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Identification of *cis*-Diols as Intermediates in the Oxidation of Aromatic Acids by a Strain of *Pseudomonas putida* That Contains a TOL Plasmid

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Pseudomonas putida **BG1 was isolated from soil by enrichment with *p*-toluate and selection for growth with *p*-xylene. Other hydrocarbons that served as growth substrates were toluene, *m*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene. The enzymes responsible for growth on these substrates are encoded by a large plasmid with properties similar to those of TOL plasmids isolated from other strains of *Pseudomonas*. Treatment of *P. putida* BG1 with nitrosoguanidine led to the isolation of a mutant strain which, when grown with fructose, oxidized both *p*-xylene and *p*-toluate to (−)-*cis*-1,2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylic acid (*cis-p*-toluate diol). The structure of the diol was determined by conventional chemical techniques including identification of the products formed by acid-catalyzed dehydration and characterization of a methyl ester derivative. The *cis*-relative stereochemistry of the hydroxyl groups was determined by the isolation and characterization of an isopropylidene derivative. *p*-Xylene-grown cells contained an inducible NAD^+-dependent dehydrogenase which formed catechols from *cis-p*-toluate diol and the analogous acid diols formed from the other hydrocarbon substrates listed above. The catechols were converted to *meta* ring fission products by an inducible catechol-2,3-dioxygenase which was partially purified from *p*-xylene-grown cells of *P. putida* BG1.**

Pseudomonas putida (*arvilla*) mt-2 can grow with toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene (pseudocumene), and 3-ethylbenzene (27, 46, 47, 50). The genes encoding the enzymes responsible for the oxidation of these aromatic hydrocarbons are carried by a transmissible plasmid that has been designated as the TOL (pWWO) plasmid (27, 46–48, 50). Several other TOL plasmids that are isofunctionally identical with pWWO have been detected in other strains of *Pseudomonas* (12, 28, 47).

The metabolic pathways for the degradation of toluene, *m*-xylene, *p*-xylene, pseudocumene, and 3-ethyltoluene have been studied in some detail (7, 27, 28, 50), and the results are summarized in Fig. 1. The enzymes encoded by TOL plasmids have a relaxed specificity which accounts for the observation that a single organism can grow with five different aromatic hydrocarbon substrates. The initial oxidative reaction occurs at a methyl substituent which is then oxidized further to form the appropriate aromatic acid (Fig. 1). In contrast, *P. putida* F1, which can grow with toluene but not the other TOL substrates, initiates degradation by incorporating one molecule of oxygen into the aromatic nucleus to form (+)-*cis*-1(*S*),2(*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol; 16). This dihydrodiol pathway for the metabolism of aromatic hydrocarbons is quite different from the pathway specified by TOL. In addition, *P. putida* F1 does not contain any detectable plasmids, and the genes responsible for the degradation of toluene and related compounds appear to be located on the chromosome (B. A. Finette, Ph.D. dissertation, the University of Texas at Austin, 1984).

The structures of the intermediates (diol carboxylic acids) involved in the conversion of aromatic acids to catechols (Fig. 1) have not been determined for an organism that contains a TOL plasmid. Diol carboxylic acids were proposed as intermediates in the degradation of *p*- and *m*-xylene by *Pseudomonas* Pxy, which contains a nonconjugative TOL-type plasmid (6, 12). This suggestion was based on the identification of (−)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (benzoate-1,2-diol) as an intermediate in the conversion of benzoate to catechol by a mutant strain of *Alcaligenes eutrophus* (40, 41). Later studies by Knackmuss and his colleagues have shown that *A. eutrophus* oxidizes a variety of halogenated and methyl-substituted benzoic acids to *cis*-diol carboxylic acids (39). These and subsequent investigations with *Pseudomonas* sp. strain B13, which can grow with 3-chlorobenzoate, revealed that the benzoate-1,2-dioxygenases in these organisms have a narrow substrate specificity and do not show significant oxidation of 4-substituted benzoates in which the substituent is larger than fluorine (37, 39). In contrast, *P. putida* mt-2, which contains the TOL plasmid (pWWO), has a broad substrate specificity and oxidizes a variety of 3- and 4-substituted benzoates. Certain disubstituted benzoates, including 3,4-dimethylbenzoate which is an intermediate in the degradation of pseudocumene, were also oxidized by this organism (37). The transfer of the TOL plasmid to *Pseudomonas* sp. strain B13 led to the isolation of an exconjugant that would grow with 4-chloro- and 3,5-dichlorobenzoate (38). Recently, DNA fragments containing the *xylD* and *xylL* genes (Fig. 1) from the TOL plasmid pWWO-161 were cloned in *Pseudomonas* sp. strain B13. The cloned *xylD* gene permitted growth with 4-chlorobenzoate, whereas both cloned genes were required for growth with 3,5-dichlorobenzoate. In addition, *Escherichia coli* K-12 strains containing hybrid plasmids bearing the *xylD* gene from pWWO were shown to oxidize benzoate to benzoate-1,2-diol. Cell extracts from

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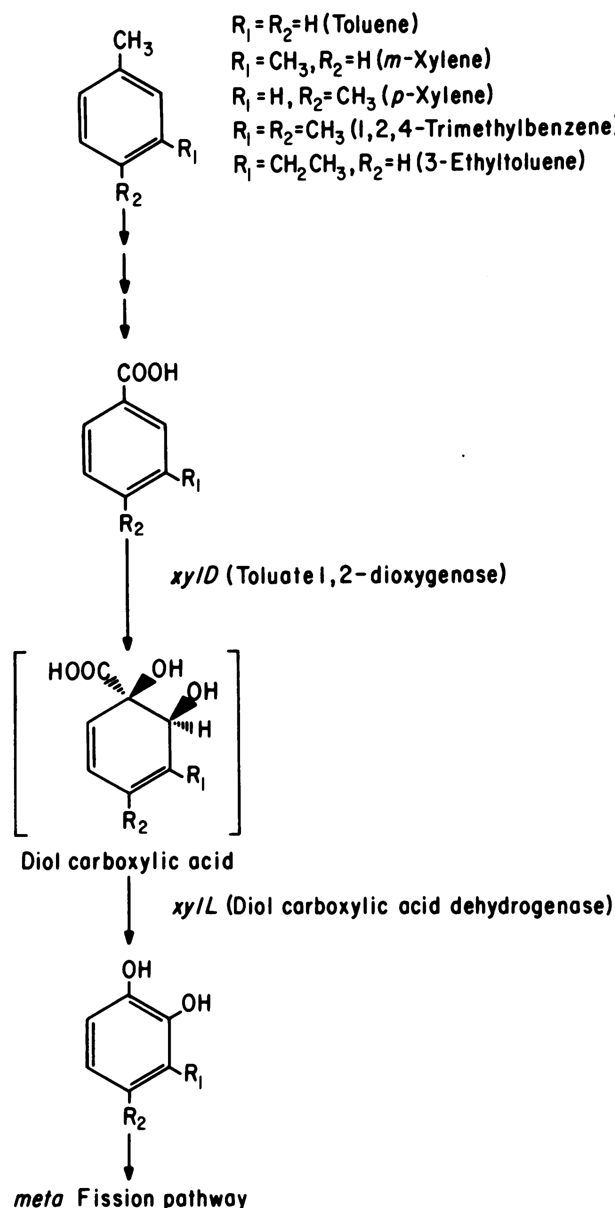

FIG. 1. Pathway for the metabolism of toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, and 3-ethyltoluene by bacteria that contain TOL plasmids. *xylD* and *xylL* refer to the genes encoding toluate dioxygenase and *cis*-toluate diol dehydrogenase, respectively. A *cis*-relative stereochemistry is assigned to the proposed diol carboxylic acid intermediates; absolute stereochemistry is not intended.

strains containing *xylD* and *xylL* were shown to catalyze an NAD^+-dependent oxidation of a mixture of 3- and 5-chlorobenzoate-1,2-diols (32).

The above observations provide strong evidence for the formation of diol carboxylic acids as intermediates in the degradation of aromatic hydrocarbons by enzymes encoded by TOL plasmids. We now report the isolation and identification of (−)-*cis*-1,2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylic acid (*cis*-*p*-toluate diol) as an intermediate in the conversion of *p*-toluate to 4-methylcatechol by a strain of *Pseudomonas* that contains a TOL plasmid. Preliminary evidence is also presented for the formation of diol carboxylic acids and catechols from benzoate, *m*-toluate, 3,4-dimethylbenzoate, and 3-ethyltoluene.

