Trichloroethylene Degradation by Escherichia coli Containing the Cloned Pseudomonas putida F1 Toluene Dioxygenase Genes

GERBEN J. ZYLSTRA,1 LAWRENCE P. WACKETT,2 AND DAVID T. GIBSON*  
Department of Microbiology and Biocatalysis Research Group, University of Iowa, Iowa City, Iowa 52242,1 and Gray Freshwater Biological Institute and Department of Biochemistry, University of Minnesota, Navarre, Minnesota 553922

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Toluene dioxygenase from Pseudomonas putida F1 has been implicated as an enzyme capable of degrading trichloroethylene. This has now been confirmed with Escherichia coli JM109(pDTG601) that contains the structural genes (todCIC2BA) of toluene dioxygenase under the control of the tac promoter. The extent of trichloroethylene degradation by the recombinant organism depended on the cell concentration and the concentration of trichloroethylene. A linear rate of trichloroethylene degradation was observed with the E. coli recombinant strain. In contrast, P. putida F39/D, a mutant strain of P. putida F1 that does not contain cis-toluene dihydrodiol dehydrogenase, showed a much faster initial rate of trichloroethylene degradation which decreased over time.

Volatile organic chemicals are widespread groundwater contaminants (24). The most ubiquitous members of this class of organic pollutants are low-molecular-weight halogenated solvents. Currently, there is concern over the presence of these compounds in drinking water supplies because they are known to persist over time and many are toxic or carcinogenic or both for experimental animals. For example, trichloroethylene (TCE) has been reported to have a half-life in one aquifer of 300 days (14) and is a suspected carcinogen in rats (10). Concern over TCE contamination has become more acute owing to the observation that some anaerobic bacteria in groundwater transform TCE to vinyl chloride (5, 21). This latter compound is a potent mutagen and carcinogen in humans (9).

Although anaerobic bacteria reductively dehalogenate TCE to produce vinyl chloride, a more toxic product, some aerobic bacteria appear to utilize alternative pathways that do not lead to the formation of vinyl chloride. Nelson and co-workers (11) have shown that certain strains of bacteria can degrade TCE after growth with mononuclear aromatic compounds such as toluene or phenol. These observations suggest that an inducible enzymatic component of the aromatic degradative pathway is involved in the gratuitous degradation of TCE. Further studies by Nelson and co-workers (13) and Wackett and Gibson (22) showed that mutants of Pseudomonas putida F1 in which toluene dioxygenase activity was absent failed to degrade TCE. In contrast, mutants defective in other enzymes of the toluene degradative pathway could still degrade TCE. These experiments clearly implicated toluene dioxygenase as playing a role in the degradation of TCE, either by itself or in concert with other enzymes.

The todCIC2BADE genes, which are responsible for the initial steps in the degradation of toluene, have been cloned from P. putida F1 on a 10.7-kilobase-pair EcoRI fragment of DNA (W. R. McCombie, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K53, p. 155). These genes form part of the tod operon (4) and encode enzymes responsible for the first three steps in the toluene degradation pathway. The genes were localized to specific regions of this DNA fragment by deletion analysis and mutant complementation (28), and their nucleotide sequence has been determined (27a). Several clones have now been constructed that overproduce specific enzymes of the toluene degradative pathway in Escherichia coli (27a). One such recombinant clone, designated pDTG601, contains the four genes necessary for a functional toluene dioxygenase: todCIC2BA. Together these three proteins catalyze the oxidation of toluene to cis-1,2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (6) (cis-toluene dihydrodiol, Fig. 1). E. coli JM109(pDTG601) produces a functionally active toluene dioxygenase under the control of the tac promoter (27a). The construction of this strain offered the unique opportunity to ascertain whether toluene dioxygenase alone can degrade TCE. In addition, we examined the effect of TCE concentration on the rate of TCE biodegradation by E. coli JM109(pDTG601) and compared the results with those obtained with P. putida F39/D. The latter is a mutant that accumulates cis-toluene dihydrodiol when grown in the presence of toluene (6).

MATERIALS AND METHODS

Strains. E. coli JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) [F' traD36 proAB lacF2ΔM15]) (26) was utilized as the host for all plasmids. The expression vector pKK223-3 (2) was obtained from Pharmacia, Inc. (Piscataway, N.J.). Plasmid pDTG601, which contains the todCIC2BA genes cloned downstream from the tac promoter in pKK223-3, has been described previously (27a). P. putida F39/D is a todD mutant of the P. putida wild-type strain F1 and thus lacks cis-toluene dihydrodiol dehydrogenase activity (6).

