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Oxidation of Trichloroethylene, 1,1-Dichloroethylene, and Chloroform by Toluene/*o*-Xylene Monooxygenase from *Pseudomonas stutzeri* OX1

SADHANA CHAUHAN,[1] PAOLA BARBIERI,[2] AND THOMAS K. WOOD[1]*

Department of Chemical and Biochemical Engineering, University of California, Irvine, California 92697-2575,[1] and Dipartimento di Genetica e di Biologia dei Microrganismi, Università degli studi di Milano, 20133 Milan, Italy[2]

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Toluene/*o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1, which oxidizes toluene and *o*-xylene, was examined for its ability to degrade the environmental pollutants trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), *cis*-1,2-DCE, *trans*-1,2-DCE, chloroform, dichloromethane, phenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,5,6-tetrachlorophenol, and 2,3,4,5,6-pentachlorophenol. *Escherichia coli* JM109 that expressed ToMO from genes on plasmid pBZ1260 under control of the *lac* promoter degraded TCE (3.3 μM), 1,1-DCE (1.25 μM), and chloroform (6.3 μM) at initial rates of 3.1, 3.6, and 1.6 nmol/(min · mg of protein), respectively. Stoichiometric amounts of chloride release were seen, indicating mineralization (2.6, 1.5, and 2.3 Cl^- atoms per molecule of TCE, 1,1-DCE, and chloroform, respectively). Thus, the substrate range of ToMO is extended to include aliphatic chlorinated compounds.

Pseudomonas stutzeri OX1 expressing toluene/*o*-xylene monooxygenase (ToMO) is one of the few microorganisms that degrade *o*-xylene (1, 4). This multicomponent enzyme has a broad substrate range in that it has been shown to oxidize *o*-xylene, *m*-xylene, *p*-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene (3). ToMO is a unique enzyme since it differs from other monooxygenases in that it has a more relaxed regio-specificity (ToMO hydroxylates toluene in the ortho, meta, and para positions as well as *o*-xylene in both the 3 and 4 positions) (3). The gene order and the deduced amino acid sequences are similar to those of the toluene 3-monooxygenase (T3MO) of *Burkholderia pickettii* PK01 (5) and toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1 (17).

Given the toxicity, recalcitrance, and prevalence of the groundwater contaminants trichloroethylene (TCE) and chloroform (12), and since other toluene monooxygenases degrade chlorinated compounds (9, 16), the ability of ToMO to degrade aromatic and aliphatic chlorinated compounds was investigated. This paper reports the ability of *Escherichia coli* JM109 expressing ToMO from pBZ1260 to degrade and mineralize TCE, chloroform, and 1,1-dichloroethylene (1,1-DCE). pBZ1260 contains a 6-kb chromosomal fragment from *P. stutzeri* OX1 encoding the ToMO gene cluster (*touABCDEF*) cloned downstream of the *lac* promoter in the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible, multicopy vector pGEM-3Z (3).

MATERIALS AND METHODS

Growth conditions. *E. coli* JM109(pBZ1260) was grown at 37°C overnight in Luria-Bertani (LB) medium (10) containing ampicillin (150 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and then subcultured in fresh medium at an optical density of 0.1 at 600 nm. When the culture attained an optical density of 0.8 to 1.0 (note that a blue, insoluble pigment was clearly seen), 1 mM IPTG (Fisher Scientific, Fairlawn, N.J.) was added, and the cultures were grown for an additional 2 h. Cells were harvested by centrifugation at 2,000 × *g* for 15 min at 4°C (model J2-21 centrifuge; Beckman; Palo Alto, Calif.). The cells were washed twice with modified minimal salts (M9) medium (10) which lacked chloride ($MgSO_4$ [0.24 g/l] and $CaSO_4$ [0.17 g/l] replaced $MgCl_2$ and $CaCl_2$) and diluted to an optical density of 1.0 at 600 nm.

* Corresponding author. Present address: Department of Chemical Engineering, University of Connecticut, Storrs, CT 06269-3222. Phone: (860) 486-4019. Fax: (860) 486-2959.