(A partial summary of these results was presented at the 84th Annual Meeting of the American Society for Microbiology, [G. M. Whited and D. T. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K52, p. 155].)

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated: silica gel (Kieselgel 60) for column chromatography and plastic-backed silica gel $60F_{254}$ sheets for thin-layer chromatography (TLC) from EM Reagents, Darmstadt, Federal Republic of Germany; Gene Screen hybridization transfer membranes from New England Nuclear Corp., Boston, Mass.; nick translation kit and Mini-Spin columns from Cooper Biomedical Inc., West Chester, Pa.; restriction endonucleases, lambda DNA, and 1.0-kilobase (kb) ladder size standards from Bethesda Research Laboratories, Gaithersburg, Md.; [^{32}P]dCTP (specific activity, 3,000 Ci/mmol) from ICN Inc., Irvine, Calif.; DEAE-cellulose (Whatman DE-52) from Whatman Ltd., Maidstone, Kent, England; Red A affinity resin dye matrix from Amicon Corp., Lexington, Mass.; Sephadex G-150, NAD^+, $NADP^+$, DNase I, and proteins used as molecular weight standards (glucose-6-phosphate dehydrogenase, conalbumin, bovine serum albumin, carbonic anhydrase, chymotrypsin, and cytochrome *c*) from Sigma Chemical Co., St. Louis, Mo.; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, 2,2-dimethoxypropane, and diazomethane (prepared from Diazold according to the manufacturer's instructions) from Aldrich Chemical Co., Milwaukee, Wis. *cis*-Toluene dihydrodiol, *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol), and 2,3-dihydroxy-1-ethylbenzene were prepared as previously described (15, 16, 25). All other chemicals used were of the highest purity commerically available.

Analytical methods. Solvents used for TLC were benzene-ethanol (9:1) (solvent A), chloroform-acetone (8:2) (solvent B), and chloroform-acetone (7:3) (solvent C). Compounds were located on chromatograms by viewing under UV light and, in some cases, by spraying the chromatogram with a 2.0% solution of 2,6-dichloroquinone-4-chloroimide in methanol (Gibbs reagent). High-pressure liquid chromatography was conducted with a Beckman model 112 chromatograph equipped with a model 421 gradient controller and a Spectra Physics 4270 integrating recorder. A DuPont Zorbax 5-μm octyldecylsilane column (6.2 mm by 25 cm) was used to separate *p*-cresol and 4-methylsalicylate. The solvent was water-acetonitrile (7:3) containing 1.0% acetic acid. 4-Methylcatechol was purified on a Beckman 5-μm octyldecylsilane column (4.6 mm by 25 cm) using a solvent of water-methanol (MeOH) with a gradient of 70 to 60% water. Absorption spectra were recorded on a Beckman model 25, Cary model 14, or Aminco model DW-2 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer. Optical rotations were measured with a Perkin-Elmer model 241C polarimeter. Mass spectra were recorded on a DuPont model 21-110C double-focusing, high-resolution spectrometer or on a DuPont model 21-491 double-focusing, low-resolution spectrometer. Proton magnetic resonance (PMR) spectra were recorded on either a Varian model EM-390 or a Nicolet

model NT-200 spectrometer. All spectra for which chemical shifts are reported were recorded at 200 MHz. Oxygen consumption was measured polarographically at 30°C with a Clark oxygen electrode. Assays were conducted in 50 mM KH_2PO_4 buffer (pH 7.2); substrates were dissolved in buffer or dimethylformamide as appropriate and supplied as saturating conditions. Results reported are corrected for endogenous oxygen consumption in the absence of substrate.

Organisms and growth conditions. *P. putida* BG1 was isolated from motor oil-saturated soil from a salvage yard in Austin, Texas, by enrichment culture in mineral salts medium (MSB; 44) with *p*-toluate (0.1%) as the original sole source of carbon and energy. After two transfers on *p*-toluate medium, cells were transferred to fresh medium and grown with *p*-xylene for 24 h. The aromatic hydrocarbon was supplied in the vapor phase as previously described (14). Serial dilutions were plated on MSB agar (2.0%) with *p*-xylene as the sole carbon source, and a single colony was purified by restreaking on the same medium. The isolated organism was identified as a strain of *P. putida* (45) and given the designation BG1. *P. putida* mt-2 (ATCC 33015) was obtained from the American Type Culture Collection. A cured derivative of *P. putida* mt-2 (strain PPO208, TOL^- Trp^-) was provided by R. H. Olsen, University of Michigan, Ann Arbor. *P. putida* F1 is the organism described by Gibson et al. (17). A mutant strain of this organism, PpFTM29 (Km^r *todABCDE*), was generated by transposon (Tn5) mutagenesis (Finette, dissertation). All organisms were stored at −70°C in Luria (L) broth medium containing (per liter) 10 g of tryptone, 5.0 g of yeast extract, 10 g of NaCl, and 25% glycerol.

Organisms were grown in MSB liquid medium or on MSB agar plates. Water-soluble substrates were added to the medium at 0.1% (wt/vol), and hydrocarbons were supplied in the vapor phase as described above. Small cultures were grown in Erlenmeyer flasks (five times the culture volume) at 30°C. Aeration was provided by placing the flasks on a rotary shaker operating at 175 rpm. Cells were harvested in the mid- to late-log phase, washed twice with 0.05 M sodium phosphate buffer (pH 7.2), and used immediately. Large quantities of cells used in enzyme purification studies were grown with forced aeration in 12-liter cultures in a New Brunswick Microferm fermentor. Cells were harvested by centrifugation in a Sharples air-driven centrifuge and were frozen at −20°C until used.

Conjugation experiments. Transfer of the plasmid (pDTG501) from *P. putida* BG1 to a cured strain (PPO208) of *P. putida* mt-2 and a Tn5 mutant (PpFTM29) of *P. putida* F1 was performed as follows. A histidine auxotroph of *P. putida* BG1 (strain PpBG14) and a tryptophan auxotroph of *P. putida* mt-2 (strain PPO208) were grown overnight on L broth. Each culture was diluted 50:1 in fresh L broth and incubated until both cultures reached the mid-logarithmic phase of growth. Equal volumes of donor and recipient cells were mixed and filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). The filter was incubated overnight on an L-agar plate at 30°C. Cells were removed from the filter with 2.0 ml of sterile saline, and after appropriate dilutions transconjugants were selected on MSB agar containing tryptophan (0.005%). The growth substrate, *p*-xylene, was provided in the vapor phase. When *P. putida* FTM29 was the recipient, transconjugants were selected on MSB agar containing kanamycin (50 μg/ml). No colonies were observed when donor and recipient cells were grown on L agar and plated separately on the selective medium.

Plasmid isolation and analysis. Plasmid DNA was isolated and purified by centrifugation in CsCl-ethidium bromide by the method of Hansen and Olsen (18). Restriction endonuclease analyses were performed as described previously (34). The sizes of DNA fragments were determined by reference to the electrophoretic mobilities of *Hin*dIII fragments of lambda and a 1.0-kb ladder size standard.

Purified plasmid DNA was labeled with ^{32}P by the nick-translation procedure of Rigby et al. (42). The enzymes and reagents for nick translation were used according to the supplier's instructions with the exception that [^{32}P]dCTP was diluted with dCTP to a level required for the activity of DNA polymerase I (34). Labeled DNA was separated from unincorporated [^{32}P]dCTP by the use of Mini-Spin columns according to the supplier's recommendations.

Southern hybridization analyses. Restriction endonuclease fragments of plasmid DNA were separated by agarose gel electrophoresis and hybridized with ^{32}P-labeled pDTG501 by a modification of the procedure described by Southern (43). The modification involved the use of Gene Screen hybridization transfer membranes which were used according to the manufacturer's instructions (New England Nuclear Manual, NEF 972). Autoradiograms were obtained by using Kodak X-Omat RP film at −70°C. Exposure and development times were varied to optimize resolution of bands on the positive hybridization control.

