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ORIGINAL PAPER

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# Tetrachloroethylene, trichloroethylene, and chlorinated phenols induce toluene-*o*-xylene monooxygenase activity in *Pseudomonas stutzeri* OX1

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**Abstract** *Pseudomonas stutzeri* OX1 naphthalene-oxidation activity is induced 3.0-fold by tetrachloroethylene (PCE) and 3.1-fold by trichloroethylene (TCE) at 100 µM. With the mutant *P. stutzeri* M1, which does not express toluene-*o*-xylene monooxygenase (ToMO, product of the *tou* operon), no naphthalene-oxidation activity induction by PCE and TCE was found; hence, PCE and TCE induce ToMO of *P. stutzeri* OX1. The chlorinated phenols 2-, 3-, and 4-chlorophenol induced ToMO expression 0.58-, 0.23- and 0.37-fold, respectively, compared to the direct inducer of the pathway, *o*-cresol. Using *P. putida* PaW340 (pPP4062, pFP3028), which has the *tou* promoter fused to the reporter catechol-2,3-dioxygenase, and the regulator gene *touR*, it was determined that the *tou* promoter was induced directly 5.7-, 7.1-, and 5.1-fold for 2-, 3-, and 4-chlorophenol, respectively (compared to an 8.8-fold induction with *o*-cresol). In addition, it was found that TCE and PCE do not directly induce the *tou* pathway and that components other than the *tou* structural and regulatory genes are necessary for induction. Gas chromatography results also showed that 100 µM TCE induced its own degradation (8–9%) in 16 h in *P. stutzeri* OX1, and all of the stoichiometric chloride from the degraded TCE was detected in solution.

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## Introduction

Chlorophenols are used extensively in the manufacture of insecticides, herbicides, and wood preservatives (Freiter 1997), and they have caused extensive environmental contamination (Maltseva and Oriel 1997). Both tetrachloroethylene (PCE) and trichloroethylene (TCE) are suspected carcinogens, regulated to levels of five parts per billion under the Safe Drinking Water Act. These compounds are the most common groundwater pollutants at hazardous waste sites (McCarty 1997). Aerobic degradation of both PCE and TCE is attractive to avoid the highly toxic vinyl chloride (VC) and *cis*-1,2-dichloroethylene (*cis*-DCE) which predominate in anaerobic degradation of TCE and PCE (McCarty 1997); VC is a known human carcinogen (McCarty 1997) whereas both VC and *cis*-DCE are U.S. EPA priority pollutants (Bradley and Chapelle 1998). Both PCE (Ryoo et al. 2000) and TCE (Chauhan et al. 1998; Shim and Wood 2000) are degraded aerobically by toluene-*o*-xylene-monooxygenase (ToMO) of *Pseudomonas stutzeri* OX1. ToMO is a multicomponent enzyme that 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*) (Bertoni et al. 1998); *P. stutzeri* OX1 is an activated sludge isolate of a wastewater treatment plant (Baggi et al. 1987).

Recently it has been shown that PCE induces ToMO expression in *P. stutzeri* OX1 (Ryoo et al. 2000), and that TCE induces expression of the toluene dioxygenase of *P. putida* F1 (Heald and Jenkins 1994; Shingleton et al. 1998), toluene 2-monooxygenase of *Burkholderia cepacia* G4 (Leahy et al. 1996), toluene 3-monooxygenase of *B. pickettii* PKO1 (Leahy et al. 1996), and toluene oxygenase of *P. mendocina* KR1 (McClay et al. 1995); hence, it was investigated here whether TCE and chlorophenols (2-, 3-, and 4-chlorophenol), which are known to act as effectors of other regulators similar to TouR (Shingler and Moore 1994), could induce ToMO expres-

sion in *P. stutzeri* OX1 and whether PCE, TCE, and the chlorophenols induce the *tou* promoter directly. If no chemical inducer is required, then these compounds can be degraded aerobically without competitive inhibition (Folsom et al. 1990).

This is the first report of TCE and chlorophenol induction of the *tou* operon. The results also indicate that TCE can induce its own degradation in *P. stutzeri* OX1 and does not require a separate inducer of the *tou* pathway, thus, competitive inhibition may be avoided.

