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ORIGINAL PAPER

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Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylenes, and vinyl chloride by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1

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Abstract A recombinant strain of *Escherichia coli* (JM109/pBZ1260) expressing constitutively toluene-*o*-xylene monooxygenase (ToMO) of *Pseudomonas stutzeri* OX1 degraded binary mixtures (100 µM each) of tetrachloroethylene (PCE) with either trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), *cis*-dichloroethylene (*cis*-DCE), *trans*-1,2-dichloroethylene (*trans*-DCE), or vinyl chloride (VC). PCE degradation was 8–20% for these binary mixtures, while TCE and *trans*-DCE with PCE were degraded at 19%, 1,1-DCE at 37%, *cis*-DCE at 97%, and VC at 27%. The host *P. stutzeri* OX1 was also found to degrade binary mixtures of PCE/TCE, PCE/*cis*-DCE, and PCE/VC when induced with toluene. Degradation of quaternary mixtures of PCE/TCE/*trans*-DCE/VC and PCE/TCE/*cis*-DCE/VC by JM109/pBZ1260 were also investigated as well as mixtures of PCE/TCE/*trans*-DCE/1,1-DCE/*cis*-DCE/VC; when all the chlorinated compounds were present, the best degradation occurred with 24–51% removal of each. For these degradation reactions, 39–85% of the stoichiometric chloride expected from complete degradation of the chlorinated ethenes was detected. The time course of PCE/TCE/1,1-DCE degradation was also measured for a mixture of 8, 17, and 6 µM, respectively; initial degradation rates were 0.015, 0.023, and 0.029 nmol/min·mg protein, respectively. This indicates that for the first time an aerobic enzyme can degrade mixtures of all chlorinated ethenes, including the once – so it was believed – completely recalcitrant PCE.

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Introduction

Both tetrachloroethylene (PCE) and trichloroethylene (TCE) are suspected carcinogens and are the most common groundwater pollutants at hazardous waste sites (McCarty 1997). Both are regulated under the Safe Drinking Water Act to a maximum contaminant level of 5 parts per billion. Through natural attenuation, these effective solvents are degraded anaerobically via reductive dehalogenation to the less chlorinated ethenes TCE, *trans*-1,2-dichloroethylene (*trans*-DCE), *cis*-1,2-dichloroethylene (*cis*-DCE), 1,1-dichloroethylene (1,1-DCE), vinyl chloride (VC), and ethene as well as to ethane (Sharma and McCarty 1996). The dechlorination of PCE is often incomplete when it does occur, with VC and *cis*-DCE formed primarily (McCarty 1997); however, dehalorespiration of PCE to ethene is possible (Maymo-Gatell et al. 1997). The potent VC generated is a known human carcinogen (McCarty 1997), and both VC and *cis*-DCE are United States Environmental Protection Agency priority pollutants (Bradley and Chapelle 1998).

We have shown recently that *P. stutzeri* OX1 through its toluene-*o*-xylene monooxygenase (ToMO) degrades PCE (Ryoo et al. 2000); this is the first report of the aerobic attack of this fully chlorinated compound. *P. stutzeri* OX1 was isolated from activated sludge of a wastewater treatment plant (Baggi et al. 1987) and grows on *o*-xylene, toluene, cresols, 2,3-dimethylphenol, and 3,4-dimethylphenol as sole carbon and energy sources (Bertoni et al. 1996). ToMO has a relaxed regiospecificity (hydroxylates toluene in the *ortho*, *meta*, and *para* positions as well as *o*-xylene in both the 3 and 4 positions) (Bertoni et al. 1996) as well as a broad substrate range, since it oxidizes *o*-xylene, *m*-xylene, *p*-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene (Bertoni et al.

