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Trichloroethylene Removal and Oxidation Toxicity Mediated by Toluene Dioxygenase of *Pseudomonas putida*

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Whole cells of *Pseudomonas putida* containing toluene dioxygenase were able to remove all detectable trichloroethylene (TCE) from assay mixtures. The capacity of cells to remove TCE was 77 μM/mg of protein with an initial rate of removal of 5.2 nmol/min/mg of protein. TCE oxidation resulted in a decrease in the growth rate of cultures and caused rapid cell death. Addition of dithiothreitol to assay mixtures increased the TCE removal capacity of cells by up to 67% but did not prevent TCE-mediated cell death. TCE induced toluene degradation by whole cells to a rate approximately 40% of that induced by toluene itself.

Trichloroethylene (TCE) is a major groundwater contaminant, and its presence in drinking water is a potential health hazard (14). Microbial metabolism of TCE under anaerobic conditions can lead to the formation of vinyl chloride and dichloroethylenes (15), which are also of concern as drinking water contaminants (11). TCE is also metabolized by several aerobic bacteria able to grow on hydrocarbons. Oxygenase enzymes have been shown to be responsible for the aerobic conversion of TCE: for example, the methane monooxygenase of methanotrophs (14), toluene-*ortho*-monooxygenase of *Pseudomonas cepacia* G4 (13), and toluene dioxygenase of *P. putida* F1 (16).

The use of aerobic bacteria for TCE bioremediation has been proposed previously (6, 12, 16). However, oxygenase-mediated metabolism of TCE can lead to the formation of toxic oxidation products, which might limit the bioremediation capacity of certain bacteria. For example, in *P. putida* F1, cytotoxicity of TCE is mediated by toluene dioxygenase, as indicated by growth inhibition and covalent modification of cellular molecules (17). This is thought to be analogous to the oxidation of hydrocarbons by cytochrome P-450 monooxygenase, leading to reaction products which alkylate DNA, RNA, and proteins (11).

We report here on the cometabolism of TCE by a wild-type strain of *P. putida* containing an inducible toluene dioxygenase enzyme. A mutant strain of this organism, *P. putida* UV4, has been shown previously to metabolize benzocycloalkenes to yield monooxygenation, dioxygenation, and trioxygenation products (2). Biotransformation of 1,2-dihydronaphthalene, indene, and 1,2-benzocyclohepta-1,3-diene by this mutant yields benzylic monols as major metabolites, exclusively with the R configuration (1). Conversely, a mutant strain of *P. putida* F1 has been shown to oxidize indene to form 1-indenol, predominantly with the S configuration (3). These observations suggest significant differences in the active sites of toluene dioxygenases from *P. putida* F1 and *P. putida* UV4 (3). The metabolism of TCE via the former mechanistic type of toluene dioxygenase has not been reported previously. The results presented here show rapid TCE removal by the strain but severe oxidation toxicity and rapid cell death. This is also the first report of enhanced capacity of bacterial cells to remove TCE in the presence of dithiothreitol. Further, we present evidence for induction of toluene degradation by TCE, which is also believed not to have been reported previously.

P. putida was grown in shaking flask cultures at 200 rpm and 30°C on a mineral medium described previously (7) but containing 0.2% (wt/vol) arginine; when required, toluene, TCE, or perchloroethylene (PCE) was supplied as a saturated vapor from side arms. Cultures were incubated for 16 to 20 h and harvested by centrifugation at 20,000 × *g* for 5 min at 30°C. Cells were resuspended to their original culture volume in fresh medium and reincubated with shaking at 30°C. The organism was also cultivated on mineral medium in a 2-liter chemostat, under nitrogen-limiting conditions, with toluene as the sole source of carbon and energy supplied via the air stream (D = 0.74/h).