Mutagenesis. *P. putida* BG1 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 μg/ml) as previously described for *P. putida* F1 (9). Small white colonies that did not reduce the indicator dyes Nitro Blue Tetrazolium and 2,3,5-triphenyl-2H-tetrazolium chloride were selected as putative mutants and examined for their ability to grow with *p*-xylene and *p*-toluate.

Enzyme assays. Catechol-2,3-dioxygenase activity was determined by measuring the formation of 2-hydroxymuconic semialdehyde at 375 nm. When 3- and 4-methylcatechols were used as substrates, enzyme activity was measured by the increase in absorbance at 388 and 382 nm, respectively (13). Reaction mixtures contained 0.34 μmol of substrate and appropriate amounts of the enzyme in a final volume of 1.0 ml of 50 mM KH_2PO_4 buffer (pH 7.5). Reactions were initiated by the addition of substrate, and the increase in absorbance at the appropriate wavelength was measured at ambient temperature on a Beckman model 25 recording spectrophotometer. The reference cuvette contained all assay components except substrate. *cis-p*-Toluate diol dehydrogenase activity was measured by following the formation of NADH at 340 nm on a Beckman model 25 recording spectrophotometer. Reaction mixtures contained, in a final volume of 1.0 ml of 50 mM Tris hydrochloride buffer (pH 8.1), 2.0 μmol of NAD^+, 0.4 μmol of *cis-p*-toluate diol, and appropriate amounts of partially purified *cis-p*-toluate diol dehydrogenase. The reaction was initiated by the addition of *cis-p*-toluate diol, and the increase in absorbance at 340 nm was measured against a reference cuvette which contained all components except the diol substrate. One unit of *cis-p*-toluate diol dehydrogenase was defined as the amount of enzyme required to reduce 1.0 μmol of NAD^+ per min. Protein concentrations in cell suspensions were measured by the method of Lowry et al. (33), and those in cell extracts were measured by the method of Bradford (5), using bovine serum albumin as a standard.

Partial purification of *cis-p*-toluate diol dehydrogenase and catechol-2,3-dioxygenase. All purification procedures were performed at 4°C. Frozen *p*-xylene-grown cells of *P. putida* BG1 were thawed and suspended in 50 mM Tris hydrochloride buffer (pH 8.0) (Tris buffer). The ratio of cells (wet

weight) to buffer was 1:3. Cells were broken in a French pressure cell at 10,000 lb/in^2, and the resulting mixture was treated with DNase I (100 μg/ml) for 20 min. Whole cells were removed by centrifugation at 10,000 × *g* for 20 min, and crude cell extract was prepared by centrifugation at 100,000 × *g* for 1 h. The clear cell extract (195 ml; 6.2 g of protein) was applied to the top of a column (2.7 by 25 cm) of DEAE-cellulose which had been previously equilibrated with Tris buffer. The column was washed with eight column volumes of Tris buffer and then eluted with a 1,600-ml gradient of 0.0 to 0.3 M NaCl in Tris buffer. Fractions (8.0 ml) were collected and assayed for the presence of *cis-p*-toluate diol dehydrogenase and catechol-2,3-dioxygenase. These enzymes eluted together in fractions 133 through 190, which were pooled and dialyzed overnight against Tris buffer. The dialyzed extract was applied to the top of a column (2.0 by 10 cm) of Red A affinity resin which had previously been equilibrated with Tris buffer. The column was washed with Tris buffer until protein could not be detected in the eluate. Catechol-2,3-dioxygenase was not retained by the Red A column. The protein in the column washings was heat treated at 50°C for 2 min, resulting in an extract which oxidized catechols without the further metabolism of the ring fission products (4).

Protein bound to the red dye column described above was eluted with 900 ml of a 0.0 to 0.4 M KCl gradient in Tris buffer. Fractions (8.0 ml) were collected, and *cis-p*-toluate diol dehydrogenase activity was located in fractions 64 through 100, which were pooled, dialyzed overnight against Tris buffer, and then concentrated approximately 10-fold by ultrafiltration. Although the dehydrogenase preparation was not homogeneous, it was devoid of catechol-2,3-dioxygenase activity and catalyzed the dehydrogenation of the carboxylic acid diols described in the Results section. The molecular weight of *cis-p*-toluate diol dehydrogenase was calculated by the gel filtration technique of Andrews (1), using a calibrated column of Sephadex G-150.

Enzymatic oxidation of carboxylic acid diols to catechols. Purified *cis-p*-toluate diol was oxidized in a reaction mixture containing 400 ml of 50 mM Tris hydrochloride buffer (pH 8.1) 750 μmol of NAD^+, 400 μmol of *cis-p*-toluate diol, and 25 U of partially purified *cis-p*-toluate diol dehydrogenase. After 30 min at room temperature, the reaction mixture was extracted twice with equal volumes of diethyl ether. The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed to leave a brown oil which contained 282 μmol of 4-methylcatechol (ϵ_{max}^{MeOH} = 2,400). Further purification by silica gel chromatography gave a pure sample of 4-methylcatechol which was identified by TLC (R_f = 0.37, solvent C), high-pressure liquid chromatography, and PMR spectroscopy.

Crude preparations of carboxylic acid diols formed from benzoate, *m*-toluate, 3-ethylbenzoate, and 3,4-dimethylbenzoate were treated in a similar manner. The reaction mixtures contained, in 50 ml of 50 mM Tris buffer (pH 8.1), 150 μmol of NAD^+, 10 U of partially purified *cis-p*-toluate diol dehydrogenase, and diol to bring the absorbance to 5.0 at 265 nm. After 30 min, the reactions were extracted twice with equal volumes of diethyl ether. The organic phases were dried over anhydrous sodium sulfate, and the solvent was removed to leave oils containing crude catechols which were either analyzed by TLC in solvent C or used for further transformations. Enzymatic cleavage of the crude catechols was carried out in 1.0-ml cuvettes containing crude catechol, (A_{280}, 0.25), 50 mM KH_2PO_4 buffer (pH 7.5), and 2.0 U of partially purified catechol-2,3-dioxygenase.

RESULTS

Isolation of organism. *P. putida* BG1 was isolated by selective enrichment with *p*-toluate as the sole source of carbon and energy. Final isolation was achieved with *p*-xylene as the growth substrate. This procedure was utilized to isolate an organism with properties similar to those reported to contain TOL plasmids (47).

Evidence for the presence of a TOL plasmid. The compounds that serve as growth substrates for *P. putida* BG1 and two strains of *P. putida* known to contain TOL plasmids, mt-2 (27, 46, 50) and HS1 (27), are as follows: toluene, benzyl alcohol, benzaldehyde, benzoate, *m*-xylene, *m*-benzyl alcohol, *m*-tolualdehyde, *m*-toluate, *p*-xylene, *p*-methylbenzyl alcohol, *p*-tolualdehyde, *p*-toluate, 1,2,4-trimethylbenzene, 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoate, 3-ethyltoluene and 3-ethylbenzoate. Substrates that did not support the growth of *P. putida* BG1 were benzene, *o*-xylene, *o*-cresol, *m*-cresol, *p*-cresol, 4-ethyltoluene, ethylbenzene, propylbenzene, naphthalene, and biphenyl.

Washed cells of *P. putida* BG1 prepared after growth with toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, or 3-ethyltoluene oxidized each hydrocarbon substrate and the corresponding carboxylic acids formed by methyl group oxidation. In contrast, cells obtained after growth with benzoate, *m*-toluate, *p*-toluate, 3,4-dimethylbenzoate, or 3-ethylbenzoate oxidized each acid growth substrate but not the corresponding aromatic hydrocarbons (Table 1). These induction patterns are similar to those reported for *P. putida* mt-2, which contains the TOL plasmid pWWO (27, 36, 46, 49). In addition, comparable *meta* ring fission activities for catechol and 3- and 4-methylcatechol were observed when *P. putida* BG1 and *P. putida* mt-2 were grown with *p*-toluate (data not shown).

It is characteristic of *P. putida* mt-2 that growth with benzoate leads to the isolation of strains that have been cured of the TOL plasmid pWWO (2, 28, 46). In five separate experiments, *P. putida* BG1 was grown with benzoate. After five passages on the same medium, cells were plated on indicator media as described in Materials and Methods. These experiments revealed than an average of 4.6% of the cells recovered had lost the ability to grow with *p*-xylene.