1996). The chlorinated aliphatics TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, VC, and chloroform have also been shown to be degraded individually and as mixtures by this enzyme (Chauhan et al. 1998; Shim and Wood 2000). Based on the gene sequence, ToMO appears to consist of a three-component hydroxylase with a catalytic oxygen-bridged dinuclear center encoded by *touABE*, a NADH-ferredoxin oxidoreductase (from *touF*), a mediating protein (from *touD*), and a Rieske-type ferredoxin (from *touC*); ToMO has greatest similarity to the aromatic monooxygenases of *Burkholderia pickettii* PKO1 and *Pseudomonas mendocina* KR1 (Bertoni et al. 1998).

Because of this ecological risk posed by soil and water-contaminated simultaneously by PCE, TCE, DCEs, and VC, the ability of JM109/pBZ1260 (Bertoni et al. 1996) expressing ToMO to degrade these mixtures has been evaluated. There are relatively few reports of degradation of mixtures of chlorinated pollutants, and most involve mixed cultures (not pure cultures expressing single degradative enzymes) and are limited to binary mixtures. Binary mixtures of TCE and chloroform (CF) have been studied for toluene-oxidizing bacteria (McClay et al. 1996) and for a mixed methanotrophic culture (Alvarez-Cohen and McCarty 1991); only the mixed methanotrophic culture successfully degraded both CF and TCE (many toluene and phenol oxidizers cannot oxidize CF at all (Chang and Alvarez-Cohen 1995)). Aziz et al. (1999) have also reported that the mutant of *Methylosinus trichosporium* OB3b which constitutively expresses soluble methane monooxygenase is able to degrade binary mixtures of TCE/*cis*-DCE, TCE/*trans*-DCE, and *cis*-DCE/*trans*-DCE. In addition, in a mixture of aromatic solvents and chlorinated aliphatics, two propane-grown pure *Rhodococcus* species degraded CF, VC, 1,1-DCE, *cis*-DCE, and 1,1,2-trichloroethane (but not significant amounts of PCE, TCE, 1,1,1-trichloroethane, and *trans*-DCE) in 2 weeks (Malachowsky et al. 1994).

Since there are no data on the simultaneous degradation of mixtures which include PCE, the degradation of PCE, TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, and VC by *E. coli* JM109/pBZ1260 expressing ToMO was investigated in a manner such that a single degradative enzyme was evaluated. This is the first report of a bacterium degrading simultaneously PCE and other less chlorinated ethenes under aerobic conditions. Further, the ability of JM109/pBZ1260 cells to completely degrade PCE mixtures by converting them to free chloride ions was investigated, as was the time course of degradation of a ternary PCE/TCE/1,1-DCE mixture.

Materials and methods

Organisms and growth conditions

From –80°C glycerol stocks, *P. stutzeri* OX1 was cultured at 30°C in Luria-Bertani (LB) medium (Sambrook et al. 1989); JM109 (Sambrook et al. 1989) and JM109/pBZ1260 were grown at 37°C in LB medium supplemented with 200 µg/ml ampicillin (Sigma Chemical Co., St. Louis, Mo.) for JM109/pBZ1260. Plasmid pBZ1260 (Bertoni et al. 1996) is a pGEM3Z derivative containing the *P. stutzeri* OX1 6 kb *touABCDEF* locus. In pBZ1260, ToMO is expressed under control of the *lac* promoter which yields constitutive expression due to high copy number.

To ensure exponential growth, overnight cultures were diluted to an optical density at 600 nm (OD) of 0.05–0.15 and grown to an OD of 0.4–1.0 (usually 0.6). The cells growing in the exponential phase were harvested by centrifugation at 13,800*g* for 5 min at 25°C (JA-17 rotor in a J2 series centrifuge, Beckman, Palo Alto, Calif.). The LB cultures were washed three times with 0.1 M potassium phosphate buffer (PPB, pH 7) to remove chloride ions. The cells were then resuspended in PPB to an OD of 2.0.

Chemicals

HPLC-grade PCE (99.9%) was obtained from Sigma Chemical Co., TCE was purchased from Fisher Scientific Company (Pittsburgh, Pa.), 1,1-DCE, *cis*-DCE, and *trans*-DCE from Aldrich Chemical Company, Inc. (Milwaukee, Wis.), and VC from Supelco (Bellefonte, Pa.). All materials used were of the highest purity available and were used without further purification.