For quantitative determination of TCE removal, *P. putida* cell suspensions were harvested, washed once in mineral salts medium, and resuspended in this medium to an A_{540} of 1.0 (0.16 g/liter of protein). A modification of the headspace assay described previously (5) was used to monitor TCE removal by cells. Cells (2 ml) were placed in 8-ml borosilicate glass vials, and 5 μl of TCE in methanol was added to give the required TCE concentration. The vials were capped with Teflon-faced silicon septa and incubated at 30°C with shaking. The bacteria were sacrificed by addition of 2 ml of pentane, and the vials were incubated for a further 30 min. Pentane (0.5 ml) was removed from the vials, and the TCE concentration was determined by capillary column gas chromatography. Dibromoethane (10 μM) was added as an internal standard immediately prior to gas chromatographic analysis. Assays were carried out in triplicate for each time point. Gas chromatographic analysis was done with a Pye Unicam 4600 Series gas chromatograph fitted with a 30-m OV1 capillary column (Phase Separations Ltd., Deeside, Clwyd, United Kingdom) and an electron capture detector. The following operating conditions were used: injector temperature, 150°C; column temperature, 100°C; detector temperature, 250°C. Under these conditions, the retention times for TCE and dibromoethane were 2.7 and 3.5 min, respectively. Assay vials for quantitative determination of toluene removal by cells were prepared as described for TCE, except that toluene at a final concentration of 1 mM replaced TCE. Measurement of toluene was done by reverse-phase high-pressure liquid chromatography with acetonitrile-H_2O (70:30) as the carrier solvent and a UV detector set at 260 nm. Catechol 2,3-oxygenase activity was determined as described by Kataeva and Golovleva (9).

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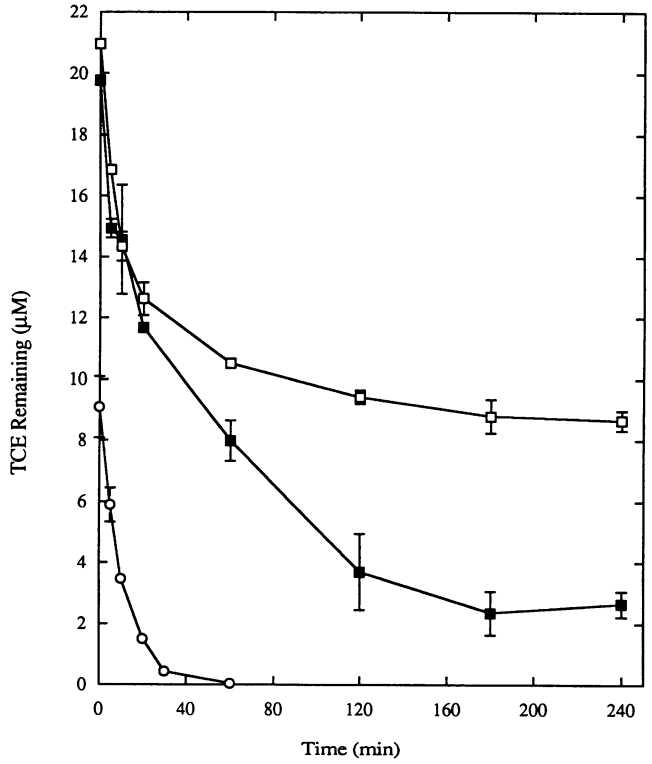

FIG. 1. Removal of TCE from culture media by *P. putida* cell suspensions. The A_{540} of cell suspensions was 1.0 (0.16 g/liter of protein). Symbols: ○, cells grown on arginine-toluene in batch culture; □, toluene chemostat-grown cells without DTT; ■, toluene chemostat-grown cells with 1 mM DTT added to assay vials. Each point is an average of results from at least three assays.