## Materials and methods

### Organisms, growth conditions, and chemicals

*Pseudomonas stutzeri* OX1 (ATCCBAA-172) and *P. stutzeri* M1 (Bolognese et al. 1999), which lacks the ability to grow on *o*-xylene due to an insertion in *touA* and inactivation of ToMO, were cultured from –80°C glycerol stocks at 30 °C in Luria-Bertani (LB) medium (Sambrook et al. 1989). *P. putida* PaW340 (pPP4062, pFP3028) (Arenghi et al. 1999) contains the wild-type *tou* promoter fused to catechol-2,3-dioxygenase (C23O) on pPP4062 (encodes kanamycin resistance) and the wild-type regulator gene *touR* on pFP3028 (encodes tetracycline resistance); this strain was grown in M9 glucose-free minimal medium (Sambrook et al. 1989) containing malate (20 mM), tryptophan (0.05 mM), kanamycin (30 µg/ml), and tetracycline (25 µg/ml). *P. putida* PaW340 (pFB1112) (Arenghi et al. 1999) contains the complete *tou* operon including *touR* on plasmid pFB1112 and was cultured in M9 glucose-free minimal medium containing malate (20 mM), tryptophan (0.05 mM), and tetracycline (25 µg/ml).

To ensure exponential growth, overnight cultures were diluted to an optical density at 600 nm (OD) to 0.1 and grown to an OD of 0.5–0.9 in 250-ml conical flasks. 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, Calif.).

TCE (99.9%) and chloroform (CF, 99.9%) were purchased from Fisher Scientific (Pittsburgh, Pa.), 1,1-dichloroethylene (1,1-DCE, 99%), *cis*-DCE (97%), and *trans*–1,2-dichloroethylene (*trans*-DCE, 98%) from Aldrich Chemical (Milwaukee, Wis.), and vinyl chloride (VC, 99.9%) from Supelco (Bellefonte, Pa.). 2- and 3-Chlorophenol (98%) and 4-chlorophenol (99%) were from Fluka Chemie (Buchs, Switzerland).

### ToMO and C23O assays

Induction of ToMO activity in *P. stutzeri* OX1 and *P. stutzeri* M1 by chlorinated aliphatics was quantified spectrophotometrically by synthesis of naphthol from naphthalene using the method of Phelps et al. (1992) with slight modifications (Ryoo et al. 2000); the naphthol produced was detected as a purple diazo dye with an absorbance maximum at 540 nm. TCE, PCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, VC, and CF in *N*,*N*-dimethylformamide (DMF, Fisher Scientific ACS-grade) were injected directly from fresh 50- or 100-mM stock solutions using a Hamilton (Reno, Nev.) liquid-tight syringe into a 10-ml cell suspension (exponential-phase, LB-grown culture washed once with 0.1 M potassium phosphate buffer, pH 7.0, PPB) in 60-ml glass vials which were then covered with a Teflon-coated septum and aluminum crimp seal to yield 50 or 100 µM of chlorinated compounds (assuming all the volatile compound is in the liquid phase). Specific activity is reported as nanomoles of naphthol produced per minute per milligram of total cell protein using a linear calibration curve derived from 1-naphthol (Aldrich Chemical).

Induction of ToMO activity by the chlorinated compounds was also assayed in *P. putida* PaW340 (pFB1112) by means of a colorimetric assay slightly modified from Martin (1949) which assayed the conversion of toluene to phenolic compounds (Arenghi et al. 1999). Cells were grown in minimal medium as described above to OD 0.5–0.6, then incubated for 3 h with 1 mM 2-, 3-, or 4-chlorophenol.

Induction of the *tou* promoter by chlorinated ethenes and chlorinated phenols was assayed spectrophotometrically at 375 nm using C23O activity that was induced in *P. putida* PaW340 (pPP4062, pFP3028) cell extracts (Baggi et al. 1987). Exponentially growing, minimal cultures of *P. putida* PaW340 (pPP4062, pFP3028) at OD 1.0 were incubated with 20–2,000 µM PCE, TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, toluene, and *o*-cresol for 3 h or with 0.5 mM *o*-cresol, 2-, 3-, and 4-chlorophenol. Crude cell extract was prepared by sonicating (Sonic Dismembrator 60, Fisher Scientific) an exponentially grown cell suspension for 2 min at 6 Watts (for the chlorinated aliphatics) or by mechanical disruption with glass beads (150–212 µm, Sigma Chemical, St. Louis, Mo.) as previously described (Arenghi et al. 1999, for the chlorophenols). The rate of formation of α-hydroxymuconic ε-semialdehyde from catechol was measured in a 1.5-ml reaction mixture containing 3 mM catechol in PPB and either 10 µl (mechanical disruption) or 200 µl (sonication) of centrifuged cell extract. The total protein concentration was determined with a protein assay kit (Sigma Chemical). Specific activity is reported as nanomoles of α-hydroxymuconic ε-semialdehyde produced per minute per milligram of total cell protein using an extinction coefficient of 33,000/M (Baggi et al. 1987).

