Monohydroxylation of Phenol and 2,5-Dichlorophenol by Toluene Dioxygenase in *Pseudomonas putida* F1

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Pseudomonas putida F1 contains a multicomponent enzyme system, toluene dioxygenase, that converts toluene and a variety of substituted benzenes to *cis*-dihydrodiols by the addition of one molecule of molecular oxygen. Toluene-grown cells of *P. putida* F1 also catalyze the monohydroxylation of phenols to the corresponding catechols by an unknown mechanism. Respirometric studies with washed cells revealed similar enzyme induction patterns in cells grown on toluene or phenol. Induction of toluene dioxygenase and subsequent enzymes for catechol oxidation allowed growth on phenol. Tests with specific mutants of *P. putida* F1 indicated that the ability to hydroxylate phenols was only expressed in cells that contained an active toluene dioxygenase enzyme system. ¹⁸O₂ experiments indicated that the overall reaction involved the incorporation of only one atom of oxygen in the catechol, which suggests either a monooxygenase mechanism or a dioxygenase reaction with subsequent specific elimination of water.

The phenomenon of enzymatic oxygen fixation was first described in 1955 (11, 15). Since that time, oxygenases have been shown to play an essential role in many aspects of cellular metabolism. Their importance in environmental science cannot be overemphasized because bacterial oxygenases have been shown to participate in the biodegradation of many natural and xenobiotic compounds.

Two general types of oxygenases have been recognized. Enzymes that incorporate one atom of molecular oxygen into an organic substrate have been termed monooxygenases, whereas enzymes that incorporate two atoms of molecular oxygen are termed dioxygenases (10). For example, in the present study, toluene dioxygenase (9, 25) from *Pseudomonas putida* F1 incorporates both atoms of molecular oxygen into the aromatic nucleus to form *cis*-1(S),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) (6, 14, 26).

Previous studies showed that toluene-grown cells of *P. putida* F1 can oxidize phenol (7). This has been confirmed recently (19; L. P. Wackett, Ph.D. dissertation, The University of Texas at Austin, 1984), and catechol and 2-hydroxymuconic semialdehyde were tentatively identified as metabolic intermediates. These observations suggest that *P. putida* F1 can catalyze the monohydroxylation of phenol to form catechol. Further evidence for the monohydroxylase activity of *P. putida* F1 was provided by studies on the hydroxylation of *p*-dichlorobenzene (*p*-DCB) and 2,5-dichlorophenol (2,5-DCP) (19). Toluene-grown cells of *P. putida* F1 oxidize both *p*-DCB and 2,5-DCP to 3,6-dichlorocatechol (Fig. 1). The chlorinated catechol accumulates in the reaction medium because it is not a substrate for the 3-methylcatechol 2,3-dioxygenase that is induced in *P. putida* F1 during growth with toluene. Toluene-grown cells of *Pseudomonas* sp. strain JS6 isolated for its ability to grow on *p*-DCB also oxidize both *p*-DCB and 2,5-DCP to 3,6-dichlorocatechol (19). When grown on *p*-DCB, this strain can mineralize both *p*-DCB and 2,5-DCP because growth on the chlorinated substrate causes induction of the modified *ortho* pathway for degradation of the 3,6-dichlorocatechol (20).

P. putida F39/D is a mutant strain of *P. putida* F1 that does not oxidize *cis*-toluene dihydrodiol to 3-methylcatechol (6). Toluene-induced cells of *P. putida* F39/D oxidized *p*-DCB to *cis*-1,2-dihydroxy-3,6-dichlorocyclohexa-3,5-diene (*cis*-*p*-dichlorobenzene dihydrodiol) as expected (Fig. 1). The same cells oxidized 2,5-DCP to 3,6-dichlorocatechol, which indicates that the dihydrodiol dehydrogenase was not required for the reaction (19). These observations suggest that toluene dioxygenase might be the enzyme responsible for the hydroxylation of phenol and 2,5-DCP to catechol and 3,6-dichlorocatechol, respectively. However, the presence of a different monooxygenase in toluene-induced cells of *P. putida* F1 and F39/D would also account for the observed results. The present study was undertaken to determine the nature of the enzyme in *P. putida* F1 responsible for the hydroxylation of phenol and substituted phenols to catechols.

MATERIALS AND METHODS

Materials. 2,5-DCP was obtained from Aldrich Chemical Co., Inc., Milwaukee Wis. *p*-DCB was from Fisher Scientific Co., Fairlawn, N.J., and ¹⁸O₂ was from MSD Isotopes, Montreal, Quebec, Canada.

