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Catabolism of Pseudocumene and 3-Ethyltoluene by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for New Functions of the TOL (pWWO) Plasmid

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Pseudocumene (1,2,4-trimethylbenzene) and 3-ethyltoluene were found to serve as growth substrates for *Pseudomonas putida* (*arvilla*) mt-2, in addition to toluene, *m*-xylene, and *p*-xylene as previously described. Similar observations were made with several additional *P. putida* strains also capable of growth with toluene and the xylenes. Additional substrates which supported the growth of these organisms included 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoate, and 3-ethylbenzoate. *P. putida* mt-2 cells grown either with toluene or pseudocumene rapidly oxidized toluene, pseudocumene, and 3-ethyltoluene as well as 3,4-dimethylbenzoate, 3-ethylbenzoate, 3,4-dimethylcatechol, and 3-ethylcatechol. Cell extracts from similarly grown *P. putida* mt-2 cells catalyzed a *meta* fission of 3,4-dimethylcatechol and 3-ethylcatechol to compounds having the spectral properties of 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate and 2-hydroxy-6-oxo-2,4-octadienoate, respectively. The further metabolism of these intermediates was shown to be independent of oxidized nicotinamide adenine dinucleotide (NAD^+) and resulted in the formation of essentially equimolar amounts of pyruvate, indicating that each ring fission product was degraded via the hydrolytic branch of the *meta* fission pathway. Treatment of cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to the isolation of a mutant, which when grown with succinate in the presence of pseudocumene or 3-ethyltoluene accumulated 3,4-dimethylcatechol or 3-ethylcatechol. Cells unable to utilize toluene, *m*-xylene, and *p*-xylene, obtained by growth in benzoate, also lost the ability to utilize pseudocumene and 3-ethyltoluene. The ability to utilize these substrates could be reacquired by incubation with a leucine auxotroph otherwise able to grow on all of the aromatic substrates.

Bacteria which grow with *m*- and *p*-xylene have been shown to oxidize these substrates via the corresponding alcohols, aldehydes, and toluic acids (9, 10, 30-32). Toluene may also be catabolized by the same route, resulting in the formation of benzoate as an intermediate (27). Davey and Gibson (9) showed that cell extracts prepared from *Pseudomonas* sp. strain Pxy cells grown with either *m*- or *p*-xylene oxidized the *m*- and *p*-isomers of tolualdehyde as well as *p*-methylbenzyl alcohol. These workers also described the isolation and identification of 3- and 4-methylcatechol as intermediates in the degradation of *m*- and *p*-xylene and showed that cell extracts catalyzed a *meta* fission of both (9).

Pseudomonas putida (*arvilla*) mt-2 metabolizes toluene, *m*-, and *p*-xylene via similar routes, involving oxidation of a methyl substituent, ring dioxygenation, and reactions of the *meta* fission pathway (44). The genes responsible for the degradation of toluene and *m*- and *p*-xylene and their oxidation products in *P. putida* mt-2 cells have been shown to reside on a transmissible plasmid, referred to as the TOL (pWWO) plasmid (42, 44, 46). Williams and Worsey (43) showed that other pseudomonads capable of utilizing toluene and the xylenes also carried plasmids which were isofunctional with the original TOL plasmid described from *P. putida* mt-2. *Pseudomonas* sp. strain Pxy was also reported to carry a plasmid isofunctional with TOL (pWWO). This plasmid, however, was shown to be nonconjugative and was originally designated the XYL plasmid, distinguishing it from the conjugative plasmid in *P. putida* mt-2 (11).