### Extent of TCE degradation and chloride ion detection

Exponential LB cultures of *P. stutzeri* OX1 were washed three times with PPB to remove chloride ions, and the cells were then resuspended in PPB to an OD of 3.5–5.0. Ten ml of the PPB cell suspension were added to 60-ml glass vials which were then covered with a Teflon-coated septum and an aluminum crimp seal. TCE was injected at 100 µM (assuming all in the liquid phase) from 10 mM dimethylformamide stock solutions; 50 µM toluene and 1 wt% glucose were added for some samples using a Hamilton liquid-tight syringe. The inverted vials were shaken at room temperature at 300 rpm on an IKA-Vibrax-VXR shaker (IKA-Works, Cincinnati, Ohio).

The concentration of TCE and toluene in the headspace was determined by gas chromatography after approximately 24 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, 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, Deerfield, Ill.). Three to five replicates (including *Escherichia coli* JM109 plus TCE negative controls) for each set of experiments were analyzed isothermally with the column and injector at 210 °C and the detector at 250 °C.

After gas chromatography, the supernatant inorganic chloride ion concentrations generated from the mineralization of TCE were measured spectrophotometrically with a modification of Bergmann and Sanik's trace chlorine determination method in naphtha (Bergmann and Sanik 1957) as previously reported (Shim and Wood 2000). 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 100 µM TCE. The minimum detectable chloride concentration with this method was 8 µM.

## Results

For the chlorinated ethenes, naphthalene oxidation activity, taken as a measure of ToMO activity in *P. stutzeri* OX1, was induced 3.0-fold by TCE and PCE at 100 µM (2.5-fold at 50 µM), whereas the first substrate of the catabolic pathway, toluene, induced 3.3-fold at 50 µM

**Table 1** Specific ToMO activity in *P. stutzeri* OX1 induced by chlorinated aliphatics

| Inducing compound | Concentration (μM)[a] | Naphthalene oxidation rate (nmol of naphthol/min per mg protein)[b, c] | |
|---|---|---|---|
| | | 2 h | 4 h |
| DMF (uninduced control) | 0 | 122±40 | 184±76 |
| Toluene | 50 (21.7) | 404±74 | 408±52 |
| Toluene + glutamate[d] | 50 (21.7) | 222±6 | 424±20 |
| PCE | 50 (8.1) | 314±10 | 300±12 |
| | 100 (16.1) | 366±18 | – |
| TCE | 50 (16.7) | 288±26 | 312±52 |
| | 100 (33.3) | 380±42 | 368±8 |
| TCE + glutamate[d] | 50 (16.7) | 138±10 | 368±8 |
| 1,1-DCE | 50 (6.3) | 156±2 | 168±24 |
| *cis*-DCE | 50 (19.7) | 156±2 | 232±12 |
| *trans*-DCE | 50 (21.4) | 156±10 | 224±8 |
| VC | 50 (8.4) | 162±6 | 132±12 |
| Chloroform | 50 (31.5) | 156±16 | 160±4 |

[a] Inducing compounds were added in 10 μl of DMF. Actual liquid concentrations shown in parenthesis based on Henry's Law
[b] Values represent average of 4–10 samples. Standard deviations shown
[c] 223 μg/ml total protein for *P. stutzeri* OX1
[d] Contains 0.04% sodium glutamate