[¹⁸O]*p*-dichlorobenzene dihydrodiol was prepared biologically from *p*-DCB (8). *P. putida* F39/D was grown on arginine in the presence of *p*-DCB and ¹⁸O₂. The resultant [¹⁸O]*p*-dichlorobenzene dihydrodiol was extracted with ethyl acetate. Extracts were dried over sodium sulfate, and the solvent was removed by flash evaporation. [¹⁸O]2,5-DCP was prepared by dehydration of [¹⁸O]*p*-dichlorobenzene dihydrodiol in 6 N H₂SO₄ for 10 min at 60°C. The dichlorophenol was extracted with ethyl acetate, and the solvent was dried over sodium sulfate and evaporated to dryness under reduced pressure.

Organisms and growth conditions. *P. putida* strains and plasmids used in these studies are listed in Table 1. Cultures were grown in minimal medium (MSB) (21) supplemented with carbon sources as indicated. Toluene and benzene were provided in the vapor phase as described previously (7). Phenol (4 μl) was added directly to the surfaces of MSB agar plates (17). Arginine was added at a final concentration of 2

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FIG. 1. Oxidation of substituted benzenes by toluene-induced cells of *P. putida*.

g/liter. Strains containing *pDTG506* were maintained in the presence of kanamycin (50 μ g/ml).

Strain construction. *pDTG506* was transferred from *Escherichia coli* JM109 (24) to *P. putida* F1 strains by triparental mating in the presence of *pRK2013* (4).

Oxidation of 2,5-DCP. Rates of conversion of 2,5-DCP to 3,6-DCC were measured as described previously (19). Cultures were grown on arginine in the presence of toluene, harvested by centrifugation, washed, and suspended in MSB-arginine to a final protein concentration of 0.26 mg/ml. 2,5-DCP was added to a final concentration of 5×10^{-5} M, and suspensions were incubated on a rotary shaker at 30°C. Samples of the suspensions were clarified by centrifugation

FIG. 2. Mass spectral analysis of 3,6-DCC produced from 2,5-DCP by toluene-grown F1 cells in the presence of $^{18}\text{O}_2$. Mass spectra of gas phase (A), 3,6-DCC produced in air (B), and 3,6-DCC produced in the enriched gas mixture (C) are shown.

TABLE 1. *P. putida* strains and plasmids

Strain or plasmid	Relevant properties ^a	Reference or source
<i>P. putida</i> strain		
F1	Tol ⁺ , prototroph	7
F3	Tol ⁻ , <i>todB</i>	5
F4	Tol ⁻ , <i>todC1</i>	5
F12	Tol ⁻ , <i>todA</i>	5
F106	Tol ⁻ , <i>todC2</i>	5
F39/D	Tol ⁻ , <i>todD</i>	6
F3(<i>pDTG506</i>)	Tol ⁺ , Km ^r	This study
F4(<i>pDTG506</i>)	Tol ⁺ , Km ^r	This study
F12(<i>pDTG506</i>)	Tol ⁻ , Km ^r	This study
F106(<i>pDTG506</i>)	Tol ⁺ , Km ^r	This study
Plasmid		
<i>pRK2013</i>	Km ^r	4
<i>pDTG506</i>	Km ^r <i>todC1</i> , <i>todC2</i> , <i>todB</i>	27

^a Abbreviations: Tol⁺, ability to grow with toluene as the sole source of carbon; *tod*, operon for toluene degradation; Km^r, kanamycin resistance.

and analyzed by high-pressure liquid chromatography at appropriate intervals.

$^{18}\text{O}_2$ incorporation. *P. putida* F1 was grown overnight in 500 ml of MSB with toluene as the carbon source. Cells were harvested by centrifugation and suspended to a density of 1.0 A_{600} (0.26 mg of protein per ml) in 500 ml of dilute (1:4) MSB-arginine medium (pH 6.5). The suspension was transferred to a 1.0-liter round-bottom flask, sealed with a stopcock, and stirred with a magnetic stirrer at 25°C. The air in the headspace of the flask was removed under vacuum and replaced with nitrogen four times. The headspace was then evacuated and refilled with air enriched with $^{18}\text{O}_2$. The gas phase in the flask was analyzed by mass spectroscopy. An identical experiment was carried out in the presence of air. The systems were allowed to equilibrate for 15 min, 2,5-dichlorophenol was added to a final concentration of 5×10^{-5} M, and the suspensions were incubated with stirring. After 50 min, the cell suspensions were sparged with nitro-