During the course of an investigation on the range of aromatic hydrocarbons which might serve as growth substrates for pseudomonads, we discovered that *P. putida* mt-2 could utilize

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pseudocumene (1,2,4-trimethylbenzene) and 3-ethyltoluene in addition to toluene and *m*- and *p*-xylene as previously reported. Similar observations were made with several additional *P. putida* strains including *Pseudomonas* sp. strain Pxy. The pathways by which pseudocumene and 3-ethyltoluene are degraded have not been established. Omori and Yamada (31, 32) suggested that pseudocumene was metabolized via 3,4-dimethylbenzoic acid and 3,4-dimethylphenol since *Pseudomonas aeruginosa* S668B2 cells accumulated these products after growth with pseudocumene or 3,4-dimethylbenzoic acid. Although 3,4-dimethylphenol was proposed as an intermediate in the oxidation of pseudocumene, this metabolite was not oxidized by *P. aeruginosa* S668B2 cells (32). Jamison et al. (19) showed that *Nocardia corallina* cells grown on *n*-hexadecane cooxidized pseudocumene to 3,4-dimethylbenzoic acid, although this organism was unable to utilize pseudocumene for growth. Jigami et al. (20) reported that a strain described as *Pseudomonas ovalis* S1B1 could grow with 3-ethyltoluene in addition to *m*- and *p*-xylene and oxidized 3-ethyltoluene to 3-ethylsalicylic acid. They did not speculate, however, on the role of this metabolite in the degradation of 3-ethyltoluene.

The ability of *P. putida* mt-2 and several other strains to grow with pseudocumene and 3-ethyltoluene prompted us to investigate further the biochemical pathways by which these two hydrocarbons are degraded. In this communication, we describe the pathways by which pseudocumene and 3-ethyltoluene are metabolized by *P. putida* mt-2 and provide evidence that the ability to degrade these substrates is encoded by the TOL (pWWO) plasmid. A preliminary account of some of this work has been reported (D. A. Kunz, P. C. Janes, and P. J. Chapman. Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, O13, p. 199).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. putida* (*arvilla*) mt-2 (PaM1) was kindly provided by G. D. Hegeman, Department of Microbiology, Indiana University. The wild-type and mutant strains of *P. putida* mt-2 used in this study have been given PaM (*P. arvilla*, Minnesota) designations to distinguish them from strains used by Williams and co-workers in previous studies (42, 44, 45), in which derivatives of *P. putida* mt-2 were given PaW (*P. arvilla*, Wales) designations. It should be pointed out, however, that the source of *P. putida* mt-2 used in Wales (PaW1) was the same as for our wild-type strain. The auxotrophic strain, PaW15, was generously provided by P. A. Williams, University College of North Wales, Bangor, United Kingdom, as was an additional culture of the wild-type (PaW1) strain for comparative purposes. *Pseudomonas* sp. strain Pxy was a kind gift of D. T. Gibson, Department of Microbiology, University of Texas. Other strains were obtained in this laboratory (Table 1).

Bacteria were maintained at −16°C in a (1:1 [vol/vol]) 0.1 M Na_2HPO_4-KH_2PO_4 buffer-glycerol mixture. The minimal medium employed was of the following composition (grams per liter): KH_2PO_4, 4.25; NaH_2PO_4, 1.0; NH_4Cl, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.012; $MnSO_4 \cdot H_2O$, 0.003; $ZnSO_4 \cdot 7H_2O$, 0.003; $CoSO_4$, 0.001; and nitrilotriacetic acid, 0.01. Growth surveys were conducted on minimal agar (2%) plates with water-soluble substrates supplied at 0.05% (wt/

TABLE 1. *P. putida* strains

Strain designation	Phenotype[a]	Genotype[b]	Parent strain	Method of production or reference[c]
PaM1 (*P. putida* mt-2, Minnesota)	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$	Wild type/TOL (pWWO)		
PaM2	Tln$^-$ Xyl$^-$ Psc$^-$ Etn$^-$ Ben$^+$ Tal$^-$	TOL^{del}	PaM1	SN
PaM3	Tln$^-$ Xyl$^-$ Psc$^-$ Etn$^-$ Ben$^+$ Tal$^-$	TOL^{del}	PaM1	SB
PaM5	Tln$^+$ Xyl$^-$ Psc$^-$ Etn$^-$ Ben$^+$ Tal$^-$	TOL-XylE	PaM1	NG
PaM300	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$	Wild type/TOL (pWWO)	PaW15 × PaM3	Conjugation
PaW1 (*P. putida* mt-2, Wales)	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$	Wild type/TOL		
PaW15	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$ Leu$^-$	*leu-1*/TOL (pWWO)	PaW1	NG
HS1	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$			*m*-Toluate enrichment
ST3	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$			*m*-Toluate enrichment
Pxy	Tln$^+$ Xyl$^+$ Psc$^+$ Etn$^+$ Ben$^+$ Tal$^+$			(11)

[a] Phenotype abbreviations: Tln, toluene; Xyl, *m*- and *p*-xylene; Psc, pseudocumene; Etn, 3-ethyltoluene; Ben, benzoate; Tal, *m*- and *p*-toluate, 3,4-dimethylbenzoate, 3-ethylbenzoate; leu, leucine.