The TOL phenotype could be transferred by conjugation to a cured strain of *P. putida* mt-2 and to a Tn*5* mutant of *P. putida* F1 (Table 2). Transconjugants which expressed the TOL phenotype were grown overnight on benzoate-mineral salts medium and plated on L agar. Two hundred colonies each of strains PPO208(pDTG501) and PpFTM29(pDTG501) were examined for their ability to grow with *p*-xylene. The results showed that 93% of the cells from the PpFTM29 transconjugants and 32% of the cells from the PPO208 transconjugants had lost the ability to grow with *p*-xylene.

The evidence described above is characteristic of organisms that contain TOL plasmids (27, 35, 46, 49). Agarose gel electrophoresis of purified plasmid preparations from *P. putida* BG1 revealed the presence of a large plasmid (pDTG501) with a molecular size greater than that reported for the archetype TOL plasmid pWWO. Restriction endonuclease analysis of pDTG501 with the enzymes *Eco*RI and *Bam*HI revealed the presence of many fragments (Fig. 2). Owing to the large number of bands and the apparent presence of several bands with identical electrophoretic properties it was not possible to determine the precise size of pDTG501. Summation of the fragment sizes given by *Eco*RI and *Bam*HI cleavage gave an approximate value of 325 kb.

TABLE 1. Oxidation of aromatic compounds by cells of *P. putida* BG1 after growth with TOL substrates[a]

Growth substrate	Oxygen consumption (nmol/min per mg of protein)									
	Toluene	*m*-Xylene	*p*-Xylene	TMB	3-ET	Benzoate	*m*-Toluate	*p*-Toluate	DMB	3-EB
Toluene	149	338	272	263	242	470	408	242	154	263
m-Xylene	221	374	387	421	339	400	400	234	76	117
p-Xylene	448	849	917	819	691	571	458	330	172	256
TMB	146	154	206	158	162	87	79	95	75	67
3-ET	293	391	376	293	341	278	348	216	160	209
Benzoate	14	42	5	9	<1	508	304	194	161	55
m-Toluate	<1	<1	8	<1	<1	189	255	107	107	86
p-Toluate	<1	<1	<1	<1	<1	284	246	195	161	59
DMB	<1	<1	6	9	<1	182	185	134	116	60
3-EB	<1	<1	<1	<1	<1	595	662	521	521	384
Fructose	18	44	26	18	22	7	22	<1	26	16

[a] Cells were grown in 50-ml cultures as described in Materials and Methods. Substrate abbreviations: TMB, 1,2,4-trimethylbenzene; 3-ET, 3-ethyltoluene; DMB, 3,4-dimethylbenzoate; 3-EB, 3-ethylbenzoate.

Several TOL plasmids have been shown to contain significant regions of homology to the archetype TOL plasmid pWWO (20). Southern hybridization analyses showed that this was also true for pDTG501. The plasmid pWWO was cleaved with *Eco*RI and *Hin*dIII, and the resulting fragments were separated by agarose gel electrophoresis (Fig. 3A). The fragments hybridizing with ^{32}P-labeled pDTG501 DNA are shown in Fig. 3B. A summary of these results is given in Fig. 4, which shows the standard restriction map of pWWO. The *Eco*RI and *Hin*dIII fragments that were found to be homologous to pDTG501 are indicated. The results show that there is considerable homology between pWWO and pDTG501 and that homology occurs in the regions of pWWO known to contain the genes responsible for hydrocarbon degradation.

Isolation of a mutant strain of *P. putida* BG1 that accumulates carboxylic acid diols. Carboxylic acid diols are presumed to be intermediates in the conversion of TOL substrates to the corresponding catechols (see Fig. 1). In an attempt to isolate mutants that accumulate the putative diol intermediates, *P. putida* BG1 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and *p*-toluate-negative colonies were selected for further examination. One mutant, when grown with fructose in the presence of *p*-xylene or *p*-toluate, accumulated an acid product (compound I) which had an absorption maximum at 266 nm. This organism, designated *P. putida* BGXM1, grew very slowly with the substrates listed in Materials and Methods. However, it oxidized *p*-toluate to compound I at a relatively rapid rate. Under the conditions described in the legend of Fig. 5, an 87% yield of compound I was obtained in 33 h. In one experiment in which *p*-toluate was added incrementally to a 1.0-liter culture, 21 mmol of compound I was formed from 44 mmol of *p*-toluate over a 100-h period. Compound I was extremely unstable; this problem was overcome by isolating it in crystalline form as the sodium salt, $[\alpha]_D^{25} = -144°$ (93.2 mM, water). Crystals of the free acid, mp 65 to 66°C (decomposition), $[\alpha]_D^{25} = -132°$ (79.2 mM, MeOH), were obtained as required by acidification of aqueous solutions of the sodium salt followed by rapid extraction with cold ether.

Characterization of compound I. Aqueous solutions of the sodium salt of compound I showed an absorption maximum at 266 nm (ϵ, 3,050 M^{-1} cm^{-1}). Upon acidification with 6 N HCl, the absorption at 266 nm decreased and a new product that absorbed maximally at 278 nm was formed. When the reaction was repeated on a larger scale, ethyl acetate extraction of the acidified reaction mixture gave a single product whose TLC properties (R_f = 0.51, solvent B) and infrared, mass, and PMR spectra were identical to those given by a synthetic sample of *p*-cresol. In addition, the PMR spectrum of compound I (sodium salt in D_2O) was changed to a spectrum indistinguishable from that of authentic *p*-cresol by the addition of P_2O_5 to the PMR sample. Phosphorus pentoxide is converted to deuterated phosphoric acid under these conditions and thus catalyzes the dehydration of compound I (Fig. 6).

Solutions of the free acid of compound I in methanol also

TABLE 2. Growth properties of *P. putida* strains

Growth substrate	Growth[a] of strain:					
	PpBG1 (pDTG501)	PpBG5[b]	PPO208[c]	PPO208 (pDTG501)	PpFTM29[d]	PpFTM29 (pDTG501)
Toluene	+	−	−	+	−	+
Benzoate	+	+	+	+	+	+
m-Xylene	+	−	−	+	−	+
m-Toluate	+	−	−	+	−	+
p-Xylene	+	−	−	+	−	+
p-Toluate	+	−	−	+	−	+
1,2,4-Trimethylbenzene	+	−	−	+	−	+
3,4-Dimethylbenzoate	+	−	−	+	−	+
3-Ethyltoluene	+	−	−	+	−	+
3-Ethylbenzoate	+	−	−	+	−	+

[a] +, Growth; −, no growth.
[b] Derivative of *P. putida* BG1 obtained after growth with benzoate.
[c] Cured strain of *P. putida* mt-2 (pWWO⁻ His⁻).
[d] Transposon mutant of *P. putida* F1 (Km^r *todABCDE*).

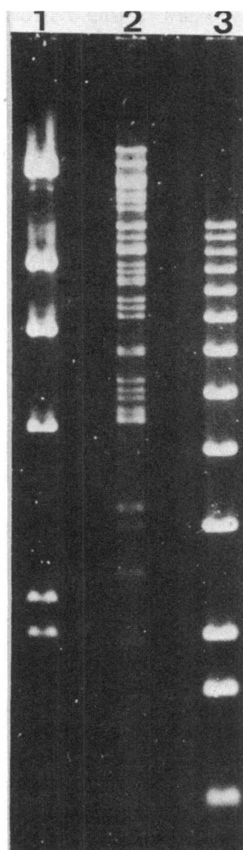

FIG. 2. Restriction endonuclease digest analysis of pDTG501 plasmid DNA. Plasmid pDTG501 was cleaved with *Bam*HI (lane 2) and subjected to agarose gel electrophoresis in a 0.5% agarose gel. The gel was then stained with ethidium bromide and photographed. Lane 1 contains lambda DNA cleaved with *Hin*dIII. Lane 3 contains 1.0-kb ladder as a size standard.

absorbed maximally at 266 nm (ε, 3,750 M^{-1} cm^{-1}). Acidification of this solution resulted in an absorption spectrum that showed maxima at 279 nm and 307 nm (Fig. 7). Analysis of the reaction products by high-pressure liquid chromatography (see Materials and Methods) revealed the presence of two compounds whose retention times were identical to those given by authentic *p*-cresol (9.2 min) and 4-methylsalicylate (12.6 min). The latter compound was isolated from a large-scale reaction mixture and shown to be identical to 4-methylsalicylate in terms of its melting point and absorption, infrared, PMR, and mass spectra. Small amounts of compounds that appeared to be esters were also detected in the large-scale reaction mixture, although their identification and mode of formation were not further investigated.