FIG. 3. Mass spectral analysis of 3,6-DCC produced from $[^{18}\text{O}]2,5\text{-DCP}$ by toluene-grown F1. Mass spectra of $[^{16}\text{O}]2,5\text{-DCP}$ (A), $[^{18}\text{O}]2,5\text{-DCP}$ (B), and 3,6-DCC produced from $[^{18}\text{O}]2,5\text{-DCP}$ in air (C) are shown.

gen for 5 min, and cells were removed by centrifugation. The supernatants were adjusted to pH 5.5 and extracted with equal volumes of ethyl acetate. The extracts were dried over anhydrous sodium sulfate, and the ethyl acetate was removed by flash evaporation. The residues were stored under nitrogen until analyzed by high-pressure liquid chromatography and gas chromatography-mass spectrometry.

Analytical methods. High-pressure liquid chromatography, capillary column gas chromatography-mass spectral analyses, and respirometry were performed as described previously (20). Protein concentrations were estimated by the method of Smith et al. (18).

RESULTS

Incorporation of ^{18}O into 2,5-DCP. The involvement of an oxygenase in the conversion of 2,5-DCP to 3,6-DCC was tested by experiments conducted in the presence of $^{18}\text{O}_2$.

TABLE 2. Oxygen consumption by washed cells

Assay substrate ^a	Rate ($\mu\text{mol/min}$ per mg of protein) of oxygen consumption after growth ^b of strain on substrate		
	F1		F39/D on phenol
	Toluene	Phenol	
Toluene	538	956	16
Phenol	83 ^c	62	405 ^c
Catechol	331	914	686
3-Methylcatechol	808	1,039	1,621
Toluene dihydrodiol	311	449	31

^a Assay substrates were added at a concentration of 100 μM .

^b Cultures were grown in MSB broth with toluene provided in the vapor as the sole carbon source or on MSB agar plates with phenol (4 μl) applied to the surface of the agar as the sole carbon source. Phenol-grown cells were washed from the plates with phosphate buffer, harvested by centrifugation, and suspended in phosphate buffer.

^c Rate fell after 1 to 2 min.

Toluene-grown cells of strain F1 were incubated with 2,5-DCP in an atmosphere that contained equal amounts (49.78 and 50.22%) of $^{18}\text{O}_2$ and $^{16}\text{O}_2$ (Fig. 2). At the end of the incubation period, 3,6-DCC was isolated and analyzed by gas chromatography-mass spectrometry. The spectrum revealed equal amounts of 3,6-DCC containing two atoms of ^{16}O and 3,6-DCC containing one atom of ^{18}O and one atom of ^{16}O . The results clearly show that one atom of molecular oxygen is incorporated into 2,5-DCP to form 3,6-DCC. There was no evidence of 3,6-DCC containing two atoms of ^{18}O , as would be expected if both atoms from a single molecule of $^{18}\text{O}_2$ were incorporated in the final product. The results indicate that the oxygen atom in the hydroxyl group in 2,5-DCP was not replaced by a different atom of oxygen derived from the incoming molecule of dioxygen. When the experiment was repeated with $[^{18}\text{O}]2,5\text{-DCP}$ and $^{16}\text{O}_2$, the ratio of ^{18}O and ^{16}O in the resultant 3,6-DCC was unchanged from that of the starting material (Fig. 3). The results confirm that the reaction mechanism does not involve the replacement of the original oxygen atom.

Oxidation of phenol and 2,5-DCP by toluene and phenol-grown cells. Previous experiments with strains F1 and F39/D involved the use of toluene-induced cells. Both of these strains will also grow slowly on phenol, but growth occurs over a very narrow concentration range (≈ 100 mg/liter).

TABLE 3. Growth of F1 derivative strains on aromatic substrates and transformation of 2,5-DCP

Strain	Growth ^a on:			Transformation ^b of 2,5-DCP
	Toluene	Benzene	Phenol	
F1	++	++	+	0.062 ^c
F3	—	—	—	<0.001
F3(pDTG506)	++	++	+	0.027
F4	—	—	—	<0.001
F4(pDTG506)	++	++	+	0.051
F12	—	—	—	0.007
F12(pDTG506)	—	—	—	0.007
F106	—	—	—	<0.001
F106(pDTG506)	++	++	+	0.019

^a Growth was tested on MSB agar plates, and substrates were provided in the vapor phase. Symbols: ++, good growth; +, little growth; —, no growth.

^b First-order rates (per minute) were measured in suspensions of cells induced with toluene. Rates were normalized to a protein concentration of 0.1 mg/ml.

