal broth was composed of potassium dihydrogen phosphate (7g), dipotassium hydrogen phosphate (2g), magnesium sulfate (0.1g), sodium citrate (0.5g) and yeast extract (2g) in distilled water (1,000 ml), adjusted to pH 7.2. Minimal agar plates were prepared by adding 1.5% agar (Wako Pure Chemical Co. Ltd.) to the minimal broth.

To determine the potentiality of PCE biodegradation, 1 g of soil was added to 10 ml of minimal broth, andthen transferred to a 26 ml vial. The head-space in the vial was flushed with deoxygenated N_2. The vial was sealed with a Teflon-coated rubber and aluminum septum-cap. PCE at a concentration between 30 and 160 $mg \cdot L_{-1}$ was inoculated into the broth using a microsyringe. Culture proceeded at 25 °C or a week.

To acclimate PCE dechlorinating bacteria, 5 g of subsoil from the lotus pond was added to 50 ml of minimal broth, which was then transferred to a 125 ml vial. PCE at a concentration between 5 and 20 $mg \cdot L^{-1}$ was inoculated into the minimal broth. Enrichment culture proceeded at 25 °C for 100 days.

The PCE-dechlorinating bacteria were isolated on minimal agar plates by smearing a 10-fold dilution of the minimal broth culture. Cotton was packed into a microtube (0.2 x 20 mm) and saturated with PCE between 1 and 1,000 mg. During incubation of the minimal agar plates, this tube was fixed with paper tape to the inside of a covered plate, thus the growing bacteria were continuously exposed to PCE vapor. Cultivation was performed in an anaerobic glass jar (Hirayama Seisakusho Co. Ltd.) at 25 °C for a week. Thereafter, 500 colonies were selected from the plate, and each was inoculated into 10 ml of broth medium to examine its ability to dechlorinate PCE. PCE at 30 $\mu g \cdot ml^{-1}$ was added to each broth medium, and the headspaces of the vials were flushed with deoxygenated N_2. Finally, nine organisms were isolated from contaminated soil, and they were called strains K-26, K-35, Y-51, F-1, F-9, F-12, F-18, F-31 and F-41. Six PCE-dechlorinating bacteria from enrichment culture of uncontaminated soil were isolated and called strains T-3, T-8, T-12, T-18, T-28 and T-37.

PCE and its products were quantified by means of gas chromatography (GC), using a column (2.6 mm i.d. x 3.1 m) packed with silicon DC-550 (chromosorb W-AW-DMCS) maintained at 120 °C with nitrogen (50 $ml \cdot min^{-1}$) as the carrier gas, equipped with an flame ionizing detector in a gas chromatographic apparatus (Shimazu GC-6A). Chemicals and their products were identified by gas chromatograph-mass spectrometry (GC-MS, Varian 3400 GC type equipped with Finnigan MAT-90). Detection limits for PCE, TCE and DCE (as *cis* - 1,2-DCE) in cultures were 0.01, 0.01 and 0.01 $mg \cdot L^{-1}$, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the time course of PCE, DCE concentration after startup for anaerobic enrichment culture with subsoil from the lotus pond. It took 40 days to dechlorinate PCE ($5mg•L^{-1}$) to DCE at the first stage of the enrichment culture. After sequential acclimation for 55 days, it took 20 days to dechlorinate PCE ($10mg•L^{-1}$), after 75 days, it took only 14 days to dechlorinate PCE at as high as 20 $mg•L^{-1}$, and DCE was accumulated.