An analysis of the PMR spectrum of compound I (sodium salt in D_2O) showed bands at: δ, 1.66, 3H (methyl group); 5.35, 1H (m); 5.66, 1H (d, J = 10 Hz); 5.91, 1H (dd, J = 10 Hz). The spectrum of compound I (free acid, pyridine-d_5) showed bands at: δ, 1.69, 3H (methyl); 5.61, 1H (m, hydroxymethine); 5.92, 1H (m, vinylic); 6.05, 1H (d, J = 10 Hz); 6.37, 1H (d, J = 10 Hz) and 9.44, 3H (bs, hydroxyl and carboxyl protons). Attempts to resolve the signal at δ 9.44 by low-temperature spectroscopy or by using dimethyl sulfoxide to disrupt possible hydrogen bonding were unsuccessful.

Further evidence for the structure of compound I was obtained by reaction with diazomethane to form a methyl ester derivative. This derivative was obtained as a colorless oil and had the following physical properties: λ_{max}^{MeOH}, 266 nm (ε, 3,250 M^{-1} cm^{-1}); λ_{max}^{oil}, 2.92 (OH), 3.42 (CH_3), 5.80 (C=O) and 6.10 (C=C)μ; $[\alpha]_D^{25}$ = −121° (76.3 mM, MeOH); calculated mass for $^{12}C_9{}^1H_{12}{}^{16}O_4$, 184.07355, found mass 184.07397. The PMR spectrum of the methyl ester in $CDCl_3$ gave bands at: δ, 1.82, 3H (s, methyl); 2.94, 2H (bs, OH); 3.86, 3H (s, methyl); 4.76, 1H (m, hydroxymethine); 5.50, 1H (m, vinylic); 5.76, 1H (d, J = 10 Hz) and 6.01, 1H (d, J = 10 Hz). The upfield shift observed for the hydroxyl protons may be due to the change in solvent or to elimination of hydrogen bonding due to ester formation, or both.

These results confirm that the structure of compound I is (−)-1,2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylic acid (*p*-toluate diol). Evidence for a *cis* configuration of the hydroxyl groups in the metabolite was obtained by reacting the methyl ester with 2,2-dimethoxypropane to form an isopropylidene derivative. This reaction product was obtained as a colorless oil. Its physical properties were as follows: λ_{max}^{MeOH}, 267 nm (ε, 4,250 M^{-1} cm^{-1}); λ_{max}^{oil}, 3.35 (CH_3), 5.78 (C=O), 6.03 (C=C), and 8.00 (C—O)μ; calculated mass for $^{12}C_{12}{}^1H_{16}{}^{16}O_4$, 224.10485, found mass 224.10422. The PMR spectrum of the isopropylidene derivative in $CDCl_3$ showed bands at: δ, 1.42, 3H (s, methyl); 1.44, 3H (s, methyl); 1.88, 3H (s, methyl); 3.78, 3H (s, ester methyl); 4.89, 1H (m, methine); 5.74, 1H (m, vinylic); 5.78, 1H (d, vinylic, J = 10 Hz), and 5.95, 1H (dd, vinylic, J = 10 Hz). These results establish that the hydroxyl groups in *p*-toluate diol have a *cis*-relative stereochemistry. The structure of this compound is shown in Fig. 8.

Enzymatic oxidation of *cis-p*-toluate diol. *P. putida* strains BG1 and mt-2, when grown with *p*-toluate, contain an inducible *cis-p*-toluate diol dehydrogenase (Table 3). As

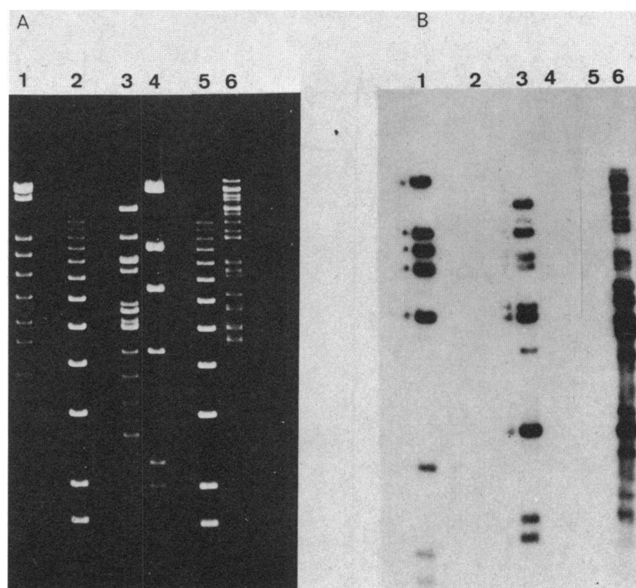

FIG. 3. DNA homology between plasmids pDTG501 and pWWO. Plasmid DNA was cleaved with restriction endonucleases, and fragments were separated by electrophoresis in a 0.5% agarose gel (A). Restriction fragments were hybridized with ^{32}P-labeled pDTG501 and analyzed by radioautography (B). Lane contents: lanes 1 and 3, pWWO cleaved with *Hin*dIII and *Eco*RI respectively; lanes 2 and 5, 1.0-kb ladder size standards; lane 4, lambda DNA cleaved with *Hin*dIII; lane 6, pDTG501 DNA cleaved with *Eco*RI.

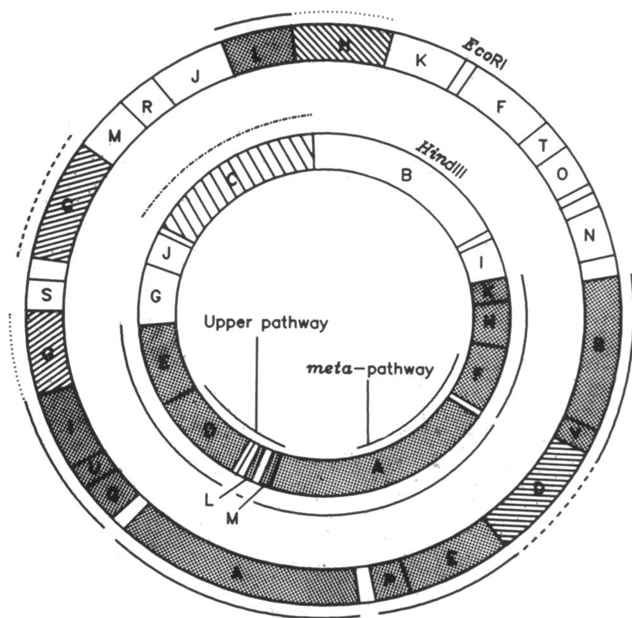

FIG. 4. Summary of hybridizations of ^{32}P-labeled pDTG501 with pWWO. *Eco*RI and *Hin*dIII restriction maps of pWWO are shown based on references 7, 11, 29, and 31. Localization of pWWO functions is shown by outlines on the inside of the circle and is based on previous work (10, 21, 24, 29, 36). Fragments filled in with dots and outlined with a solid line are fragments homologous to pDTG501. Fragments with dense cross-hatching represent pairs of pWWO *Eco*RI fragments that comigrate; one or both of the fragments are homologous with pDTG501. These fragment pairs are C and D (outlined with dashed line) and G and H (outlined with dotted line). Weak hybridization was observed with *Hin*dIII fragment C, shown with broad cross-hatching and a dot-dash outline pattern. Unlabeled areas represent fragments that were below the minimum size visualized on our gels and autoradiographs.

described previously, each of these strains contains a TOL plasmid. The enzyme was purified 38-fold from induced cells of *P. putida* BG1 (Materials and Methods). An apparent molecular weight of 96,000 was determined by gel filtration chromatography. The enzyme utilized NAD^+ but not $NADP^+$ as an electron acceptor and catalyzed the stoichiometric reduction of NAD^+ in the presence of *cis-p*-toluate diol. No activity was observed when *cis*-toluene dihydrodiol, *cis*-naphthalene dihydrodiol, or the methyl ester of *cis-p*-toluate diol was used as a substrate.