[b] Genotype abbreviations: pWWO, TOL plasmid from *P. putida* mt-2; TOL^{del}, plasmid deletion; TOL-XylE, denotes catechol-2,3-oxygenase defective after notations of Williams et al. (45).

[c] SN, Spontaneous selection on nutrient broth; SB, spontaneous selection on benzoate; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis.

vol). Where liquid hydrocarbons and aldehydes served as growth substrates, these were supplied in the vapor form by placing a narrow glass tube filled with the volatile compound in the lid of a petri dish as described by Gibson (12).

For oxidation studies and enzyme assays, cells were grown at 30°C on agar plates. These cultures were harvested after 24 h for toluene-grown cells or after 48 h for pseudocumene-grown cells. Succinate-grown cells were obtained from 1-liter batch cultures containing 0.05% succinate and incubated at 30°C on a rotary shaker for 14 to 16 h. Cell crops were harvested by centrifugation at 20,000 × *g* at 4°C for 10 to 15 min, and washed once with 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0). Cells not used immediately for enzyme analysis were stored at −16°C before use.

Isolation of mutants. Mutants unable to utilize pseudocumene or 3-ethyltoluene were isolated after nitrosoguanidine mutagenesis and enrichment with penicillin G (Sigma Chemical Co., St. Louis, Mo.) and D-cycloserine (Nutritional Biochemicals Corp., Cleveland, Ohio) as follows. A 50-ml overnight culture of *P. putida* mt-2 grown with 0.05% succinate was harvested and washed once with 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0). These cells were then suspended in 50 ml of 0.1 M sodium citrate buffer (pH 5.5) containing 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml and incubated for 1 h at 30°C on a rotary shaker. Cells were then removed by centrifugation and washed with 0.1 M sodium citrate buffer (pH 5.5), followed by an additional washing with minimal medium. Washed cells were then suspended in 50 ml of minimal medium containing 0.05% succinate and allowed to grow for 12.5 h. After the cells were harvested and washed once with minimal medium, they were grown on 0.1% *m*-toluate in 50 ml of medium at 30°C with shaking for 8 h before 10^4 U of penicillin G per ml and 0.2 mg of D-cycloserine per ml were added. After a 9-h enrichment to allow cell lysis, the cells were harvested, washed twice with minimal medium, and plated. Selection of appropriately blocked mutants was achieved by plating cells on minimal medium containing 3,4-dimethylbenzoate as the principal carbon source (0.05%) with succinate present at 0.005%. Small colonies which produced a brown color in the surrounding medium could be detected and were purified and classified as putative mutants defective in the degradation of 3,4-dimethylbenzoate.

Measurement of oxidation rates. Oxidation rates were measured by oxygen consumption both manometrically with constant volume manometers (Braun, Melsungen, Germany) and polarographically with a Gilson oxygen electrode (Gilson Medical Electronics, Middleton, Wis.) at 30°C.

Preparation of cell extracts and enzyme assays. Cell-free extracts were prepared by passing 1 to 3 g of a frozen cell paste through a Hughes press (19) at 10,000 lb/in^2 and suspending the broken-cell paste in 0.05 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) containing 10% acetone. DNase (1 mg) (Sigma Chemical Co.) was added to reduce the viscosity, and the suspension was centrifuged at 20,000 × *g* for 30 min at 4°C to give a supernatant referred to as crude extract.