Table 1 shows characteristics of PCE-degrading bacteria isolated from contaminated soils and enrichment culture of uncontaminated soil. Total organic carbons in the soils varied from 0.8 to 29.7 $mg•dryg^{-1}$. PCE-dechlorinating bacteria from contaminated soil were isolated and called strains K-26 and K-35 isolated from the electronic factory; Y-51 from the dry cleaning facility Y; and F-l, F-9, F-12, F-18, F-31 and F-41 from dry cleaning facility F. Strain K-26 was a gram-positive rod bacterium that was catalase and oxidase positive and fermented a various carboxyhydrates, glucose, xylose, mannose, arabinose, fructose, maltose and rhamnose. The organism did not ferment lysine, arginine, or ornithine, nor did it reduce nitrate to nitrite. Strain Y-51 was a gram-negative rod bacterium, and it fermented a variety of carbohydrates, glucose, xylose, mannose, arabinose, fructose, and rhamnose. In addition, the organism anaerobically fermented catalase, oxidase and urease, and reduced nitrate to nitrite. K-35 was a gram-positive coccus which had Staphylococcus-like form. The organism anaerobically fermented catalase and oxidase, and it reduced nitrate to nitrite. PCE-dechlorinating bacteria from uncontaminated soil were isolated and called strain T-3, T-8, T-12, T-18, T-28, and T-37. Stain T-3 was a gram-negative rod bacterium, and the others were gram-positive rod or coccus bacteria.

We found that PCE was completely converted to TCE in the K-26 cell culture and to DCE *via* , TCE in the Y-51 cell culture. PCE was slowly dechlorinated to TCE in the K-35 cell culture. Figure 2 shows the mass balance on PCE and dechlorinated degradation products. In the Y -51 cell culture, the concentration of 181μM ($30mg•L^{-1}$) of PCE was converted to 34μM of TCE and 51μM of DCE (1,1-DCE; 7%, *cis* -1,2-DCE; 92%, *trans* -1,2-DCE; 1%) within 1.5 days, to 180μM of DCE within 2 days.

Figure 3 indicates the results of dechlorination of PCE when K-26, K-35 and Y-51 cells were cultured in the presence of 30, 60 and 160 $mg•L^{-1}$ of PCE in the broth medium at 25 °C for a week. At various periods, the head-space in the vial was collected with a microsyringe and analyzed by the GC system. The dechlorinating ability of the three isolated strains varied with the length of the incubation period. PCE was partially converted to TCE by K-35 cells after incubation for 4 days. PCE was readily dechlorinated to DCE within 3 days when Y-51 cells were cultured at concentrations of 30, 60 and 160 $mg•L^{-1}$ of PCE. The K-26 cells also dechlorinated PCE, which was completely converted to TCE within 3 days, even at concentrations of 30, 60 and 160 $mg•L^{-1}$. Strains K-26 and Y-51 can thus readily dechlorinate high concentrations of PCE. In contrast, PCE dechlorination by the K-35 cells was comparably slow and required over 5 days to complete the production of TCE.

Table 1. Characteristics of PCE-dechlorinating bacteia isolated from contaminated and uncontaminated soils

Sample	TOC	Contaminant (μg•dry g^{-1})			Isolate	Cell	Gram	Sporulation	Motility	End
	(mg•dry g^{-1})	PCE	TCE	*cis* -1,2-DCE		morphology	stain			product
Contaminated soil										
Electronic factory	0.8	23	3.8	0.0	K-26	Rod	+	+	+	TCE
					K-35	Coccus	+	-	-	TCE
Dry cleaning facility Y	3.9	130	11	0.1	Y-51	Rod	-	-	+	DCE
Dry cleaning facility F	5.0	17	0.1	0.1	F-1	Rod	+	+	+	DCE
					F-9	Rod	+	+	+	DCE
					F-12	Rod	-	-	+	DCE
					F-18	Rod	+	+	+	DCE
					F-31	Rod	+	+	+	DCE
					F-41	Rod	-	-	+	DCE
Uncontaminated soil										
Pond planted with lotus root	29.7	0.0	0.0	0.0	T-3	Rod	-	-	-	DCE
					T-8	Rod	+	+	+	DCE
					T-12	Rod	+	±	+	DCE
					T-18	Coccus	+	-	-	DCE
					T-28	Rod	+	±	+	DCE
					T-37	Coccus	+	-	-	DCE