Growth of organisms. P. putida F39/D was grown in liquid culture at 30°C on arginine in the presence of toluene vapors, harvested, and suspended as previously described for P. putida F1 (22). E. coli JM109(pDTG601) and JM109 (pKK223-3) were grown with shaking at 37°C on mineral salts basal medium (16) supplemented with 20 mM glucose, 1 mM thiamine, and 100 μg of ampicillin per ml. When the culture attained a turbidity of 0.7 at 600 nm, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM and cell growth was continued for 1 h (one doubling time). Cells were harvested by centrifugation at 8,000 × g at 4°C, suspended at the appropriate turbidity in fresh mineral
FIG. 1. (A) Oxidation of toluene (I) to cis-toluene dihydrodiol (II) by toluene dioxygenase. This three-component enzyme system transfers electrons from NADH to the terminal component that catalyzes the formation of cis-toluene dihydrodiol from toluene (27). A flavoprotein designated reductase_TOL (18), initially accepts electrons from NADH. Subsequently, electrons are transferred to ferredoxin_TOL (19), which then reduces the terminal dioxygenase, ISP_TOL (17). (B) Diagrammatic representation of recombinant clone pDTG601. This plasmid contains the genes for toluene dioxygenase (todC1C2BA) under control of the tac promoter.

Results

Toluene dioxygenase alone conferred TCE degradation activity on E. coli. The toluene dioxygenase genes in E. coli JM109(pDTG601) were induced by isopropyl-β-D-thiogalactopyranoside for 1 h during the log phase of growth. Cells were harvested by centrifugation, suspended in fresh media at various cell densities, and incubated with TCE (200 nmol per vial). Under a number of different conditions, strain JM109(pDTG601) was observed to biodegrade TCE (Fig. 2). The ability to degrade TCE depended on the cloned toluene dioxygenase genes since the negative control strain JM109 (pKK223-3), which contained the vector without the cloned genes, did not show degradation of TCE. The low level of decrease shown in the negative control culture of Fig. 2 was due to TCE removal for gas chromatography analysis and was the same as that observed in control experiments that lacked cells.

Effect of E. coli JM109(pDTG601) cell density on TCE degradation. Figure 2 shows that the rate at which whole cells of E. coli JM109(pDTG601) degraded TCE depended on the cell density. A significant difference was seen between cells at a turbidity of 1.0 (600 nm) compared with those at a turbidity of 2.0. However, cells suspended at turbidities above 2.0 did not show a proportional increase in the rate of degradation of TCE, and a decrease in the extent of the linear range of the progress curve was observed. Consequently, cells in succeeding experiments were always suspended at a turbidity of 2.0. This corresponded to a protein concentration of 0.61 mg/ml.

Comparison of TCE degradation by P. putida F39/D and E. coli JM109(pDTG601). E. coli JM109(pDTG601) contains the toluene dioxygenase genes from P. putida F1, and the rate of oxidation of TCE by this strain was compared with the rate of oxidation by P. putida F39/D. The latter organism contains a mutation in the structural gene for cis-toluene dihydrodiol dehydrogenase and thus accumulates cis-toluene dihydrodiol in the culture medium when grown on arginine in the presence of toluene vapors (6). JM109(pDTG601) also oxidizes toluene to cis-toluene dihydrodiol (27a). Thus, the rate of formation of cis-toluene dihydrodiol from toluene for these two bacterial strains is an indication of the amount of

salts basal medium supplemented with 20 mM glucose, and kept on ice until used (usually within 1 h).

Measurement of TCE degradation. The appropriate cell suspension (2 ml) was added to glass vials (total volume, 14 ml) which were then sealed with a Teflon-coated rubber septum and an aluminum crimp seal. TCE (10 μl of an appropriate stock solution in N,N-dimethylformamide) was added to triplicate sets of vials for each experiment. The vials were incubated at 37°C (30°C for P. putida) in a shaking water bath. Samples (100 μl) of the gas phase contained in each sealed vial were removed every hour and injected into a Hach Carle AGC100 gas chromatograph equipped with a flame ionization detector and fitted with a Graphpak AT-1000 column (Alltech Associates, Inc., Applied Science Div., State College, Pa.) operated at 150°C with a nitrogen carrier gas flow of 30 ml/min. TCE had a retention time of 2.0 min in this system and was quantitated by peak height in comparison with TCE standards. Values plotted in the figures are the means of at least two independent experiments with triplicate sets of vials in each experiment. The theoretical calculated rate of loss of TCE due to the removal of 100-μl samples every hour was similar to that observed experimentally for the negative control strains E. coli JM109(pKK223-3) and uninduced P. putida F39/D. Protein concentrations of cell suspensions were determined as described previously (22).