Catechol-2,3-oxygenase (EC 1.13.11.2; catechol:oxygen 2,3-oxidoreductase) was assayed either spectrophotometrically or polarographically with a Gilson O_2 electrode (28). The spectrophotometric assay consisted of measuring the rates of formation of the respective ring fission products of catechol (375 nm; ϵ = 33,000 M^{-1}) (2), 3-methylcatechol (388 nm; ϵ = 13,400 M^{-1}) (2), 4-methylcatechol (382 nm; ϵ = 28,100 M^{-1}) (2), 3-ethylcatechol (390 nm; ϵ = 10,900 M^{-1}), and 3,4-dimethylcatechol (320 nm; ϵ = 13,200 M^{-1}) in a 3-ml reaction mixture which contained a 0.3 mM concentration of the respective catechol, 50 mM KH_2PO_4 buffer (pH 7.5) and cell extract. The polarographic assay was carried out by measuring the rate of O_2 consumption at 30°C in a 1.5-ml reaction mixture which contained 0.3 mM catechol or alkyl-substituted catechol, 50 mM KH_2PO_4 (pH 7.5), and cell extract. To measure catechol-2,3-oxygenase in cell extracts which also contained catechol-1,2-oxygenase, extracts were heated at 55°C for 10 min, as described by Murray and Williams (24), to destroy catechol-1,2-oxygenase, which could otherwise interfere with the assay. This procedure destroyed 90 to 95% of the catechol-1,2-oxygenase activity without affecting the catechol-2,3-oxygenase activity.

The activity of *meta* ring fission substrate-metabolizing enzymes was determined by measuring the rate of decrease in absorbance at the λ_{max} of the respective ring fission products of catechol (2-hydroxymuconic semialdehyde), 3-methylcatechol (2-hydroxy-6-oxo-2,4-heptadienoate), 4-methylcatechol (2-hydroxy-5-methylmuconic semialdehyde), 3-ethylcatechol (2-hydroxy-6-oxo-2,4-octadienoate), and 3,4-dimethylcatechol (2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate). The 3-ml reaction mixtures contained: a 0.03 mM concentration of the respective ring fission product prepared by incubating the respective catechol in a cuvette with 5 to 10 μl of heat-treated (55°C for 10 min) *P. putida* mt-2 or *Pseudomonas* sp. strain U cell extract grown on phenol (2). Heat-treated cell extracts from either of these organisms retained catechol-2,3-oxygenase activity but had lost the activities of ring fission-degrading enzymes. When rates of disappearance of ring fission products derived from catechol and 4-methylcatechol were measured, cell extracts were treated with NAD^+ nucleosidase (NADase) (2.7 U/ml, Sigma Chemical Co.) at room temperature to destroy endogenous NAD^+ which might otherwise affect measurements of hydrolase activity made in its absence (23, 40). 2-Hydroxymuconic semialdehyde dehydrogenase was assayed by following the disappearance of 2-hydroxymuconic semialdehyde in a 3-ml reaction mixture which contained 0.03 mM 2-hydroxymuconic semialdehyde, 0.33 mM NAD^+, 50 mM KH_2PO_4 buffer (pH 7.5), and untreated cell extract. The activity of 2-hydroxymuconic semialdehyde dehydrogenase measured in the presence of NAD^+ was corrected for 2-hydroxymuconic semialdehyde hydrolase activity measured in its absence (23, 40).

Catechol-1,2-oxygenase (EC 1.13.11.1; catechol:oxygen 1,2-oxidoreductase) was measured by following the formation of *cis,cis*-muconate at 260 nm (16), and *cis,cis*-muconate-lactonizing enzyme was assayed by following the disappearance of *cis,cis*-muconate as described by Ornston (33). To measure catechol-1,2-oxygenase in extracts containing high levels of catechol-2,3-oxygenase, cell extracts were first treated with

50 mM H_2O_2 for 10 min as described by Nakazawa and Yokota (25), which destroyed from 90 to 95% of the catechol-2,3-oxygenase activity.

All spectrophotometric assays were carried out at room temperature in a Hitachi, Perkin-Elmer model 124 spectrophotometer. Specific activity was expressed as that amount of activity required to convert 1 nmol of substrate per min per mg of protein. Protein determinations were conducted by the method of Lowry et al. (22), with bovine serum albumin (Sigma Chemical Co.) as a standard.