(Table 1). Note that in some experiments induction was as high as 10-fold for TCE and PCE. 1,1-DCE, *cis*-DCE, *trans*-DCE, VC, and CF slightly induced ToMO expression (Table 1, ca. 30%). The ability of both TCE and PCE to induce ToMO significantly is surprising, as previous reports for induction were 60% for TCE and 3% for PCE for *P. mendocina* KR1 at 20 μM (McClay et al. 1995) as well as 77% for TCE at 80 μM for *P. putida* TVA8 (Shingleton et al. 1998). The data for a 4-h incubation with the chlorinated aliphatics served to corroborate the 2-h incubation data except for those experiments with 0.04% sodium glutamate present. For *P. mendocina* KR1, it was reported previously that glutamate enhanced induction of toluene oxygenase activity by TCE (McClay et al. 1995). However here, sodium glutamate initially repressed induction of ToMO in *P. stutzeri* OX1 by both TCE and toluene for the first 2 h, then induction was restored after 4 h of incubation (presumably after the glutamate was consumed).

To verify that the chlorinated ethenes were inducing ToMO in *P. stutzeri* OX1, *P. stutzeri* M1 was incubated with these compounds at 50 μM (M1 lacks active ToMO due to an insertion in *touA*). There was no induction of naphthalene oxidation activity after 2–4 h by TCE, PCE, or toluene relative to samples that contained DMF alone.

For the chlorinated phenols, induction of ToMO activity was monitored using the heterologous host *P. putida* PaW340, which is unable to degrade hydrocarbons and phenols and which harbors pFB1112 (contains the complete *tou* operon and *touR* regulator in the wild-type configuration). In this strain, ToMO activity is regulated tightly and is undetectable in the absence of inducers (Arenghi et al. 1999). Induction of ToMO activity was highest with 2-chlorophenol (58% of the direct inducer of the pathway, *o*-cresol), and 3-chlorophenol and 4-chlorophenol induced at 24% and 38% of *o*-cresol, respectively (Table 2).

To corroborate these results and to see if the chlorinated compounds directly induce the *tou* pathway, *P. putida* PaW340 (pPP4062, pFP3028) was used. After exposure for 3 h, it was found that the chlorophenols at 500 μM directly induced the ToMO promoter 5.1- to 7.1-fold and nearly as well as *o*-cresol, which induced 8.8-fold (Table 3); hence, the mono-chlorinated phenols are efficient effectors. These results are consistent with the clear induction of the *tou* promoter by other phenolic compounds such as dimethyl phenols and cresols (Arenghi et al. 1999). In contrast, after 3 h, TCE and PCE (as well as 1,1-DCE, *cis*-DCE, *trans*-TCE, and CF) did not act as *Tou*R effectors as they did not directly induce the *tou* pathway (107–160 nmol semialdehyde formed/min per mg protein for all these compounds vs 196±5 nmol/min per mg protein for DMF). As expected, toluene also did not induce the *tou* promoter (90±25 nmol/min per mg protein) directly whereas

**Table 2** Specific ToMO activity in *P. putida* PaW340 (pFB1112) induced by chlorinated phenols

| Compound | ToMO specific activity (nmol phenolic/min per mg of protein)[a] |
|---|---|
| None | 0 |
| *o*-Cresol | 4.2±0.3 |
| 2-Chlorophenol | 2.5±0.8 |
| 3-Chlorophenol | 1.0±0.6 |
| 4-Chlorophenol | 1.6±0.5 |

[a] Values represent average of 3 independent experiments. Standard deviations shown

**Table 3** Induction of the *tou* promoter in *P. putida* PaW340 (pPP4062, pFP3028) by chlorinated phenols (500 μM)

| Compound | C23O specific activity (nmol/min per mg of protein)[a] |
|---|---|
| None | 41±3.5 |
| 2-Chlorophenol | 232±11 |
| 3-Chlorophenol | 291±65 |
| 4-Chlorophenol | 211±49 |
| *o*-Cresol | 360±85 |

[a] Values represent average of 3 samples. Standard deviations shown

**Table 4** Degradation of TCE and conversion to stoichiometric chloride by *P. stutzeri* OX1

| Compound[a] | Degradation (%)[b,c] | Chloride detected (%)[d] |
|---|---|---|
| TCE | 9.3±1 | 100±3 |
| | 8.0±1 | 100±2 |
| TCE+toluene | 25±4 | 82±6 |
| | 28±1 | 100±13 |
| TCE+glucose | 19±1 | 100±6 |