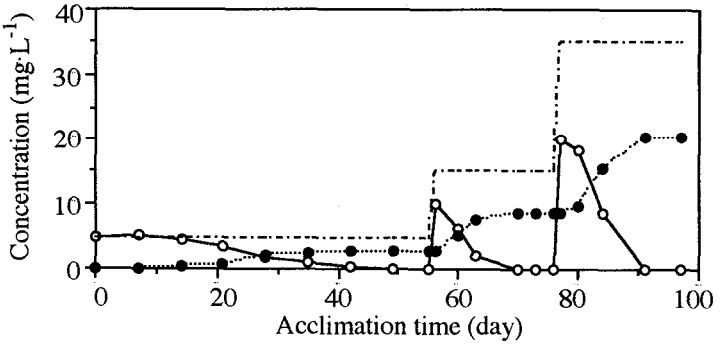

Figure 1. Changes in the PCE, DCE concentration after startup of anaerobic enrichiment culture
(Remaining PCE; o—o , sum of addditional PCE; ——— cumulative DCE; ●·····●)

The ability to dechlorinate seems to be associated with the production of dechlorinating enzymes. PCE dechlorination by all 3 isolated strains varied according to the temperature. For example, Y-51 effectively dechlorinated PCE ($160 mg \cdot L^{-1}$) within a temperature range from 15 - 40 °C (Fig. 4).
PCE was readily dechlorinated to DCE *via* , TCE by the Y-51 cells, and the mechanism seems to be due to active enzyme(s) produced by the cells between cell-free lysates from the Y-51 strain efficiently degraded PCE to DCE *via* , TCE. However, the enzyme was readily inactivated by oxygen because the cell culture did not dechlorinate PCE in aerobic conditions (data not shown). Accordingly, the active enzyme may be vulnerable to oxygen or may not be produced in the presence of PCE under aerobic conditions.

It has been reported that toluene induced *Pseudomonas putida* , to degrade TCE and three isomeric DCFs in growth media, but PCE, vinyl chloride and ethylene were not removed from the incubation mixtures (Wackett and Gibson 1988; Furukawa et al. 1994). This degradation is due to the biodegradative activity of toluene dioxygenase (Wackett and Gibson 1988). Y-51 cells readily dechlorinated PCE to DCE *via* , TCE by an active enzyme in anaerobic conditions. However, DCE was not reduced to vinyl chloride. In anaerobic methanol-PCE enriched cultures, PCE was dechlorinated to vinyl chloride (Distefano et al. 1991). It was also shown that by using isotopically labeled TCE with a single ^{13}C atom, TCE is definitely dechlorinated in the soil to *cis* -1,2-DCE not 1,1-DCE under anaerobic conditions (Kleopfer et al. 1985). However, no evidence that PCE was completely biodegraded by the bacteria or that an effective organism was isolated was found in this study. We are further investigating the biosynthesis of the enzyme.

PCE and TCE are common soil and ground water contaminants in various areas. These chemicals are also environmental mutagens and carcinogens. Therefore, it is important to remove and degrade these toxic agents in soil and ground water. In this study, fifteen organisms that dechlorinate PCE were isolated, and their application in the environment was examined. Finally, nine organisms were isolated from contaminated soil, and they were called strains K-26, K-35, Y-51, F-1, F-9, F-12, F-18, F-31 and F-41. Six PCE-dechlorinating bacteria from enrichment culture of uncontaminated soil were isolated and called strains T-3, T-8,