Chemicals. TCE, chloroform, dichloromethane, and phenol were purchased from Fisher Scientific (Tustin, Calif.), and 1,1-DCE, *cis*-1,2-DCE, and *trans*-1,2-DCE were purchased from Supelco, Inc. (Bellefonte, Pa.). The chlorinated phenols 2,4,5- and 2,4,6-trichlorophenol, and 2,3,4,5,6-pentachlorophenol were purchased from Sigma Chemical Co. 2,3,5,6-Tetrachlorophenol was purchased from AccuStandard, Inc. (New Haven, Conn.), and 2,4-dichlorophenol was purchased from Eastman Kodak Co. (Rochester, N.Y.). All materials used were of the highest purity available and were used without further purification.

Extents of degradation and initial degradation rates. To determine the extents of degradation of the chlorinated aliphatics and the initial degradation rates of TCE, chloroform, and 1,1-DCE, these compounds were added at an initial concentration of 10 μM (assuming all substrates to be in the liquid phase) from a 0.1 M stock solution in *N*,*N*-dimethylformamide (Fisher Scientific). Ten milliliters of IPTG-induced cells in M9 medium containing no chloride was added to 60-ml glass vials to yield the actual liquid concentrations shown in Table 1 (calculated with Henry's law constants of 0.4, 1.39, and 0.11 for TCE [6], 1,1-DCE [13], and chloroform [13], respectively). The vials were shaken at room temperature at 200 rpm on an IKA-Vibrax-VXR shaker (IKA-Works, Inc., Cincinnati, Ohio). The concentrations of the chlorinated aliphatics were determined by gas chromatography after 24 h (extent of degradation performed in duplicate) and at 0, 5, 10, and 15 min after substrate addition (initial degradation rate) by injecting a 10-μl headspace sample by using a 50-μl gas-tight syringe (Hamilton, Reno, Nev.) into a 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with an electron capture 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 isocratically with the column and injector at 180°C and the detector at 210°C. To determine the initial degradation rates, two independent experiments were used for TCE, 1,1-DCE, and chloroform, and each of these experiments had duplicate or triplicate vials. Both abiotic (no cells plus chlorinated aliphatic) and biotic (live JM109 plus chlorinated aliphatic) controls were used.

To determine the extents of degradation of phenol and the chlorinated phenols, these compounds were added at 10 μM initial concentrations (assuming all substrates to be in the liquid phase) to 25-ml liquid cultures containing IPTG-induced cells in 250-ml Erlenmeyer flasks. 2,4-Dichlorophenol stock was prepared in ethanol, whereas the other phenol stocks were prepared in water. After 4, 8, and 12 h, phenol and 2,4-dichlorophenol were assayed by a colorimetric assay (11), and the disappearance of the other phenols was monitored by measuring the absorption at 310 nm for 2,4,5- and 2,4,6-trichlorophenol, 306 nm for 2,3,5,6-tetrachlorophenol, and 318 nm for 2,3,4,5,6-pentachlorophenol. For each assay, cells were removed by centrifugation prior to monitoring the absorbance change.

Plasmid stability. After 2 h of induction with IPTG, segregational plasmid stability of pBZ1260 in *E. coli* JM109 was measured at the time of the addition of the substrates TCE, 1,1-DCE, or chloroform and 15 min later (to cover the period of the initial degradation experiments). Appropriate dilutions of the culture were plated on LB plates and LB plates containing ampicillin (150 μg/ml). The colonies were enumerated after incubating the plates at 37°C overnight.

Chloride analysis and total protein. Chloride released by the degradation of TCE, 1,1-DCE, and chloroform was measured in duplicate by a colorimetric

TABLE 1. Summary of compounds degraded by T.MO[a]

Compound	Initial concn (μM)	Removal in 24 h (%)	Initial degradation rate (nmol/[min · mg of protein])[b]	Stoichiometric Cl^- released (atoms)	Plasmid-bearing cells (%)
TCE	3.3	>95	3.1 ± 0.3	2.6	85
1,1-DCE	1.3	>95	3.6 ± 1	1.5	85
$CHCl_3$	6.3	>95	1.6 ± 0.5	2.3	85

[a] Overnight removal, initial degradation rate (in the first 5 min, with actual liquid concentrations shown as calculated by using Henry's law), and overnight chloride release for TCE, 1,1-DCE, and chloroform by ToMO expressed in *E. coli* JM109(pBZ1260) are given. Total protein levels were calculated as 0.522 mg/ml at an A_{600} of 1.0.