Measurement of cis-toluene dihydrodiol formation. P. putida F39/D and E. coli JM109(pDTG601) were grown, induced, harvested, and suspended as described above. Cells (50 ml) were placed in 250-ml Erlenmeyer flasks, and toluene was introduced in the vapor phase as described previously (3). The flasks were incubated with shaking at 30°C for P. putida F39/D and at 37°C for E. coli JM109 (pDTG601). Samples (0.5 ml) were removed every hour, the cells were removed by centrifugation, and the absorbance of the clear supernatant solution was measured at 265 nm, which is the absorbance maximum for cis-toluene dihydrodiol (6). The reported extinction coefficient of 5,220 cm⁻¹ M⁻¹ (6) was used to calculate the concentration of cis-toluene dihydrodiol. Control strains E. coli JM109(pKK223-3) and uninduced P. putida F39/D did not accumulate any material that absorbed at 265 nm.
toluene dioxygenase that is induced. JM109(pDTG601) accumulated cis-toluene dihydrodiol at the linear rate of \(0.95 \pm 0.13\) (\(n = 4\)) \(\mu\text{mol/h}\) per mg of protein over a 6-h period. Over the same time period, \(P.\ putida\) F39/D accumulated cis-toluene dihydrodiol at a uniform rate of \(1.07 \pm 0.10\) (\(n = 4\)) \(\mu\text{mol/h}\) per mg of protein. Thus, both strains metabolized toluene and produced cis-toluene dihydrodiol at rates that are equivalent within statistical error. Consistent with these data was the observation that cell extracts prepared from either \(E.\ coli\) JM109(pDTG601) or \(P.\ putida\) F1 showed similar levels of toluene dioxygenase activity (data not shown).

The linear production of cis-toluene dihydrodiol from toluene was mirrored by the linear rate of disappearance of TCE over at least 6 h catalyzed by JM109(pDTG601) (Fig. 3). In contrast, \(P.\ putida\) F39/D oxidized TCE at a rapid initial rate that decreases with time (Fig. 3). This result differed markedly from the experiments with toluene in which \(P.\ putida\) F39/D produced cis-toluene dihydrodiol at a uniform rate. However, this nonlinear time course of TCE degradation was also seen previously for \(P.\ putida\) F1 (22), the parent strain of \(P.\ putida\) F39/D. There is evidence that TCE oxidation mediates cytotoxic effects on \(P.\ putida\) F1, and this may underlie the rate curves observed with both \(P.\ putida\) F1 and F39/D (23). It is interesting that TCE oxidation by toluene dioxygenase proceeded differently in a foreign background.

**Effect of concentration on TCE degradation by \(E.\ coli\) JM109(pDTG601) and \(P.\ putida\) F39/D.** The amount of TCE degraded by \(P.\ putida\) F39/D and \(E.\ coli\) JM109(pDTG601) varied in proportion to the concentration of TCE to which the cells are exposed. The data shown in Fig. 4 were derived

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**FIG. 2.** Dependence of TCE degradation on cell density. Cells of \(E.\ coli\) JM109(pDTG601) grown as described in Materials and Methods were incubated with TCE (200 nmol) at the following cell densities (600 nm): 1.0 ( ), 2.0 ( ), 4.0 ( ), and 6.0 ( ). A control experiment in which JM109(pKK223-3) was incubated with TCE at a cell density of 2.0 is shown by open circles.

**FIG. 3.** Degradation of TCE by \(E.\ coli\) JM109(pDTG601) and \(P.\ putida\) F39/D. Cells were grown and suspended as described in the text, TCE (200 nmol) was added to each vial, and the amount of degradation was determined at 1-h intervals. Symbols: ●, JM109(pDTG601); ■, \(P.\ putida\) F39/D. Control experiments ( ○) were conducted with JM109(pKK223-3) and uninduced cells of \(P.\ putida\) F39/D.
by measuring the total amount of TCE that had been degraded in a 4-h period. At each of the TCE concentrations examined (50 to 1,000 nmol per vial), JM109(pDTG601) showed a linear rate of degradation (similar to that seen in Fig. 3) over a 4-h period. P. putida F39/D, however, showed a nonlinear rate of degradation, as represented in Fig. 3, for all concentrations tested. This result indicated that the observed rate decay is not due to substrate depletion at concentrations below the \( K_m \) of toluene dioxygenase for TCE. In all experiments with P. putida F39/D, a fast initial rate of TCE degradation was seen in the first 30 min to 1 h with a much slower rate in the succeeding 3-h incubation period.