^c Data from reference 19.

FIG. 4. Proposed mechanism of dichlorocatechol formation from 2,5-DCP by the toluene dioxygenase enzyme system in strain F1.

Cells grew readily on arginine in the presence of phenol and on MSB agar plates with phenol as the sole source of carbon and energy. Respirometry revealed that patterns of enzyme induction in cells grown on phenol were similar to those in cells grown on toluene (Table 2). In F39/D cells, stimulation of oxygen uptake by toluene was less than with phenol because the *cis*-toluene dihydrodiol cannot be oxidized. Cells of strain F1 grown with phenol or toluene oxidized toluene faster than phenol. Phenol, toluene, or 3-methylcatechol did not stimulate oxygen uptake by arginine-grown cells. Thus, growth with phenol seems to lead to induction of the sequence of enzymes necessary for degradation of toluene. Conversely, growth with toluene induced the enzymes necessary for phenol oxidation. Furthermore, F1 cells induced with either phenol or toluene readily converted 2,5-DCP to 3,6-DCC.

Growth of F1 and mutant derivatives on toluene and phenol. Toluene dioxygenase is a multicomponent enzyme system that requires the products of four structural genes (*todA*, *todB*, *todC1*, and *todC2*) for enzymatic activity (5). Strains defective in any one of these genes (F12, F3, F4, and F106) were unable to grow with toluene or phenol (Table 3). They also were unable to convert 2,5-DCP to 3,6-DCC. Plasmid pDTG506 encodes the structural genes *todB*, *todC1*, and *todC2* of the toluene dioxygenase system (27). When pDTG506 was transferred to *P. putida* strains F3, F4, and F106, each strain regained the ability to grow with toluene and phenol. The exconjugants also regained the ability to convert 2,5-DCP to 3,6-DCC. The failure of pDTG506 to complement the *todA* mutation in F12 supports the contention that toluene dioxygenase is the enzyme responsible for the monohydroxylation of phenol and 2,5-DCP.

DISCUSSION

We reported previously (19) that *P. putida* F106, a mutant in *todC*, is unable to convert substituted phenols to the corresponding catechols. The preliminary results suggested the involvement of the toluene dioxygenase enzyme system in the transformation of phenols. We have now extended the observations to include a series of mutants in the genes for toluene oxidation. All strains blocked in toluene dioxygenase were unable to grow on toluene or phenol or to transform 2,5-DCP. Restoration of the ability to grow on toluene also restored the ability to grow on phenol and to convert 2,5-DCP to 3,6-DCC. These results, taken with the evidence concerning induction of toluene dioxygenase by phenol, provide proof that the toluene dioxygenase enzyme system carries out the hydroxylation of phenols.

The results of $^{18}\text{O}_2$ studies indicate that only one atom of oxygen is incorporated into 2,5-DCP to form 3,6-dichlorocatechol. The mechanism of this reaction remains unknown. The simplest explanation would be the direct insertion of a

hydroxyl group into the aromatic nucleus. Phenol hydroxylase has been shown to catalyze this type of reaction with substituted phenols in several systems (2, 12, 13). Alternatively, an arene oxide might be the initial oxygenated product which could then isomerize to yield the catechol reaction product. However, in light of the evidence implicating toluene dioxygenase as the enzyme responsible for chlorinated phenol oxidation, the most likely explanation for the observed results in dioxygenation of the aromatic nucleus followed by elimination of water (Fig. 4). Brilon et al. (1) offered a similar explanation for the conversion of 2-hydroxynaphthalene to 2,3-dihydroxynaphthalene by pseudomonads grown on naphthalene or 2-naphthalenesulfonic acid. They suggested that a nonspecific naphthalene dioxygenase might catalyze the insertion of two hydroxyl groups and that the product of the reaction could rearomatize by spontaneous elimination of water.

The retention of ^{18}O during conversion of [^{18}O]2,5-DCP to [^{18}O]3,6-DCC indicates that the hydroxyl group of 2,5-DCP is not eliminated during the reaction. Therefore, if the reaction involves elimination of water from a trihydroxy intermediate, the elimination must be extremely specific.

The results suggest that *P. putida* F1 does not use a classical phenol hydroxylase (monooxygenase) but relies on the activity of toluene dioxygenase for growth on phenol. The toluene dioxygenase system exhibits not only a very broad substrate specificity but also a broad inducer specificity. This combination allows the transformation of a wide range of organic compounds, including substituted benzenes (8), heterocyclic compounds (3), indan and indene (23), and trichloroethylene (16, 22).

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