Aerobic degradation of tetrachloroethylene by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1

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Received 12 January 2000; accepted 25 April 2000

Tetrachloroethylene (PCE) is thought to have no natural source, so it is one of the most difficult contaminants to degrade biologically. This common groundwater pollutant was thought completely nonbiodegradable in the presence of oxygen. Here we report that the wastewater bacterium *Pseudomonas stutzeri* OX1 degrades aerobically 0.56 μmol of 2.0 μmol PCE in 21 h (V_{max} ≈ 2.5 nmol min^{-1} mg^{-1} protein and K_M ≈ 34 μM). These results were corroborated by the generation of 0.48 μmol of the degradation product, chloride ions. This degradation was confirmed to be a result of expression of toluene-*o*-xylene monooxygenase (ToMO) by *P. stutzeri* OX1, since cloning and expressing this enzyme in *Escherichia coli* led to the aerobic degradation of 0.19 μmol of 2.0 μmol PCE and the generation of stoichiometric amounts of chloride. In addition, PCE induces formation of ToMO, which leads to its own degradation in *P. stutzeri* OX1. Degradation intermediates reduce the growth rate of this strain by 27%.

Keywords: PCE degradation, monoxygenase, *Pseudomonas*

Highly chlorinated compounds are refractory and threaten the environment. For example, tetrachloroethylene (PCE, perchloroethylene) is one of the five most frequently detected volatile organic compounds found in municipal groundwater supplies[1], since it has been used extensively as an industrial degreasing solvent and fumigant[2]. In addition, PCE is one of 14 volatile organic compounds on the United States Environmental Protection Agency (US EPA) Priority Pollutant List[2]. Because this solvent is toxic and is a suspected human carcinogen[3], PCE is regulated under the Safe Drinking Water Act to a maximum contaminant level of 5 p.p.b.

Bacterial degradation of PCE has never been achieved in the presence of oxygen[4–6]. However, degradation through oxygen attack of fully halogenated chlorotrifluoroethylene has been achieved by purified soluble methane monooxygenase of *Methylosinus trichosporium* OB3b; the lack of PCE degradation by this enzyme was suggested to be due to steric hindrance[7]. Since fluorine is more electronegative and more tightly bound than chlorine, chlorotrifluoroethylene should be more difficult to oxidize than PCE. Therefore it is reasonable that a monooxygenase can degrade PCE.

It is very well established that PCE is degraded anaerobically by means of reductive dehalogenation to the less chlorinated ethenes trichloroethylene (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[8]. The dechlorination of PCE is often incomplete when it does occur, with VC and *cis*-DCE formed primarily[4]; however, dehalorespiration of PCE to ethene is possible[5]. Vinyl chloride is a known human carcinogen[4] and both VC and *cis*-DCE are US EPA priority pollutants[9].

Because of the ecological risk posed by soil and water contaminated by PCE, the ability of a monooxygenase to degrade PCE aerobically has been evaluated in a strain recently shown to degrade chlorinated aliphatics well. *Pseudomonas stutzeri* OX1 was isolated from activated sludge of a wastewater treatment plant[10] and grows on *o*-xylene, toluene, cresols, 2,3-dimethylphenol, and 3,4-dimethylphenol as sole carbon and energy sources[11]. Toluene-*o*-xylene monooxygenase (ToMO) from *P. stutzeri* OX1 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)[11] as well as a broad substrate range. It oxidizes *o*-xylene, *m*-xylene, *p*-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene[11]. 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[12,13]. 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[14].

There have been no previous examples of a bacterium degrading PCE under aerobic conditions. We showed that whole *P. stutzeri* OX1 cells could degrade PCE by converting it to free chloride ions. In addition, we identified the method of oxidative attack on this recalcitrant molecule by evaluating PCE degradation with the cloned *P. stutzeri* OX1 *touABCDEF* locus in *Escherichia coli*. Furthermore, the ability of PCE to induce its own degradation was investigated.