The reaction product formed from *cis-p*-toluate diol by the partially purified dehydrogenase was isolated in pure form (Materials and Methods) and shown to have chromatographic properties (TLC and high-pressure liquid chromatography) and absorption and PMR spectra identical to those given by a synthetic sample of 4-methylcatechol.

Oxidation of aromatic acids by *P. putida* BGXM1. Growth of *P. putida* BGXM1 in the presence of aromatic acids known to be growth substrates and inducers of enzymes encoded by TOL plasmids led to the accumulation of putative diol carboxylic acids. The absorption spectra of the metabolites formed from benzoate, *m*-toluate, *p*-toluate, 3-ethylbenzoate, and 3,4-dimethylbenzoate under neutral and acidic conditions are shown in Fig. 9. Each metabolite showed significant spectral changes in the presence of acid, and the reaction products were identified by TLC (Table 4).

Oxidation of diol intermediates by partially purified *cis-p*-toluate diol dehydrogenase. The carboxylic acid diols listed in Table 4 were incubated with a partially purified preparation of *cis-p*-toluate diol dehydrogenase and NAD^+ as described in Materials and Methods. The reaction products were isolated and analyzed by TLC in solvent C. The results showed that catechols were formed from each carboxylic acid diol. The tentative identification of each catechol was determined by enzymatic ring cleavage to *meta*-fission products which were characterized by their absorption spectra under acid and alkaline conditions (Table 5).

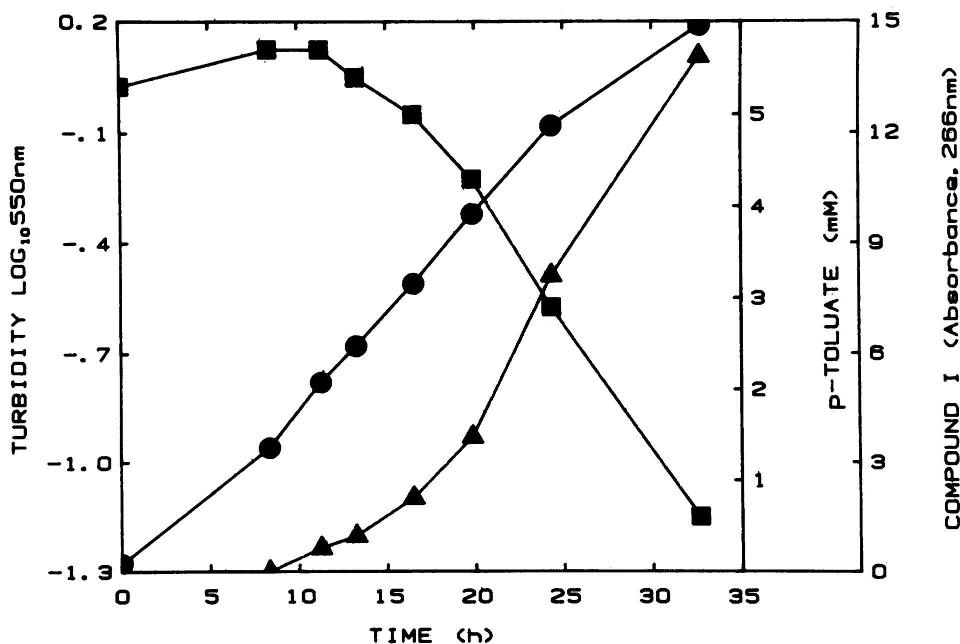

FIG. 5. Oxidation of *p*-toluate to compound I by strain PpBGXM1. A 1.0-liter culture was grown with aeration in a 2.8-liter Fernbach flask. The MSB medium contained fructose (10 mM) and *p*-toluate (0.1%). Samples were taken at the time intervals indicated and monitored for growth (●), *p*-toluate concentration (■), and formation of compound I (▲).

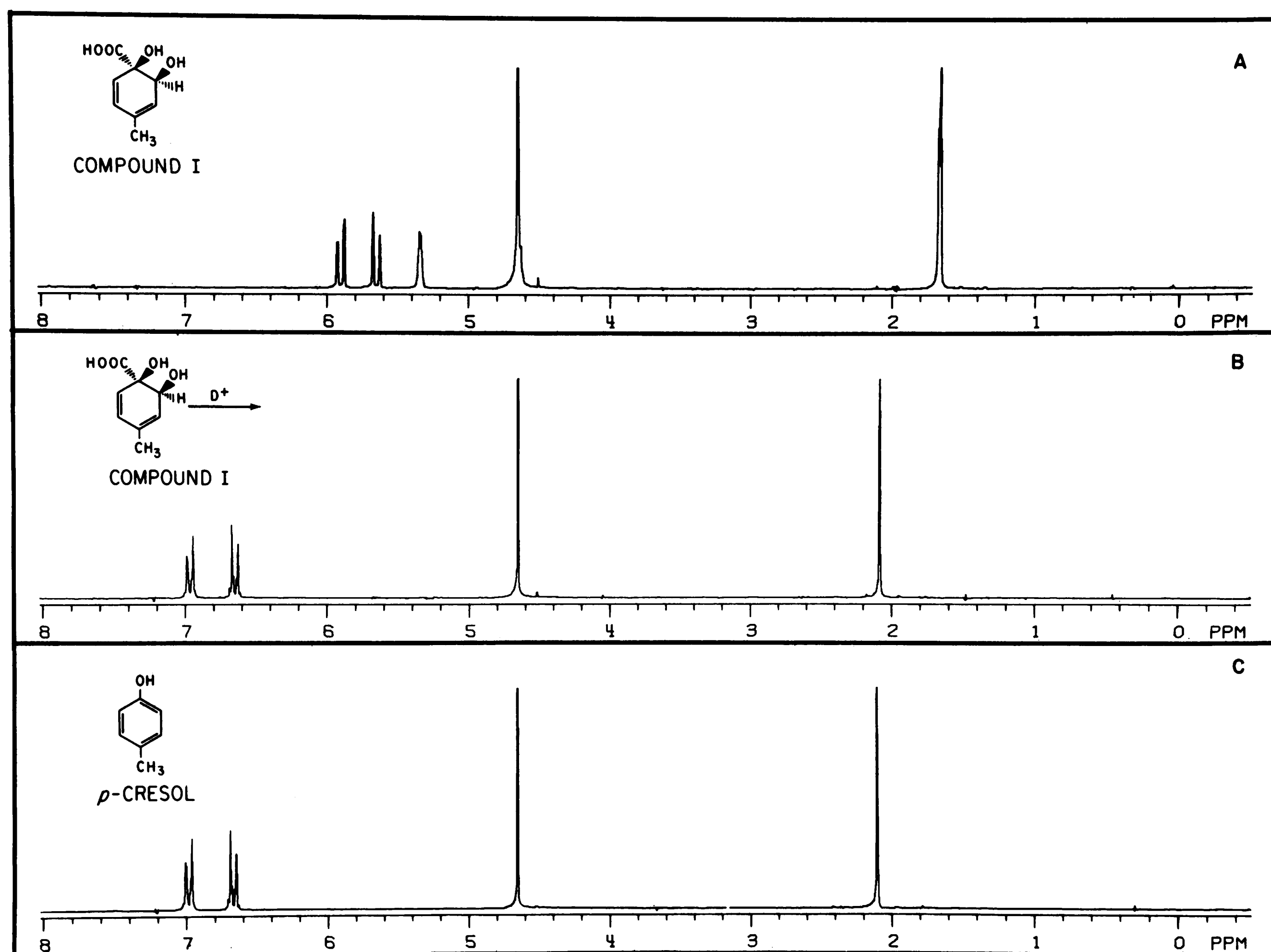

FIG. 6. PMR spectra of compound I (sodium salt) in D_2O before (A) and after (B) the addition of P_2O_5. Authentic *p*-cresol gave the spectrum shown in panel C. All spectra were recorded at 200 MHz in D_2O. The structure shown for compound I was determined by several different methods which are described in the text.