Extents of chlorinated ethene degradation

Ten milliliters of the PPB cell suspension (OD 2) were added to 60 ml glass vials which were then covered with a Teflon-coated septum and aluminum crimp seal. The six chlorinated compounds were injected directly to the cell suspension from 10 mM dimethylformamide (Fisher Scientific) stock solutions (except for VC at 32 mM in methanol) using a Hamilton (Reno, Nev.) liquid-tight syringe to yield all compounds at 100 µM (assuming all the volatile compound is in the liquid phase). For *P. stutzeri* OX1, 50 µM toluene was added to induce ToMO expression (also added to the JM109 negative controls). The inverted vials were shaken at room temperature at 300 rpm on an IKA-Vibrax-VXR shaker (IKA-Works, Inc., Cincinnati, Ohio). The concentrations of the chlorinated compounds in the headspace were determined by gas chromatography after 20 h by injecting a 50 µl headspace sample with a 100 µl Hamilton gas-tight syringe into a 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector and fitted with a 0.1% AT-1000 on an 80/100 Graphpac packed column (Alltech Associates, Inc., Deerfield, Ill.). The samples were analyzed with the column and injector at 200°C and the detector at 250°C. JM109 plus chlorinated compound negative controls were used (in triplicates), and each set of aliphatic compounds was degraded three times to confirm the results (percentage removal of the chlorinated ethenes indicated above that of losses of the negative controls, which was usually 15%).

For the time course degradation of 50 µM PCE, TCE, and 1,1-DCE, triplicate preparations of cells were used, which were washed only once and suspended in PPB at OD 10. The degradation was determined relative to the average of triplicates of JM109 plus the chlorinated compounds (negative controls) which were measured at the same time.

Chloride ion generation

After gas chromatography, the inorganic chloride ion concentrations generated from the degradation of PCE were measured spectrophotometrically with the procedure of Bergmann and Sanik (1957) by adding 200 µl 0.25 M $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ in 9 M HNO_3 and 200 µl $Hg(SCN)_2$ in 95% ethanol to 0.6 ml of the supernatant. After 5 min, the absorbance of the $Fe(SCN)^{2+}$ product at 460 nm was measured with a DU640 spectrophotometer (Beckman Instruments). The colored $Fe(SCN)^{2+}$ product and $HgCl_2$ are both formed as free chloride ions and displace the thiocyanate ion of $Hg(SCN)_2$. Three replicates were used for each set of experiments, and chloride ion concentrations were calculated relative to

the average of three replicates of JM109 negative controls, which contained the same concentration of chlorinated ethenes.

Results

To determine the ability of JM109/pBZ1260 to degrade PCE with the other less chlorinated ethenes, degradation was evaluated initially with binary mixtures as shown in Table 1. For all five combinations, PCE was degraded from 8 to 20%; however, the other less chlorinated ethenes were degraded at 19% for TCE and *trans*-DCE, 27% for VC, 37% for 1,1-DCE, and 97% for *cis*-DCE. VC appeared to inhibit the degradation of PCE.

To corroborate the binary degradation results with ToMO expressed in JM109/pBZ1260, *P. stutzeri* OX1 was induced to express ToMO and used to degrade binary mixtures of PCE/TCE, PCE/*cis*-DCE, and PCE/VC (Table 1). 50 µM toluene was added to the cell suspensions to induce expression of ToMO since the highest levels of PCE degradation occurred with toluene present (Ryoo et al. 2000). As with degradation with ToMO expressed in *E. coli*, VC appeared to inhibit the degradation of PCE in this binary mixture, and *cis*-DCE was degraded to the greatest extent (96%). Since *cis*-DCE is the most prevalent dichloroethylene after natural attenuation of PCE and TCE (McCarty 1997), it is encouraging that is it is so readily degraded by ToMO. For all of these experiments, the toluene was removed below the detection limits, indicating induction of ToMO.