Results and discussion

During experiments to investigate whether TCE and PCE induce ToMO expression in *P. stutzeri* OX1, we observed that PCE was degraded. Additional experiments (Table 1, 40 replicates) showed that *P. stutzeri* OX1 in the presence of 50 μM toluene degraded 0.56 μmol of PCE. Toluene was completely removed during these

experiments, suggesting that ToMO was expressed and is responsible for the PCE degradation. Since monooxygenase attack of chlorinated aliphatics such as TCE usually yields inorganic chloride ions[15,16], the PCE degradation by *P. stutzeri* OX1 was corroborated through analysis of the chloride ions generated: 21% of the stoichiometric chloride from the degraded PCE was detected in solution (expect 4 mol of chloride released per mole of PCE degraded). Note that PCE was not degraded (nor was chloride detected) for the negative controls *Pseudomonas putida* F1 and *Burkholderia cepacia* G4 induced with toluene even though both strains completely degraded the toluene (Table 1).

The initial rates of PCE degradation by *P. stutzeri* OX1 were determined to be 0.48, 0.86, and 1.16 nmol min^{-1} mg^{-1} protein at 8.1, 16.1, and 32.3 μM, respectively, which yield $V_{max} \approx 2.5$ nmol min^{-1} mg^{-1} protein and $K_M \approx 34$ μM. JM109/pBZ1260 degraded PCE at about one half this rate ($V_{max} \approx 0.9$ nmol min^{-1} mg^{-1} protein and $K_M \approx 20$ μM). These rates are comparable to the degradation of the less recalcitrant TCE by aerobes expressing aromatic monooxygenases (V_{max} of 8–20 nmol min^{-1} mg^{-1} protein)[15]. Taken together, the extents of degradation experiments and these initial degradation rate reactions indicate that PCE is degraded continuously from 0 to 30 h, as was shown previously for less chlorinated ethenes[13].

These PCE degradation results with *P. stutzeri* OX1 were verified by four additional sets of experiments in which different media and inducers were utilized to find the best conditions for PCE degradation. Exponentially growing *P. stutzeri* OX1 cells cultured in M9 glucose minimal medium lacking chloride ($M9Cl^-$) and resuspended in phosphate buffer with toluene were also found to degrade 0.18 μmol of PCE (Table 1), and cells cultured in Luria–Bertani (LB) medium and then suspended in phosphate that lacked toluene degraded 0.17 μmol of PCE (Table 1). Culturing cells upon *o*-xylene vapors and *o*-xylene addition during degradation (*o*-xylene was completely removed during degradation) did not significantly enhance PCE degradation relative to growth in LB and addition of toluene during degradation (Table 1). For these experiments, 33–71% of the stoichiometric chloride expected from PCE degradation was detected, a result similar to that generated by aromatic monooxygenase attack of less chlorinated TCE (51–85% for four pseudomonads)[15]. By analogy with TCE mineralization in those systems, it is likely that PCE undergoes complete degradation (in addition to dechlorination) in *P. stutzeri* OX1.

Assuming oxidative PCE degradation is analogous to degradation of TCE, *cis*-DCE, *trans*-DCE, and VC with a monooxygenase, PCE epoxide is likely to be formed[17,18]; hence, we expected that the intermediates of the degradation of PCE would be somewhat toxic to the *P. stutzeri* OX1 cells. Similar toxicity is well documented for TCE degradation; for example, a reduction in OD_{450} of 75% was seen for *B. cepacia* G4 grown on toluene in a fed-batch reactor[19], and a 66% reduction in specific growth rate was seen for *P. putida* F1 in the presence of TCE vapors[20]. Unoxidized PCE is not toxic, as was shown with *P. putida* F1 expressing toluene dioxygenase in the presence of PCE vapors[20]. Evidence of PCE toxicity was seen here with *P. stutzeri* OX1, since the maximum specific growth rate was reduced by 27% in the presence of PCE (0.23 ± 0.03 h^{-1} with PCE vs. 0.32 ± 0.02 h^{-1} without PCE in $M9Cl^-$ supplemented with 100 μM toluene but lacking glucose); toluene degradation was confirmed during these experiments with gas chromatography (GC).