DISCUSSION

Enrichment culture with *p*-toluate as the sole carbon source and selection for growth with *p*-xylene led to the isolation of *P. putida* BG1, which exhibited phenotypic properties identical to those reported for organisms that contain TOL plasmids (22, 23, 28, 46–48). These properties included growth with toluene, *m*-xylene, *p*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene and their corresponding aromatic acids formed by oxidation of a methyl group; separate regulation of aromatic hydrocarbon and aromatic acid degradation (Table 1); and *meta* fission of catechol intermediates.

P. putida BG1 contains a large plasmid (pDTG501) which was detected by agarose gel electrophoresis. Restriction endonuclease analyses gave a molecular size of approximately 325 kb, which is considerably larger than the 117 kb reported for the TOL plasmid (pWWO) from *P. putida* mt-2 (8). However, TOL plasmids ranging in size from about 40 to 300 kb have been isolated from different strains of *Pseudomonas* (8, 47).

Attempts to cure *P. putida* BG1 of its plasmid by rigorous benzoate selection (Materials and Methods) gave a low yield of putative TOL^- strains. Several of these isolates which had lost the entire Tol phenotype contained a large plasmid with approximately the same molecular size as pDTG501 (data not shown). Studies are being conducted to see whether this is due to deletion of a small DNA fragment as has been reported for pWWO (2).

Plasmid pDTG501 was transferred by conjugation to a cured strain of *P. putida* mt-2 and a Tn*5* mutant of *P. putida* F1. All transconjugants isolated showed the same growth properties as *P. putida* BG1 and *P. putida* mt-2. When the transconjugants were grown with benzoate, significant curing mass was observed as determined by loss of the Tol phenotype.

The results discussed above show that pDTG501 is similar to previously described TOL plasmids with the exception of its large size and the failure of growth with benzoate to lead to the isolation of cured strains. Southern hybridization experiments revealed considerable homology between the archetype TOL plasmid pWWO and pDTG501 (Fig. 3). Similar extensive homology between the NAH and SAL plasmids has also been reported (20). The homology observed between pWWO and pDTG501 appears to be in the regions responsible for the Tol phenotype (Fig. 4), although limited homology was also observed in regions of pWWO that have been implicated in replication and transfer (10).

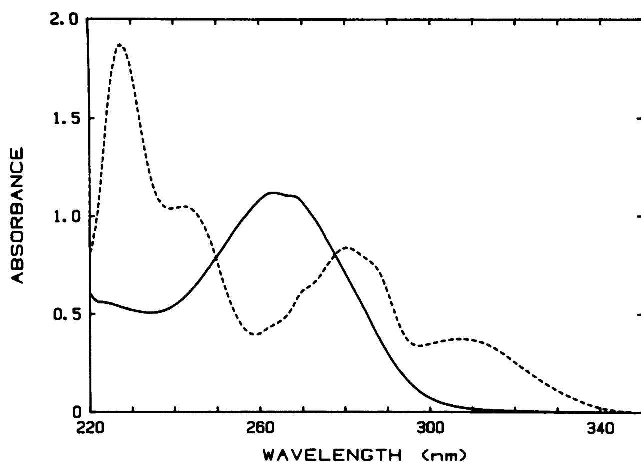

FIG. 7. Absorption spectrum of compound I (free acid) in methanol before (——) and after (– – –) acidification. The cuvette contained a 300 μM solution of compound I in methanol and was acidified with 20 μl of 12 N HCl. Spectra were recorded on an Aminco DW-2 recording spectrophotometer.

The *Eco*RI F fragment of pWWO has been suggested to contain a "core" of IncP9 replication functions (3, 30). Plasmid pDTG501 does not appear to share this homology, a feature that has also been reported for some other TOL plasmids (20).

The TOL plasmid designation is based primarily on the biochemical properties encoded by the plasmid, which include the metabolic pathways utilized for the degradation of toluene and *m*- and *p*-xylene, the presence of two operons which encode the enzymes for the upper and lower pathways, and the use of *meta* fission for the degradation of catechol intermediates. Benzoate curing has also been used to implicate the involvement of TOL plasmids in the degradation of toluene, *m*- and *p*-xylene, and their corresponding acid derivatives. However, the mechanism of plasmid curing by growth on benzoate is not fully understood (26). Thus DNA homology and phenotypic properties may be more useful in the classification of TOL plasmids. By these criteria pDTG501 is a TOL plasmid, and this designation would not change even if subsequent analyses showed that the molecular structure of phenotypically cured derivatives of pDTG501 was different from that of cured pWWO derivatives. This is a reasonable expectation based on the different "curing" event seen in some reported TOL plasmids (2, 47, 51).

Organisms which contain TOL plasmids, *P. putida* strains mt-2 (46) and HS1 (27) and *Pseudomonas* Pxy (12), oxidize benzoates to the corresponding catechols during growth, presumably through carboxylic acid diol intermediates.

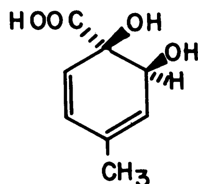

FIG. 8. Compound I, (−)-*cis*-1,2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylic acid (*cis*-*p*-toluate diol; absolute stereochemistry is not intended).

TABLE 3. *cis*-*p*-Toluate diol dehydrogenase activity in *P. putida* strains that contain TOL plasmids[a]

Strain	Plasmid	Carbon source	Sp act[b]
BG1	pDTG501	Glucose	0.001
		p-Toluate	0.067
mt-2	pWWO	Glucose	0.016
		p-Toluate	0.353

[a] Each strain of *P. putida* was grown on MSB medium containing 0.1% (wt/vol) of the indicated carbon source. Cell extracts were prepared and enzyme activity was determined as described in Materials and Methods.
[b] Micromoles of NADH formed per minute per milligram of protein.

Davey and Gibson (6) suggested that carboxylic acid diols were intermediates in the conversion of *m*- and *p*-toluate to 3- and 4-methylcatechol, respectively, by *Pseudomonas* Pxy. This suggestion was based on analogous reactions reported for the oxidation of benzoate to catechol by *A. eutrophus* (40, 41). Figure 10 shows the pathway used by *P. putida* BG1 for the oxidation of *p*-toluate to 4-methylcatechol with the derivatives used to identify *cis*-*p*-toluate diol.

The structural and physical characteristics of (−)-*cis*-1,2-dihydroxy-4-methyl-cyclohexa-3,5-diene-1-carboxylic acid

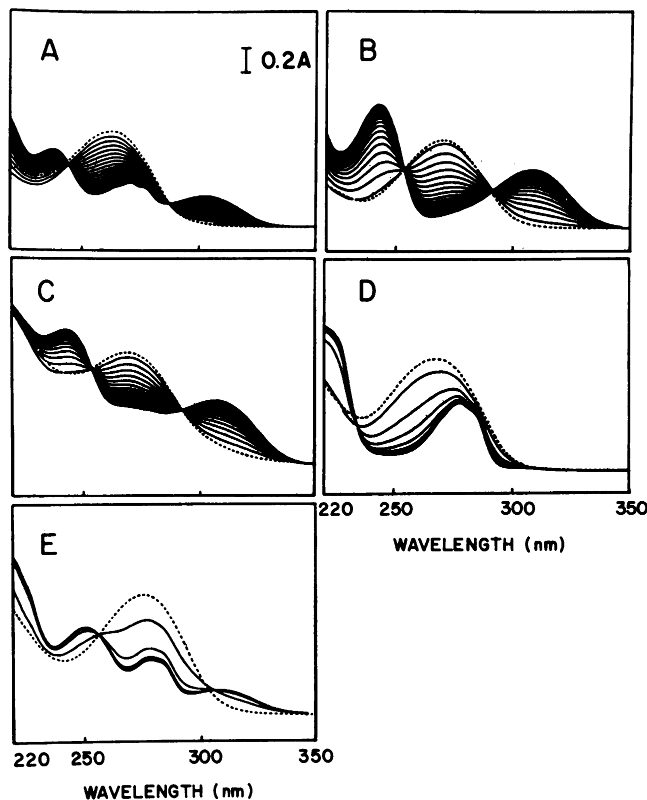

FIG. 9. Spectral changes observed during the acid-catalyzed dehydration of diols formed from aromatic acids by *P. putida* BGXM1. Crude salts of the diols formed from benzoate (A), *m*-toluate (B), 3-ethylbenzoate (C), *p*-toluate (D), and 3,4-dimethylbenzoate (E) were diluted to 1.0 ml with distilled water, and the absorption spectra were recorded on an Aminco DW-2 recording spectrophotometer (---). Spectral changes were recorded (——) at 71-s (B through E) and 284-s (A) intervals after the addition of 20 μl of 12 N HCl to each cuvette.