[b] Reported as averages ± standard deviations.

assay after 24 h (2). After cells were removed from a 1-ml sample by centrifugation, 200 μl of 0.25 M ferric ammonium sulfate in 9 N HNO_3 and 200 μl of saturated mercuric thiocyanate in ethanol were added and incubated for 10 min, and then the absorbance at 460 nm was measured. Total protein of cells at an absorbance of 1.0 (at 600 nm) was determined with a Sigma Diagnostics Protein Assay Kit (catalog no. P 5656).

RESULTS AND DISCUSSION

ToMO expressed in *E. coli* JM109 was tested for the ability to oxidize TCE, 1,1-DCE, *cis*-1,2-DCE, *trans*-1,2-DCE, chloroform, dichloromethane, phenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,5,6-tetrachlorophenol, and 2,3,4,5,6-pentachlorophenol by looking for the disappearance of the starting compounds (extents of degradation). TCE, 1,1-DCE, and chloroform were degraded overnight (>95% removed), whereas there was no significant degradation of the other compounds. For these three compounds, the degradation was further characterized by calculating the initial rates of degradation (Table 1). The initial degradation rates (at 0 to 5 min) were also corroborated in all cases by the results at later time points; for example, the degradation rates at 0 to 10 min and 0 to 15 min corroborated those at 0 to 5 min. During these initial degradation rate experiments, the plasmid was present in about 85% of the cells, and there was no significant loss of these chlorinated aliphatics in the abiotic controls as well as after 15 min or 20 h in the presence of live JM109 cells which lacked the ToMO genes (no plasmid-negative control; 20-h losses of 0, 13, and 0% for TCE, 1,1-DCE, and $CHCl_3$, respectively).

To our knowledge, this is the first report of 1,1-DCE oxidation by an aromatic monooxygenase (note: purified toluene dioxygenase [TDO] has recently been shown to oxidize 1,1-DCE [8]). The initial rate of degradation for 1,1-DCE by ToMO was found to be 1.5 times higher than that by soluble methane monooxygenase (sMMO) from *Methylosinus trichosporium* OB3b (adjusted to 1.3 μM by using the reported maximum initial velocity and K_m) (15). Furthermore, a comparison of the initial rates of degradation of TCE (3.3 μM) by ToMO from *E. coli* JM109(pBZ1260) induced with 1.0 mM IPTG with those of other monooxygenases shows that the ToMO rate is comparable to that of T4MO of *P. mendocina* KR1 (5.98 nmol/[min · mg of protein] at 3.7 μM) (16), toluene *o*-monooxygenase (T2MO) of *Burholderia cepacia* (4.18 nmol/[min · mg of protein] at 3.7 μM) (16), TDO of *Pseudomonas putida* F1 (3.49 nmol/[min · mg of protein] at 3.7 μM) (16), and T3MO of *B. pickettii* PKO1 (2.4 nmol/[min · mg of protein] at 40 μM, but measured over 2 h) (9) when these other organisms are induced with toluene or phenol.

Very few organisms are capable of degrading chloroform under aerobic conditions, and it has been shown that T2MO, TDO, and T3MO do not degrade chloroform whereas T4MO and sMMO of *M. trichosporium* OB3b are capable of oxidizing it (7, 12); hence, there is only one other report of oxidation of chloroform by an aromatic oxygenase (12). Degradation of chloroform by ToMO at 6.3 μM was 3 times faster than that by T4MO at 16 μM (a concentration 2.5 times higher) and 100 times slower than that by sMMO at 6.3 μM (12, 15).

To quantify the extent of mineralization, chloride release was assayed and found to be 2.6 Cl^- atoms per TCE molecule, 1.5 Cl^- atoms per 1,1-DCE molecule, and 2.3 Cl^- atoms per chloroform molecule (no chloride was released in JM109 (pBZ1260) controls which lacked a chlorinated substrate). This indicates stoichiometric amounts of inorganic chloride were released; hence, ToMO is capable of completely mineralizing these compounds to a degree seen previously for TCE with T2MO of *B. cepacia* (14, 16), T4MO of *P. mendocina* KR1 (16), and TDO of *P. putida* F1 (16).

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