To further verify that ToMO was at least partially responsible for the PCE degradation, ToMO was expressed in *E. coli* JM109. Table 1 indicates, for both sets of conditions investigated, that PCE was degraded by JM109 when ToMO was expressed (0.194 and 0.146 μmol of PCE degraded for initial cultivation in LB and $M9Cl^-$, respectively). Further, stoichiometric amounts of chloride were generated from the degraded PCE by *E. coli* JM109/pBZ1260. Hence, not only was one bacterium identified capable of degrading PCE aerobically, but another (*E. coli* JM109/pBZ1260) was created by expressing the *P. stutzeri* enzyme that is responsible for its degradation. In contrast, toluene *o*-monooxygenase (TOM) of *B. cepacia* G4 expressed in JM109/pMS64 did not degrade PCE. Expression of both ToMO and TOM in JM109 were comparable based on the naphthalene to naphthol assay ($1,340 \pm 75$ nmol mg^{-1} protein vs. $5,400 \pm 250$ nmol mg^{-1} protein, respectively).

We also investigated whether PCE induces its own degradation in *P. stutzeri* OX1. Using naphthalene oxidation as a measure of oxygenase activity in *P. stutzeri* OX1, it was found that 50 μM PCE induces oxygenase activity by 4.2-fold relative to uninduced cells with *N*,*N*-dimethylformamide (DMF) alone (for comparison, 50 μM toluene addition induced oxygenase activity by 5.3-fold). Note that this assay works well with ToMO, since under the same conditions this monooxygenase produces 19-fold more naphthol in JM109/pBZ1260 as compared to JM109. These PCE induction results were corroborated by the degradation results shown in Table 1, in which 0.17 μmol of PCE was degraded and 0.44 μmol of chloride was generated by *P. stutzeri* OX1 resuspended in potassium phosphate buffer (PPB) and lacking any other inducer. Further evidence that ToMO is the enzyme that is induced by PCE was provided using strain *P. stutzeri* M1, which has inactivated ToMO; PCE addition to this strain failed to induce oxygenase activity. Hence, in the

Table 1. Degradation of 2.0 μmol of PCE and generation of chloride ions in phosphate buffer by ToMO[a]

Strain	Growth medium	Energy source[b]	Contact time (h)	PCE Degraded[c] (μmol)	Cl^- Generated[c] (μmol)	Cl^- Generated[d] (%)	Replicates
P. stutzeri OX1	LB	50 μM Toluene	21	0.56 ± 0.11	0.48 ± 0.04	21	40
	LB	None	24	0.17 ± 0.012	0.44 ± 0.03	65	10
	LB	50 μM *o*-Xylene	24	0.41 ± 0.02	0.54 ± 0.03	33	5
	$M9Cl^-$	50 μM Toluene	24	0.18 ± 0.014	0.51 ± 0.04	71	10
	M9 *o*-xylene	None	25	0.15 ± 0.01	0.26 ± 0.02	43	5
JM109/pBZ1260	LB	1% Glucose	22	0.194 ± 0.006	0.78 ± 0.09	100	10
	$M9Cl^-$	1% Glucose	29	0.146 ± 0.004	0.47 ± 0.02	80	10
B. cepacia G4	LB	50 μM Toluene	24	0.0	0.0	0	3
P. putida F1	LB	50 μM Toluene	24	0.0	0.0	0	3

[a]Averages and standard deviations shown for replicate vials. Positive controls for chlorinated aliphatic biodegradation and chloride ion generation were conducted with readily degraded TCE using *Pseudomonas fluorescens* 2-79TOM (which degrades TCE as a result of constitutive expression of TOM[29]); with this control, it was found that 100% of 1.0 μmol of TCE was degraded overnight and 3.0 μmol of chloride ions were detected (100% degradation), whereas TCE was not degraded and chloride was not generated by *P. fluorescens* 2-79.
[b]Energy source indicates organic present in PPB during PCE degradation.
[c]PCE degraded and chloride concentrations are relative to JM109 replicates at the same conditions.
[d]Percentage of stoichiometric chloride that was generated from PCE degradation.

wild-type strain *P. stutzeri* OX1, PCE induces ToMO expression and its own degradation (although more significant oxidation occurs if toluene, which induces ToMO indirectly[21], is present). Precedents for chlorinated aliphatics inducing their own degradation include TCE degradation for *Pseudomonas mendocina* KR1[22] (PCE induced much lower toluene oxidation activity with this strain and was not degraded), and TCE degradation by means of induction of toluene dioxygenase in *P. putida* F1[23,24].