For the degradation of quaternary mixtures of PCE/TCE/*cis*-DCE/VC by JM109/pBZ1260, the large extent of degradation of *cis*-DCE was repeated as 96% was degraded along with 11% removal of PCE; however, TCE and VC degradation was inhibited as negligible degradation of each occurred (Table 1). For PCE/TCE/*trans*-DCE/VC, PCE itself was not degraded well.

Most significantly, when mixtures of all the chlorinated ethenes were present (PCE/TCE/*trans*-DCE/*cis*-DCE/1,1-DCE/VC), the best degradation of all components occurred (Table 1). Twenty-four percent of the PCE, 28% of the TCE, 46–51% of the *cis*-, *trans*-, and 1,1-DCE, and 48% of the VC was degraded. This indicates that the presence of all of the chlorinated compounds tends to stimulate degradation.

Since monooxygenase attack of chlorinated aliphatics such as TCE usually yields inorganic chloride ions (Nelson et al. 1987; Sun and Wood 1996), the degradation of the chlorinated ethenes by both JM109/pBZ1260 expressing ToMO and *P. stutzeri* OX1 was corroborated through analysis of the chloride ions generated (Table 1). For these experiments, 39–85% of the stoichiometric chloride expected from complete degradation of the chlorinated ethenes was detected. For example, for the overnight degradation of 100 µM PCE and 100 µM *cis*-DCE, 10% of the PCE was degraded and 97% of the *cis*-DCE was degraded; hence, 40 µM of chloride is expected from the PCE degradation and 194 µM of chloride from the *cis*-DCE degradation; since 199 µM were measured, 85% of the stoichiometric chloride was detected (Table 1).

The time course for the degradation of a ternary mixture was also measured and is shown for PCE/TCE/1,1-DCE at 50 µM in Fig. 1. TCE was degraded to the largest extent but clearly all three compounds were degraded simultaneously (as has been seen with non-PCE mixtures, Shim and Wood 2000). The initial degradation rates were calculated as 0.015, 0.023, and 0.029 nmol/min·mg protein, for PCE, TCE, and 1,1-DCE, respectively (using 0.522 mg protein/ml, Chauhan et al. 1998).

Table 1 Degradation of mixtures of tetrachloroethylene (PCE), trichloroethylene (TCE), three dichloroethylenes (DCEs), and vinyl chloride (VC) (at 100 µM each) by toluene-*o*-xylene monooxygenase (ToMO) in *E. coli* JM109/pBZ1260 and *P. stutzeri* OX1. Actual initial liquid chlorinated ethene concentrations (based on Henry's Law, Bradley and Chapelle 1998, Mercer and Cohen 1990) are 16.3 µM for PCE, 35 µM for TCE, 39.5 µM for *cis*-DCE, 42.7 µM for *trans*-DCE, 12.6 µM for 1,1-DCE, and 16.8 µM for VC

Strain	Mixture	% Removal[a]	Cl^- generated (µM)
E. coli JM109/pBZ1260	PCE/TCE	17±9[b]/19±4	39 (49)
	PCE/1,1-DCE	20±10/37±4	54 (84)
	PCE/*cis*-DCE	10±2/97±2	85 (199)
	PCE/*trans*-DCE	17±5/19±6	39 (41)
	PCE/VC	8±4/27±5	73 (43)
	PCE/TCE/*trans*-DCE/VC[c]	2±2/8±3/16±2/36±1	45 (45)
	PCE/TCE/*cis*-DCE/VC	11±4/2±4/96±1/2±1	60 (146)
	PCE/TCE/3DCEs/VC	24±6/28±7/51±3[d]/46±2[e]/48±5	51 (260)
P. stutzeri OX1[f]	PCE/TCE	21±4/10±4	71 (81)
	PCE/*cis*-DCE	23±5/96±1	68 (193)
	PCE/VC	7±2/25±6	58 (32)