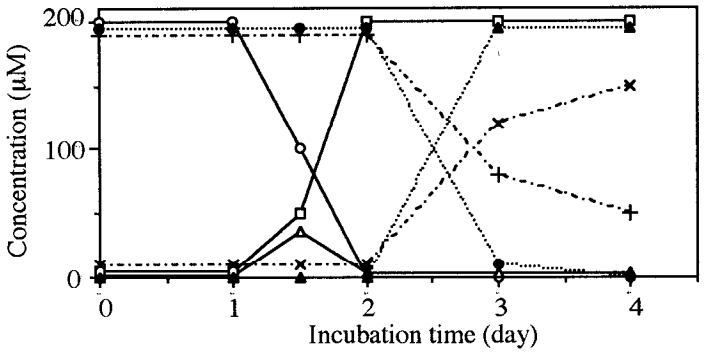

Figure 2. Dechlorination of PCE by Y-51, K-26 and K-35 cell culture. (PCE; ○ Y-51, ● K-26, + K-35, TCE; △ Y-51, ▲ K-26, × K-35, DCE; □ Y-51)

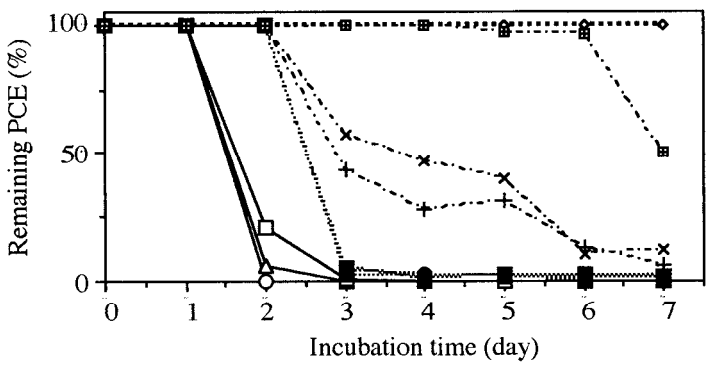

Figure 3. Dechlorination of PCE by Y-51, K-26 and K-35 cell culture.
Y-51 cell culture was incubated in broth containing 30 (○), 60 (△), 160 (□) $mg \cdot L^{-1}$ of PCE, K-26 was incubated in broth containing 30 (●), 60 (▲), 160 (■), K-35 was incubated in broth containing 30 (+), 60 (×), 160 (⊞), and control (◇).

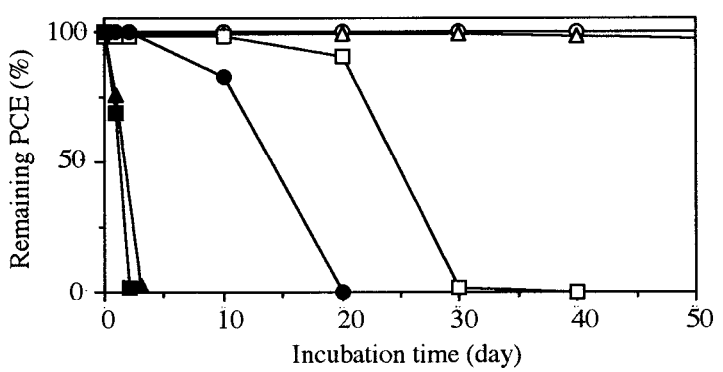

Figure 4. Relationship between the incubation temperature and the dechlorination of PCE by Y-51 cell culture.
(5°C; ○ , 10°C; △ , 15°C; □ , 20°C; ● , 30°C; ▲ , 40°C; ■)

T-12, T-18, T-28 and T-37. K-26 and K-35 cell cultures dechlorinated 60mg•L^{-1} of PCE to TCE within a week. Y-51 cell culture dechlorinated 160mg•L^{-1} of PCE to DCE *via* , a TCE intermediate within 3 days, and product of all other cell cultures was DCE. PCE was dechlorinated only in an anaerobic culture system by these fifteen organisms but not in an aerobic culture system. Accordingly, the mechanism of dechlorination of PCE may be associated with a degradative enzyme that is activated only under anaerobic condition and may be vulnerable to oxygen. This enzyme may also actively degrade PCE to DCE in cell-free lysates.

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