Although *P. stutzeri* OX1 can grow under denitrifying conditions[10], the PCE degradation experiments reported here were not conducted under denitrifying conditions (no nitrate or any nitrogen source was present during the degradation with washed cells). Furthermore, it is unlikely that the PCE degradation seen with *P. stutzeri* OX1 was the result of anaerobic attack, since the pure strains isolated to date that degrade PCE anaerobically are very fastidious and require not only an electron donor such as hydrogen, but also acetate or formate, vitamin B_{12}, and unidentified fermentation components[5,25]; in contrast, the PCE degradation experiments with *P. stutzeri* OX1 that lacked toluene did not include any organic matter to supply electrons. Also, oxygen was present in excess in the air of the 50 ml of headspace in the 60 ml vials that contained the nongrowing, PCE-degrading cells (roughly 500 times more oxygen in the headspace than required for growth on 50 μM toluene).

In summary, PCE degradation by *P. stutzeri* OX1 under aerobic conditions was corroborated through analysis of more than 100 vials in 13 separate experiments, and the enzyme ToMO was confirmed to be involved in the degradation. The implications of these results include that some waste sites might be remediated by adding *P. stutzeri* OX1 since PCE induces its own degradation. Further, given that ToMO has been shown recently to degrade simultaneously mixtures of TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, and VC[13], it appears that this powerful enzyme holds promise for degrading mixtures of PCE and all of its less chlorinated degradation products, which are present at many hazardous waste sites.

Experimental protocol

Organisms and growth conditions. *Pseudomonas stutzeri* OX1 was cultured at 30°C in LB medium[26], M9Cl$^-$[27], or M9 minimal medium with *o*-xylene vapors. *Pseudomonas stutzeri* M1[28], which lacks the ability to grow on *o*-xylene because of an insertion in *touA* and inactivation of ToMO, was cultured at 30°C in LB medium. *Pseudomonas putida* F1[20], *Pseudomonas fluorescens* 2-79[29], and *B. cepacia* G4[30] were cultivated in LB medium and used as negative controls for PCE degradation; *P. fluorescens* 2-79TOM[29], which degrades TCE without an inducer as a result of constitutive expression of TOM, was cultured in LB supplemented with 50 μg ml^{-1} kanamycin (Fisher Scientific, Fair Lawn, NJ) and used as a positive control for chloride generation and TCE degradation. Plasmids pBZ1260[11], a pGEM3Z derivative containing the *P. stutzeri* 6 kb *touABCDEF* locus, and pMS64[30], a pGEM4Z derivative containing the *B. cepacia* G4 11.2 kb *tomA012345* locus, were used with *E. coli* JM109[26]. In pBZ1260, ToMO is expressed under control of the *lac* promoter (constitutive because of high copy number), and in pMS64, TOM is expressed under control of the constitutive IS*50* promoter of Tn5. From -80°C glycerol stocks, JM109, JM109/pBZ1260, and JM109/pMS64 were grown at 37°C in LB medium or M9Cl$^-$ containing 0, 200, and 100 μg ml^{-1} ampicillin (Sigma Chemical Co., St. Louis, MO), respectively.

To ensure exponential growth, overnight cultures were diluted to an OD_{600} of 0.05–0.15 and grown to an OD_{600} of 0.5–0.9. The exponentially grown cells 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, CA). The LB cultures were washed three times with 0.1 M PPB (pH 7) to remove chloride ions. The cells were then resuspended in PPB to an OD_{600} of 3.5–5.0. For JM109/pBZ1260 and JM109/pMS64, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Fisher Scientific) was added to the PPB cell suspension (to ensure induction of the *lac* promoter) along with 1% (wt/vol) glucose, and for *P. stutzeri* OX1, 50 μM toluene was added where indicated.