[a] Compared to controls (*E. coli* JM109) after the same time period (~20 hr). Percentages are based on three replicates
[b] Mean±standard deviation
[c] At 32 µM VC
[d] For 1,1-DCE only
[e] For both *cis*- and *trans*-1,2-DCEs (combined peak)
[f] 50 µM toluene added during degradation

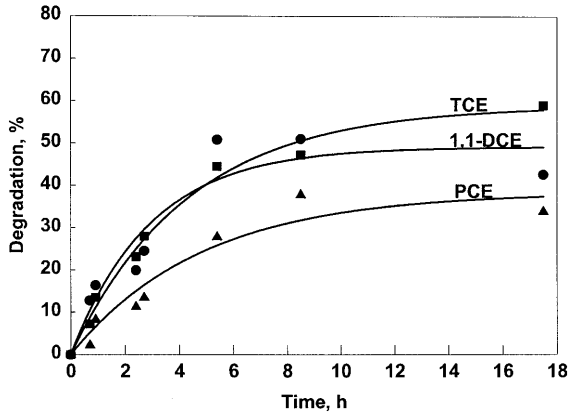

Fig. 1 Tetrachloroethylene (PCE, ▲), trichloroethylene (TCE, ■), and 1,1-dichloroethylene (1,1-DCE, ●) degradation by JM109/pBZ1260. Actual initial liquid concentrations were 8, 17, and 6 µM, respectively. Degradation shown is relative to JM109 negative controls, and *solid lines* indicate trends

Discussion

The initial rates of chlorinated ethene degradation by ToMO for the PCE/TCE/1,1-DCE ternary mixture are significantly slower than that for TCE degradation in the presence of chloroform with a mixed, methanotrophic, resting-cell culture (0.023 vs. 1.2 nmol/min·mg protein, Alvarez-Cohen and McCarty 1991) and lower than those for *cis*-DCE degradation with soluble methane monooxygenase in the presence of TCE and in the absence of formate (0.029 for 1,1-DCE vs. 14 nmol/min·mg protein, Aziz et al. 1999); however, they are 250 times faster than those for TCE degradation in mixtures by *Rhodococcus* species (Malachowsky et al. 1994). What is exceptional here is that all six chlorinated ethylenes (including PCE) were degraded simultaneously by ToMO (Table 1); the slower degradation rates (compared to methane monooxygenase) may be due to competitive inhibition of ToMO since Aziz et al. (1999) report significant inhibition of TCE and *cis*-DCE degradation by methane monooxygenase in the presence of *trans*-DCE.

The chloride concentrations generated by the degradation of the chlorinated ethenes with ToMO (Table 1) compare well to those reported for oxygenase attack of TCE (51–85% of the expected stoichiometric chloride was detected for four pseudomonads expressing monooxygenases and a dioxygenase) (Sun and Wood 1996). Using bacteria expressing two similar monooxygenases for the degradation of TCE, it has been shown that chloride release indicates complete mineralization to CO_2 (Nelson et al. 1986, 1987; Winter et al. 1989); hence, it is likely that the chlorinated ethenes here are also mineralized. Using the Bergmann and Sanik method (1957), we found the minimum detectable chloride concentration was 8 µM (0.28–0.36 mg/l) in 15 separate chloride standardization tests; this threshold is *at least* four times smaller than the minimum chloride concentration reported in Table 1 (32 – 260 µM Cl^- detected).

Since both PCE and TCE induce ToMO expression in *P. stutzeri* OX1 and the induced ToMO degrades both compounds (Ryoo et al. 2000, 2001), and given that it has now been demonstrated that ToMO can degrade mixtures of all chlorinated ethenes, it appears that *P. stutzeri* OX1 is capable of degrading mixtures of chlorinated ethenes without chemical inducers. Hence, *P. stutzeri* OX1 expressing ToMO holds promise for degrading mixtures of PCE and all of its less chlorinated degradation products which are present at many hazardous waste sites. For successful degradation, it should be recognized that an additional carbon source will have to be added as well as oxygen, and these may be supplied perhaps through perforated pipes or by utilizing a rhizoremediation system (Shim et al. 2000).

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