[a] TCE and toluene were added in 10 µl of DMF to a final concentration of 100 µM (33.3 µM based on Henry's Law) and 50 µM (21.7 µM based on Henry's Law), respectively, and the glucose concentration was 1%
[b] Degradation and mineralization obtained in 15 h
[c] Values represent average of 3–5 samples. Standard deviations shown
[d] Values greater than 100% rounded to 100%

*o*-cresol did by 3.7-fold (718±18 nmol/min per mg protein). The primary substrates toluene and *o*-xylene induced ToMO expression after they were oxidized to phenolics by basal ToMO levels in *P. stutzeri* OX1 (Arenghi et al. 1999). Note that the relative induction of the *tou* promoter as assayed by the C23O assay was consistent for the two classes of chlorinated compounds since the positive control *o*-cresol induced roughly 2.4-fold better at a 2.5-fold higher concentration.

To determine if, besides *Tou*R, ToMO activity itself is required to induce expression from the ToMO promoter in the presence of PCE and TCE, expression of ToMO in *P. putida* PaW340 (pFB1112) in minimal medium was evaluated after 2–4 h (recall ToMO expression was seen in *P. stutzeri* OX1, Table 1, but not seen with inactive ToMO from *P. stutzeri* M1). Whereas in this strain both toluene and *o*-cresol induced ToMO activity 3.5-fold after 2 h (407±22 and 423±38 nmol naphthol/min per mg protein, respectively, vs 117±0 nmol naphthol/min per mg protein for DMF), again both TCE and PCE failed to induce the *tou* pathway (131±7 and 120±5 nmol naphthol/min per mg protein, respectively), which indicates that some other element beyond active ToMO and TouR is required for induction by these compounds. These results were corroborated by similar results seen for growth in LB medium in which toluene and *o*-cresol induced ToMO expression 6.8- to 8.3-fold but TCE and PCE did not induce.

It was also investigated whether TCE could induce its own degradation in *P. stutzeri* OX1 (with no other inducer present). Upon addition of 100 µM TCE, 9 and 8% TCE was degraded in two independent experiments (Table 4), which indicates that TCE does indeed induce ToMO and its own degradation in this strain. These results were corroborated by the detection of inorganic chloride ions at the stoichiometric amounts expected (Table 4). Note that TCE was more efficiently degraded when either 50 µM toluene or 1 wt% glucose was added (as much as three-fold greater degradation). Toluene was completely removed during these experiments so it served both as an energy source as well as an inducer of ToMO. These results agree well with those of aerobic PCE degradation with *P. stutzeri* OX1 (Ryoo et al. 2000) in that PCE also induced its own degradation, but three times greater degradation occurred upon the addition of 50 µM toluene.

## Discussion

We have shown that both chlorinated aliphatics (TCE and PCE) and chlorinated phenols (2-, 3-, and 4-chlorophenol) induce ToMO activity in *P. stutzeri* OX1. Whereas the chlorinated phenols induce directly the *tou* promoter, TCE and PCE induce indirectly. However, the oxygenase that was induced in *P. stutzeri* OX1 by TCE and PCE was clearly ToMO, since the chlorinated aliphatics were unable to induce oxygenase activity in the mutant that lacks active ToMO, *P. stutzeri* M1. Further studies are needed to understand the mechanism of induction of ToMO activity by the chlorinated aliphatics in the wild-type strain *P. stutzeri* OX1 and to understand the relationship between induction of ToMO and the degradation of these chlorinated aliphatics.

*P. stutzeri* OX1 is able to mineralize TCE (and PCE, Ryoo et al. 2000) since chloride ions were detected here, and other groups have routinely detected chloride ions and carbon dioxide when TCE is degraded by whole cells expressing similar monooxygenases (Little et al. 1988; Oldenhuis et al. 1989; Uchiyama et al. 1992; Winter et al. 1989). Based on the degradation of TCE by related monooxygenases, the intermediates formed by the oxidation of TCE by *P. stutzeri* OX1 are probably unstable TCE epoxide (van Hylckama Vlieg et al. 1996), which leads to glyoxylate, CO, and formate (Newman and Wackett 1997). Significantly, we have shown that *P. stutzeri* OX1 may be used directly without an additional inducer to remediate both PCE and TCE.

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