Pyruvate was formed by incubating cell extracts with the respective catechol for 60 min at room temperature in a 3-ml reaction mixture containing 50 mM KH_2PO_4, pH 7.5. The amount of pyruvate formed was determined from the decrease in absorbance at 340 nm when reduced NAD^+ was oxidized upon addition of lactate dehydrogenase (Sigma Chemical Co.) (41).

Production, detection, and isolation of metabolic intermediates. PaM5 was grown overnight at 30°C with 0.05% succinate. Five milliliters of the cell suspension was then used to inoculate a 2-liter Erlenmeyer flask containing 500 ml of minimal medium with 0.05% succinate and aromatic hydrocarbon supplied as the vapor, as described by Gibson (12). After a 24-h incubation on a rotary shaker at 30°C, the cells were removed by centrifugation, and the supernatant was extracted with 3 volumes of diethyl ether, which removed neutral products, or alternatively, the supernatant was first acidified with 6 N H_2SO_4 before extraction to remove acidic products. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure at 30°C. The residue was then analyzed by thin-layer chromatography by using thin-layer silica gel plates (Eastman chromatogram sheets, type 130R, silica gel fluorescent indicator). The solvents used for chromatography were petroleum ether-acetone-acetic acid (75:25:1 [vol/vol], solvent A) and chloroform-acetone (80:20 [vol/vol], solvent B). Compounds were located on chromatograms by viewing under UV light or by spraying with 2,6-dibromoquinone-4-chloroimide (2% in methanol, Gibb reagent) followed by 5% $NaHCO_3$.

To produce a compound in sufficient quantities for isolation, a series of cultures were incubated, and the supernatant was pooled and extracted. After removal of solvent, the products were purified by preparative thin-layer chromatography or vacuum sublimation or both. Identification of metabolic products was accomplished by melting-point determination, UV and infrared spectrophotometry, gas chromatography-mass spectrometry, and nuclear magnetic resonance spectroscopy. Samples for infrared spectrophotometry were prepared in KBr pellets (2%, wt/wt), and the spectra were recorded with a Beckman Acculab spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin-Elmer R12B instrument with samples dissolved in $CDCl_3$ (5%) containing 10% tetramethylsilane as an internal standard. Preparation of samples and analysis by gas chromatography-mass spectrometry was conducted as previously described (41).

Plasmid curing and conjugation experiments. Cured *P. putida* mt-2 strains were isolated as spontaneous mutants after growth in nutrient broth or with 0.05% benzoate as described by Williams and Worsey (43). After incubation, cells were diluted and spread onto plates containing *m*-toluate (0.05%) and succinate (0.005%). Colonies selected for their inability to form large colonies with *m*-toluate as a sole carbon source were isolated and subsequently replicated to agar plates supplied with aromatic substrates which normally supported growth of the wild-type strain. Clones no longer able to utilize those hydrocarbons, alcohols, aldehydes, and acids which served as carbon sources for the wild type were assumed to be cured of the TOL (pWWO) plasmid.

Conjugation experiments were performed by mixing 0.75 ml of the donor and 0.75 ml of the recipient which had been grown overnight in nutrient broth to a cell density of 10^8 to 10^9 cells per ml. Control tubes containing 0.75 ml of the donor or recipient and 0.75 ml of minimal medium were incubated simultaneously. After a 90-min incubation at 30°C, the mating mixtures and controls were vigorously blended in a Vortex mixer, quickly diluted, and plated to selection plates which were incubated for 2 to 5 days at 30°C. No fewer than 25 exconjugants were picked and twice purified on selection plates before their ability to utilize aromatic substrates was assessed. Selection for exconjugants was achieved on minimal plates containing *m*-toluate as the selective carbon source and lacking amino acids to select against an auxotrophic donor.