**DISCUSSION**

In two previous studies (13, 22), mutants defective in the ISP \(_{TOL}\) component of toluene dioxygenase were shown to lack the ability to biodegrade TCE. Revertant strains that regained toluene dioxygenase activity simultaneously regained the ability to oxidize TCE. These results demonstrated a role for toluene dioxygenase in TCE degradation. However, the possibility that other constituents in P. putida F1 act in concert with toluene dioxygenase to degrade TCE was not precluded by these observations. The strain used in the present study, E. coli JM109(pDTG601), contains 3.1 kilobases of DNA from P. putida F1 that encode for only four gene products: reductase \(_{TOL}\), ferredoxin \(_{TOL}\), and both subunits of ISP \(_{TOL}\) (27a). These results clearly demonstrate that the P. putida F1 genes for toluene dioxygenase alone are sufficient to confer TCE-oxidizing ability onto E. coli. Furthermore, the recombinant organism also oxidizes toluene and a wide range of other aromatic compounds (G. J. Zylstra, S. Chauhan, and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K61, p. 255). Thus, toluene dioxygenase shows identical in vivo substrate specificity whether it is expressed in P. putida or E. coli.

It is of interest that toluene dioxygenase showed different properties with respect to in vivo TCE oxidation in E. coli than in P. putida. The rates of cis-toluene dihydrodiol formation by P. putida F39/D and E. coli JM109(pDTG601) were similar, whereas initial TCE oxidation rates differed substantially for the two organisms. It is unclear why the TCE degradation rates differed. These data indicate that in vivo biodegradation rates might be influenced by factors other than the \( K_m \) and \( V_{max} \) parameters of a given catabolic enzyme. Even though the recombinant E. coli showed slower initial biodegradation, linear rates of TCE oxidation were maintained for much longer periods than with Pseudomonas cultures. Previously, it had been shown that oxidation of TCE by mammalian liver microsomes results in the formation of electrophilic intermediates that may react with intracellular biomolecules (7, 10). TCE oxidation by growing cultures of P. putida F1 leads to a dramatic diminution in growth rate and to the incorporation of \(^{14}\)C\(^{-}\)TCE into macromolecules which may be the result of alkylation of nucleophilic biomolecules (23). The observation that TCE oxidation rates are more sustained in E. coli could reflect less damage to metabolism in this organism. Thus, the matching of a biodegradative enzyme activity with the proper host strain might be an important theme in the use of recombinant DNA technology in bioremediation.

*Pseudomonas fluorescens* PFL12 was reported to utilize TCE and several other volatile halogenated hydrocarbons as growth substrates (20). However, no data were presented to support this claim. There are no confirmed reports of an organism that can utilize TCE as the sole source of carbon and energy for growth. In addition to toluene dioxygenase, other oxygenases can initiate TCE degradation. For example, a recent report by Winter et al. (25) describes the oxidation of TCE by toluene-grown cells of *Pseudomonas mendocina* KR. This organism utilizes a multicomponent enzyme system (toluene 4-monoxygenase) to oxidize toluene to \( p \)-cresol (G. M. Whited, Ph.D. thesis, University of Texas, Austin, 1986; G. M. Whited and D. T. Gibson, submitted for publication). The genes encoding toluene 4-monoxygenase were cloned into strains of E. coli, and the recombinant organisms were shown to degrade TCE (25).

*Pseudomonas cepacia* G4, the first organism reported to
degrade TCE (12), initiates toluene degradation by hydroxylation at the 2-position to yield o-cresol (15). Thus, three different oxygenases, each one induced by toluene, are capable of catalyzing the degradation of TCE. Methane monooxygenase (8; B. G. Fox, W. A. Froland, J. G. Borneman, L. P. Wackett, and J. D. Lipscomb, submitted for publication), propane monooxygenase (L. P. Wackett, G. A. Brusseau, S. R. Householder, and R. S. Hanson, submitted for publication), and ammonia monooxygenase (1) have also been implicated in TCE degradation. The molecular mechanisms involved await studies with purified enzyme systems.

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LITERATURE CITED