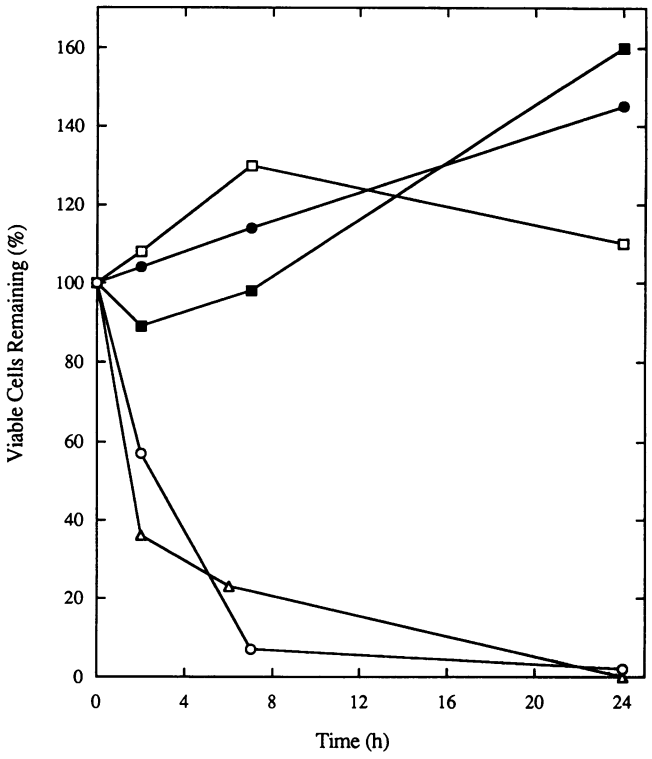

FIG. 2. Effect of TCE on the viability of *P. putida*. Symbols: ○, arginine-toluene batch-grown cells incubated in assay mixtures with TCE; ●, cells grown similarly without TCE; □, arginine batch-grown cells incubated in assay mixtures with TCE; ■, cells grown similarly without TCE; △, arginine-toluene batch-grown cells in the presence of TCE and 10 mM DTT. Viability at 100% was 1.9×10^9 CFU/ml. TCE was supplied as air-saturated vapor in the flask culture headspace, and DTT was added directly to the assay medium.

The results show that wild-type cells, after growth in batch culture in the presence of toluene, completely removed TCE from assay mixtures when it was supplied at a concentration of 10 μM (Fig. 1). At higher supplied concentrations, the rate of TCE removal declined rapidly, with little or no removal after 30 min of incubation. Doubling the amount of biomass in the assay resulted in a commensurate doubling of the total amount of TCE removed. However, increasing the supplied TCE concentration over the range 10 to 100 μM had little influence on the total amount of TCE removed by batch-grown cells. The maximum initial rate of TCE removal was 11 nmol/min/mg of protein when TCE was supplied at an initial concentration of 40 μM (data not shown).

Cells grown continuously on toluene were able to remove approximately 77 μM TCE per mg of protein at an initial rate of 5.2 nmol/min/mg of protein when supplied with TCE at an initial concentration of 20 μM. Addition of 1 mM dithiothreitol (DTT) to these assay mixtures increased the total amount of TCE removed by approximately 40% (Fig. 1), while addition of 10 mM DTT increased the amount removed by 67%. There was no loss of TCE from assay mixtures containing DTT in the absence of cells.

Incubation of toluene-induced cells with TCE resulted in rapid loss of viability, while noninduced cells maintained viability in the presence of TCE (Fig. 2). Addition of 10 or 100 mM DTT to culture media did not improve the survival of toluene-induced cells in the presence of TCE.

Supply of TCE as a saturated vapor during batch growth decreased the specific growth rate (μ) in the exponential phase from 0.46 to 0.09/h for arginine-toluene-grown cells (Table 1). Conversely, PCE, which has solvent properties similar to those of TCE but is not significantly removed by arginine-toluene-grown cells, had little effect on the growth of induced cells. This suggests that biotransformation of TCE via toluene dioxygenase had a growth-inhibitory effect. However, provision of TCE during growth on arginine alone also decreased μ but to a far lesser extent than for arginine-toluene-grown cells

TABLE 1. Specific growth rates of *P. putida* during exponential growth in batch culture on mineral medium with various supplements

Medium supplement(s)[a]	μ (h^{-1})	Relative specific growth rate (%)
Arginine	0.38	83
Arginine, TCE	0.20	44
Arginine, toluene	0.46	100
Arginine, toluene, TCE	0.09	20
Toluene, TCE	0.06	13
Toluene, PCE	0.47	102

[a] Toluene, TCE, or PCE was supplied as a vapor from side arm reservoirs on the batch-growth flasks; arginine was supplied directly to growth media at a concentration of 0.2% (wt/vol). Inocula for flasks containing toluene were grown on mineral medium supplemented with arginine and toluene, and inocula for flasks that did not contain toluene were grown on this medium supplemented with arginine only.

TABLE 2. Substrates tested for the ability to induce indole metabolism, catechol 2,3-oxygenase activity, toluene-stimulated oxygen uptake, and degradation of toluene when supplied to batch cultures of *P. putida* grown in liquid mineral medium containing 0.2% (wt/vol) arginine

Supplement	Indole reaction[a]	Catechol 2,3-oxygenase activity[b]	Toluene-stimulated oxygen uptake[c]	Toluene degradation[d]
None	−	<2	0	0
Toluene	+	100	100	100
Phenol	+	64	86	ND[e]
TCE	+	20	38	39
PCE	+	27	65	ND

[a] Ability of culture to convert indole to the blue dye indigo. −, negative result (no color production); +, positive result (production of blue color) when supplied with 0.25 mM indole.