Chemicals. 3-Ethylbenzoic acid (melting point, 47°C) was prepared by carboxylation of the Grignard derivative of 3-bromo-1-ethylbenzene, which was obtained from 3-ethylaniline by a Sandmeyer reaction on its diazonium salt. 3,4-Dimethylbenzyl alcohol was synthesized by the reduction of the ethyl ester of 3,4-dimethylbenzoic acid, using sodium-bis(2-methoxyethoxy)aluminum hybride (Vitride; Eastman Organic Chemical Bulletin, vol. 46, p. 1-7, 1974). 3-Methylbenzyl alcohol was prepared by sodium borohydride reduction of *m*-methyltolualdehyde (97%; Aldrich Chemical Co., Milwaukee, Wis.). 3,4-Dimethylcatechol was prepared by the oxidation of 2-hydroxy-3,4-dimethylacetophenone as described by Baker et al. (1). 3-Ethylcatechol was synthesized by oxidation of 3-ethyl-2-hydroxybenzaldehyde with alkaline H_2O_2 as described by Dakin (8). The requisite 3-ethyl-2-hydroxybenzaldehyde was obtained from 2-ethylphenol by formylation with α,α'-dichloromethyl methyl ether in the presence of $TiCl_4$, using a procedure based on that of Gross et al. (15). 3-Ethylsalicylate was the gift of D. J. Hopper (University of Wales, Aberystwyth, United Kingdom), obtained by carboxylating 2-ethylphenol (17), and *cis,cis*-muconic acid was prepared by peracetic oxidation of phenol as described by Pandell (35).

RESULTS

Growth characteristics of *P. putida* strains. As shown in Table 2, *P. putida* mt-2 (PaM1) was able to utilize pseudocumene (1,2,4-trimethylbenzene) and 3-ethyltoluene in addition to toluene, *m*-, and *p*-xylene as previously reported (44). Additional aromatic compounds which supported growth included 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoic acid, 3-

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

Growth substrate	Growth[a]						
	PaM1	PaM3	PaM5	PaM300	HS1	ST3	Pxy
Toluene	+	−	+	+	+	+	+
m-Xylene	+	−	−	+	+	+	+
p-Xylene	+	−	−	+	+	+	+
2-Ethyltoluene	−	−	−	−	−	−	−
3-Ethyltoluene	+	−	−	+	+	+	+
4-Ethyltoluene	±	−	−	±	−	−	−
1,2,3-Trimethylbenzene	−	−	−	−	−	−	−
1,2,4-Trimethylbenzene	+	−	−	+	+	+	+
1,3,5-Trimethylbenzene	−	−	−	−	−	−	−
Benzyl alcohol	+	−	+	+	+	+	+
3,4-Dimethylbenzyl alcohol	+	−	−	+	+	+	+
Benzaldehyde	+	+	+	+	+	+	+
Benzoate	+	+	+	+	+	+	+
3-Ethylbenzoate	+	−	−	+	+	+	+
4-Ethylbenzoate	±	−	−	±	−	−	−
3,4-Dimethylbenzoate	+	−	−	+	+	+	+

[a] +, good growth; ±, slight growth; −, no growth.

ethylbenzoic acid, and the respective alcohols, aldehydes, and acids derived from the metabolism of toluene and *m*- and *p*-xylene. 4-Ethyltoluene and 4-ethylbenzoate supported slow growth of PaM1, whereas 2-ethyltoluene, 1,2,3-, and 1,3,5-trimethylbenzene were not utilized (Table 2). Identical growth properties were observed with a secondary but separate *P. putida* mt-2(PaW1) culture made available to us. Two additional strains in our collection, HS1 and ST3, were found to have growth characteristics similar to those of *P. putida* mt-2, including the ability to grow with pseudocumene and 3-ethyltoluene. Similar results were also observed for *Pseudomonas* sp. strain Pxy.

PaM2 and PaM3, spontaneous putative TOL (pWWO) plasmid-cured *P. putida* mt-2 strains, were unable to grow with any of the aromatic compounds which supported growth of the wild-type strain, PaM1, with the exception of benzaldehyde and benzoate. In contrast, a representative exconjugant obtained after PaW15 was mated with PaM3 (PaM 300) reacquired the ability to utilize all of the aromatic substrates (Table 2).

PaM5, a mutant selected for its inability to grow with 3,4-dimethylbenzoate, was also unable to utilize *m*- and *p*-xylene, pseudocumene, 3-ethyltoluene, or the corresponding oxidation products thereof. PaM5 still retained, however, the ability to grow with toluene and benzyl alcohol, suggesting that this strain contained a mutation in the TOL (pWWO) plasmid preventing growth on the alkyl-substituted compounds (Table 2).