Extents and initial rates of PCE degradation. To determine the extent of PCE degradation, 10 ml of the PPB cell suspension were added to 60 ml glass vials, which were then covered with a Teflon-coated septum and aluminum crimp seal. Two micromoles of HPLC-grade PCE (99.9%; Sigma Chemical Co.) were injected directly to the cell suspension from fresh 200 mM DMF (ACS grade; Fisher Scientific) stock solutions using a Hamilton (Reno, NV) liquid-tight syringe (yielding 200 μM PCE based on liquid volume). The inverted vials were shaken at 32°C at 300 r.p.m. on an IKA-Vibrax-VXR shaker (IKA-Works, Inc., Cincinnati, OH). The headspace PCE concentrations were determined by GC after 20–30 h by injecting a 50 μl headspace sample with a 50 μl Hamilton gas-tight syringe into a 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) 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, IL). Ten replicates for each set of experiments were analyzed with the column and injector at 210°C and the detector at 250°C. Ten replicates of JM109 plus 2.0 μmol of PCE (supplemented with IPTG and glucose to match conditions used with JM109/pBZ1260 and supplemented with toluene to match *P. stutzeri* OX1) for each set of experiments were used as the negative control for nonenzymatic loss of PCE. By comparing the JM109 negative controls to no-cell negative controls (PPB + PCE), it was found that 0–10% of the PCE was lost in 24 h as a result of abiotic decay and cell adsorption (values in Table 1 indicate PCE degradation above these losses).

To determine initial PCE degradation rates, 0.5, 1.0, or 2.0 μmol of PCE were added to unwashed PPB cell suspensions of *P. stutzeri* OX1 (containing 50 μM toluene) and JM109/pBZ1260 at OD_{600} 2.0–5.0, and the PCE concentrations (relative to JM109 plus PCE) were determined every 7 min with the GC for up to 2 h. Two replicates were used for each PCE concentration, and a Henry's law value of 1.0[31] was used to determine the actual liquid concentrations.

Chloride ion generation. After GC, the inorganic chloride ion concentrations generated from the degradation of PCE were measured spectrophotometrically with the procedure of Bergmann and Sanik[32] 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 $Hg(SCN)_2$. Ten replicates were used for each set of experiments, and chloride ion concentrations were calculated relative to the average of 10 replicates of JM109 negative controls which contained 2.0 μmol of PCE as well as relative to *P. stutzeri* OX1 that lacked PCE. Dead cells were not used as controls, since their lysis affected the chloride assay. To assess interference by extracellular metabolic products, calibration curves were constructed by adding NaCl to JM109/pBZ1260 and *P. stutzeri* OX1 in PPB at OD_{600} 5.0, contacting for 24 h at 32°C at 300 r.p.m., and then assaying for chloride (resulting calibration curves were linear). The minimum detectable chloride concentration with this method was 8 μM (0.08 μmol here).

Specific growth rate. M9Cl$^-$ cultures of *P. stutzeri* OX1 were inoculated into 60 ml glass vials containing M9Cl$^-$ supplemented with 100 μM toluene and lacking glucose. The vials were then sealed as above and 200 μM PCE added. The OD_{600} was followed by taking 200 μl culture samples with a syringe, and the presence of toluene was monitored with GC as above.

Oxygenase induction. Induction of oxygenase activity in *P. stutzeri* OX1 and *P. stutzeri* M1 was quantified by synthesis of naphthol from naphthalene using the method of Phelps and colleagues[33] with slight modifications; the naphthol produced was detected as a purple diazo dye with an absorbance maximum at 540 nm. Cells grown in LB medium were harvested and suspended in 10 ml PPB at OD_{600} = 1 in sealed 60 ml glass vials containing either DMF, 50 μM PCE in DMF, or 50 μM toluene in DMF. After 2– 4 h to allow oxygenase induction, a 6 μl aliquot of 1% naphthalene in DMF was added to 0.5 ml of cell suspension in a 1.5 ml microcentrifuge tube and incubated for 1 h at 37°C to allow oxidation of naphthalene. After centrifugation at 13,000 r.p.m. for 10 min, 20 μl of 0.1% tetrazotized *o*-dianisidine (Sigma Chemical Co.) and 24 μl of glacial acetic acid were added to 240 μl of the supernatant. After 2 min, the absorbance at 540 nm was obtained.

Acknowledgments

This study was supported by the E. I. du Pont de Nemours and Company Educational Aid Program and the National Science Foundation (BES-9807146). We are grateful for the comments on the manuscript provided by Prof. Kenneth Reardon and Prof. Barth Smets.

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