(*cis-p*-toluate diol) are similar to those reported by Reineke et al. (39) for a series of *cis*-1,2-diol-3,5-cyclohexadiene carboxylic acids formed from the analogous halogen- and methyl-substituted benzoic acids by *A. eutrophus* B9. However, *para*-substituted benzoic acids were poor substrates for the *A. eutrophus* dioxygenase. Consequently, not enough material was obtained for the detailed analyses which were reported for the *meta*- and unsubstituted compounds.

Acid treatment of *cis-p*-toluate diol in water led to the formation of *p*-cresol. This same treatment of the diol formed from benzoate by *P. putida* BGXM1 resulted in the formation of both phenol and salicylate, as Reiner and Hegeman (41) have reported for (−)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid formed by *A. eutrophus* B9. It is of interest that *P. putida* BGXM1 accumulated a carboxylic acid diol from benzoate. Since strains of *P. putida* BG1 which have lost the Tol phenotype still grow with benzoate, it appears that strain BGXM1 may be a double mutant in which the plasmid and chromosomal diol carboxylic acid dehydrogenase are inactive. Davey and Gibson (6) reported the accumulation of 3-methylsalicylate from a mutant of *Pseudomonas* Pxy when grown in the presence of *m*-xylene. In addition, Kunz and Chapman (27) reported the accumulation of 3-ethylsalicylate from cultures of a *P. putida* mt-2 mutant grown in the presence of 3-ethyltoluene. In the present study, acid treatment of the diols formed by *P. putida* BGXM1 from *m*-toluate and 3-ethyltoluene resulted in the formation of 3-methylsalicylate and 3-ethylsalicylate, respectively, as major products (Table 4). It is of interest that these products were not metabolized by the respective organisms.

P. putida strains BG1 and mt-2, when grown with *p*-toluate, were shown to contain an inducible NAD^+-dependent dehydrogenase which oxidizes *cis-p*-toluate diol to 4-methylcatechol (Table 3). In *P. putida* BG1 this reaction is catalyzed by a 96,000-dalton protein which requires NAD^+ as an electron acceptor. Preliminary investigations indicate that the carboxylic acid functional group is required for activity as the methyl ester of *cis-p*-toluate diol, *cis*-toluene dihydrodiol, and *cis*-naphthalene dihydrodiol were not oxidized by partially purified *cis-p*-toluate diol dehydrogenase prepared from cells grown with *p*-xylene.

The genes encoding both the toluate dioxygenase and the diol dehydrogenase from the archetype TOL plasmid pWWO have been cloned (22), and the location of the genes

TABLE 4. Products formed from carboxylic acid diols after treatment with acid[a]

Substrate	Diol[b]	Reaction Products[c]
Benzoate	HOOC, OH, OH, H	Phenol Salicylate
m-Toluate	HOOC, OH, OH, H, CH_3	3-Methylsalicylate (major) *m*-Cresol[d] (minor)
p-Toluate	HOOC, OH, OH, H, CH_3	*p*-Cresol
3-Ethylbenzoate	HOOC, OH, OH, H, CH_2CH_3	3-Ethylsalicylate (major) 3-Ethylphenol[d] (minor)
3,4 Dimethylbenzoate	HOOC, OH, OH, H, CH_3, CH_3	3,4-Dimethylphenol 3,4-Dimethylsalicylate[d]

[a] Crude salts of the putative *cis*-diols which accumulated after growth with the substrate indicated were diluted in 20 ml of water to an approximate absorbance of 20 at 265 nm. Dehydration was initiated with 0.5 ml of 12 N HCl, and the reaction was allowed to proceed overnight at room temperature.

[b] Proposed structures of *cis*-diols (absolute stereochemistry not intended).

[c] Products were extracted with ether and analyzed by TLC in solvent A. Each reaction product gave R_f values and colorimetric reactions with Gibbs reagent identical to those given by authentic compounds.

[d] Identified by R_f only.

TABLE 5. Products formed from carboxylic acid diols after treatment with *cis-p*-toluate diol dehydrogenase and catechol-2,3-dioxygenase[a]

Diol[b]	Diol dehydrogenase product[c]	λ_{max} of catechol 2,3-dioxygenase product[d]	
		Acid	Alkaline
HOOC, OH, OH, H	OH, OH	316	376
HOOC, OH, OH, H, CH_3	OH, OH, CH_3	314	388
HOOC, OH, OH, H, CH_3	OH, OH, CH_3	322	382
HOOC, OH, OH, H, CH_2CH_3	OH, OH, CH_2CH_3	314	392
HOOC, OH, OH, H, CH_3, CH_3	OH, OH, CH_3, CH_3	314	393

[a] *cis-p*-Toluate diol dehydrogenase and catechol-2,3-dioxygenase were partially purified as described in Materials and Methods.

[b] Proposed structure of *cis*-diols formed from analogous aromatic acids.

[c] Proposed structure of catechols formed from *cis*-diol intermediates.

[d] Reported λ_{max} values at acid (2.0) and alkaline (12.0) pHs were almost identical to those reported for ring fission products formed from catechol (4), 3-methylcatechol (4), 4-methylcatechol (4), 3-ethylcatechol (27), and 3,4-dimethylcatechol (27).

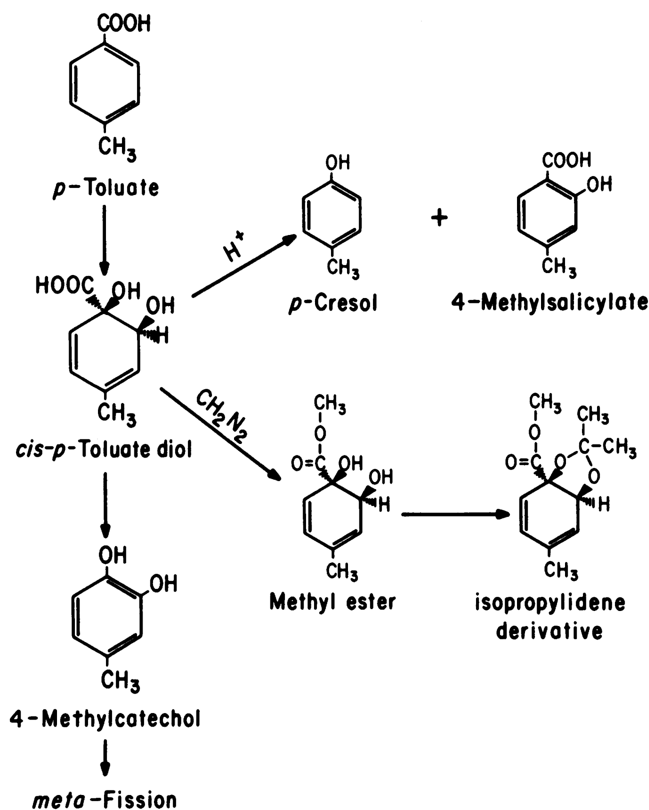

FIG. 10. Pathway proposed for the oxidation of *p*-toluate to 4-methylcatechol. The derivatives used to identify *cis-p*-toluate diol are also shown. It should be noted that *p*-cresol is the only product detected when aqueous solutions of *cis-p*-toluate diol are acidified. Acidification of methanolic solutions of *cis-p*-toluate diol leads to the formation of *p*-cresol and 4-methylsalicylate.

has been mapped (19). In addition, these genes have been transferred to other pseudomonads, by conjugal transfer of pWWO or by introduction of the cloned genes, to extend the catabolic diversity of aromatic substrates which were novel for these bacteria (24, 32, 38). Growth of *P. putida* BGXM1 in the presence of each of the TOL substrates led to the accumulation of diol carboxylic acids which are expected intermediates in the degradation of the respective hydrocarbon substrates. Evidence that these diols were intermediates was demonstrated by the sequential conversion of each of them to catechols and further oxidation to *meta* ring fission products by partially purified enzymes prepared from cells grown on *p*-xylene. These reactions are consistent with other reports on the metabolism of aromatic acids by organisms that contain TOL plasmids (6, 27, 46).

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