Oxidation of aromatic compounds by intact *P. putida* mt-2 cells. Washed *P. putida* mt-2 cell suspensions grown with toluene or pseudocumene rapidly oxidized toluene and *m*- and *p*-xylene, pseudocumene, and 3-ethyltoluene (Table 3). Additional substrates readily oxidized included 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoate, and 3,4-dimethylcatechol, 3-ethylbenzoate, and 3-ethylcatechol. 4-Ethyltoluene was also oxidized at a low rate by toluene-grown cells. Succinate-grown cells showed little oxidation of these compounds except for 3,4-dimethylcatechol and 3-ethylcatechol, which were oxidized at only about 10% the rate observed for cells grown with toluene or pseudocumene.

Isolation and identification of metabolic products. Treatment of *P. putida* mt-2 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to the isolation of two mutants which could no longer utilize *m*- and *p*-xylene, pseudocumene, 3-ethyltoluene, or the corresponding intermediates in their metabolism (e.g., PaM5) (Table 2). Furthermore, when these mutants were incubated in the presence of these substrates on agar plates, brown products accumulated in the medium which were presumed to represent the corresponding catechols or their oxidation products. One such mutant, PaM5, was grown on 0.05% succinate with pseudocumene supplied in the vapor as described by Gibson (12), and the culture filtrate was extracted at neutral pH (pH 6.5) and then at acidic pH (pH 2.0). Analysis of each extract by thin-layer chromatography showed the presence of acidic and neutral products. The acidic product was shown to have an identical R_f value with that of authentic 3,4-dimethylbenzoic acid (R_f, 0.13 in solvent A; R_f, 0.09 in solvent B), whereas the neutral compound resembled 3,4-dimethylcatechol by its migration (R_f, 0.21 in solvent A; R_f, 0.33 in sol-

TABLE 3. *Oxidation of aromatic compounds by intact cells of P. putida (arvilla) mt-2*[a]

Assay substrate	Rates of oxygen consumption after growth with[b]:		
	Toluene	Pseudocumene	Succinate
Toluene	70	30	5
m-Xylene	80	49	3
p-Xylene	69	35	3
Pseudocumene	61	41	6
3-Ethyltoluene	75	41	4
4-Ethyltoluene	33	ND[c]	ND
3,4-Dimethylbenzyl alcohol	85	ND	ND
3,4-Dimethylbenzoate	60	77	4
3-Ethylbenzoate	74	70	5
3,4-Dimethylcatechol	83	75	11
3-Ethylcatechol	70	100	13
Succinate	ND	ND	107

[a] Oxygen consumption was measured manometrically with constant volume manometers. Each Warburg flask contained: 20 mM phosphate buffer (pH 7.0) containing 140 μg of chloramphenicol (1 ml); cell suspension (1 ml, 20 mg [wet weight]); 1.0 mM substrate contained in 0.3 ml of water or 0.06 ml of *N,N'*-dimethylformamide and water to 2.8 ml. The center well contained 20% KOH (0.2 ml). All results have been corrected for endogenous respiration (10 μl of O_2 consumed per h).

[b] Rates are expressed as microliters of O_2 consumed per hour per milligram of cell dry weight.

[c] ND, Not determined.

vent B) and by its color reaction with Gibb reagent. When four separate cultures were incubated simultaneously with pseudocumene, and the culture filtrates were extracted after 24 h at neutral pH (pH 6.5), 32 mg of a crude brown residue was obtained. A portion of this residue was sublimed under reduced pressure at 65°C to give a white solid (mp, 81 to 83°C), which upon mass spectral analysis (trimethylsilyl derivative) gave a parent ion mass of 282, with a fragmentation pattern identical to that of the trimethylsilyl derivative of 3,4-dimethylcatechol. Confirmation of the identity of the metabolite was provided by its UV spectrum in cyclohexane and its infrared spectrum, both of which were identical to that of authentic material. Further proof of the metabolite structure was obtained by demonstrating that cell extracts of toluene-grown *P. putida* mt-2 cells converted the biologically formed 3,4-dimethylcatechol to a ring fission product with identical spectral properties (λ_{max}, 322 nm at pH 7.5) as those observed with authentic 3,4-dimethylcatechol.