[b] Catechol 2,3-oxygenase activity in crude cell extract. 100% rate = 0.365 μmol of product per min/mg of protein.

[c] Rate of toluene-stimulated oxygen uptake measured with an oxygen electrode. 100% rate = 623 ± 26 nmol of O_2 per min/mg of protein.

[d] Rate of degradation of 1 mM toluene in headspace assay. 100% rate = 244 nmol/min mg of protein.

[e] ND, not determined.

(Table 1). Induction of toluene dioxygenase by TCE could account for this observation; this is supported by the data presented in Table 2. Provision of TCE during growth of the organism on arginine conferred the ability to convert indole to the blue dye indigo and induced catechol 2,3-oxygenase activity, toluene-stimulated oxygen uptake, and toluene degradation (Table 2). The formation of indigo and expression of the *meta* ring fission enzyme catechol 2,3-oxygenase are consistant with induction of toluene dioxygenase in this strain (7, 8). Similar results were obtained with cells grown on arginine in the presence of PCE, although for both chlorinated ethylenes the apparent levels of induction were lower than those for either toluene or phenol. Neither TCE nor PCE was able to support growth of the organism when provided as the sole source of carbon and energy.

The results presented here suggest that the bioconversion and cytotoxicity of TCE were mediated by toluene dioxygenase, which is consistent with reports concerning *P. putida* F1 (17). However, the initial rate of TCE removal by batch-grown cells, when supplied at 10 μM, was on the order of 20-fold higher than that reported for *P. putida* F1 (16). A higher rate of toxic oxidation product formation may account for the severe inhibition of growth and cell death reported here. It is interesting that for *P. cepacia* G4, relatively little apparent cytotoxicity results from metabolism of TCE via toluene *ortho*-monooxygenase; kinetics of TCE removal by this organism were linear over 3 h of incubation for a supplied TCE concentration of 20 μM (5). The kinetics of TCE removal has also been shown to be linear for *P. stutzeri* IBB_2 (6). Mechanistic differences between the dioxygenase and monooxygenase routes of TCE oxidation may account for these findings. Li and Wackett (10) reported that for *P. putida* F1, reaction products of TCE oxidation are diffusible and modify proteins and reduced nucleotides in solution. The major products of TCE oxidation by this organism were reported to be formate and glyoxylic acid, with formyl chloride and glyoxylyl chloride being proposed as initial oxidation products, respectively (10). Since the toluene dioxygenase of the strain reported on here is also known to function as a monooxygenase (1), other possible initial products of TCE oxidation are TCE-epoxide, 2,2,2-trichloroacetaldehyde, TCE-dioxetane, and 1,2-dihydroxy-TCE. In *P. putida* F1, the mechanism of TCE oxidation is thought not to involve these intermediates (10), although they cannot be discounted for the strain reported on here.

DTT is known to protect against deleterious effects of chemical agents operating via free-radical or oxidative stress. The increased capacity of cells to degrade TCE in the presence of DTT reported here (Fig. 1) may, therefore, be explained by chemical reduction of reactive intermediates affording protection from inactivation. Indeed, DTT has been used previously to protect liver cells from oxidative stress injury arising from carbon tetrachloride metabolism (4). Regardless of the nature of reactive intermediates of TCE oxidation, their involvement in cytotoxicity and cell death would require their release from the surface of toluene dioxygenase. The high TCE oxidation rates of the strain reported on here should benefit further work on the elucidation of stable oxidation products arising from reactive intermediates. The nature of such products should help to elucidate the mechanism of TCE oxidation in this strain, which may differ significantly from that of *P. putida* F1.

Significant induction of toluene dioxygenase by chlorinated ethylenes has not been reported previously and may be an important consideration for potential use of the strain in bioremediation processes. The results suggest that, for this strain at least, PCE is a gratuitous inducer of toluene dioxygenase and that TCE should not be regarded solely as a fortuitous substrate of an aromatic pathway.

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