Evidence that 3,4-dimethylbenzoic acid was formed during incubation of PaM5 in the presence of pseudocumene was obtained by growing this strain in 0.05% succinate for 8 h at 30°C in 1-liter batch cultures at which time pseudocumene was added directly to the cell suspension (0.025%). After 1.5 h, the cells were centrifuged, and the culture supernatant was acidified to pH 2.0 and extracted with ether. Removal of the solvent led to the isolation of 240 mg of a light-brown residue from which 3,4-dimethylbenzoic acid could be recrystallized from water and ethanol to give a white solid with a melting point of 166°C. Authentic 3,4-dimethylbenzoic acid also gave a melting point of 166°C. Additional evidence for the identity of this product was provided by its mass spectrum (*m/e*, 222 for its trimethylsilyl derivative) and its infrared spectrum which were the same as those of authentic 3,4-dimethylbenzoic acid.

PaM5 was grown on succinate with 3-ethyltoluene replacing pseudocumene in the vapor phase as described earlier. Acidification of the culture filtrate at pH 2.0 and extraction with diethyl ether led to the isolation of 62 mg of a brown, oily residue. A portion of this crude residue was subjected to preparative thin-layer chromatography, using solvents A and B, successively, resulting in the isolation of acidic and neutral products. The neutral product gave a color reaction with Gibb reagent on thin-layer chromatography and R_f values (R_f, 0.23 in solvent A; R_f, 0.33 in solvent B) identical to those of authentic 3-ethylcatechol. Further evidence for the identity of this metabolite as 3-ethylcatechol was provided by its mass spectrum which gave a parent molecular ion of 138 when underivatized and 282 as its trimethylsilyl derivative, and by its nuclear magnetic resonance spectrum in $CDCl_3$ which showed major signals at (τ) 3.31 (3H, complex multiplet), 7.37 (2H, quartet; J, 1.2 Hz), and 8.79 (3H, triplet; J, 1.12 Hz). The chemical shifts of the hydroxyl protons were approximately 5.0 ppm in this solvent. These properties were identical to those obtained with an authentic sample of 3-ethylcatechol and also with that reported earlier for this compound (14). Additional proof of structure was obtained by demonstrating that *P. putida* mt-2 cell extracts converted the biologically formed 3-ethylcatechol to a ring fission product having identical spectral properties (λ_{max}, 390 nm at pH 7.5) as those obtained with authentic 3-ethylcatechol.

The acidic product obtained during growth of PaM5 in the presence of 3-ethyltoluene showed fluorescent properties under ultraviolet light after thin-layer chromatography, gave a similar color reaction with Gibb reagent, and had the same R_f values (R_f, 0.08 in solvent A; R_f, 0 in solvent B) as did authentic 3-ethylsalicylic acid. Further evidence which suggested that this product might be 3-ethylsalicylate was provided

by mass spectral analysis which gave a parent molecular ion of 166 (underivatized) and 310 as its trimethylsilyl derivative, with a fragmentation pattern identical to that of authentic 3-ethylsalicylate.

Induction of degradative enzymes. *P. putida* mt-2 growth on toluene or pseudocumene resulted in the induction of the *meta* fission enzymes (Table 4), as evidenced by high levels of catechol-2,3-oxygenase, 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase, and 2-hydroxymuconic semialdehyde dehydrogenase in cell extracts from cell grown on these hydrocarbons. Growth on toluene led not only to induction of the *meta* pathway enzymes but resulted also in activities of catechol-1,2-oxygenase and *cis,cis*-muconate-lactonizing enzyme, representative enzymes of the *ortho* fission pathway, which were significantly higher than basal levels of these enzymes obtained from succinate-grown cells (Table 4). No significant induction of catechol-1,2-oxygenase and *cis,cis*-muconate-lactonizing enzyme occurred in pseudocumene-grown cells. Growth of the mutant strain (PaM5) on toluene resulted in the induction of catechol-1,2-oxygenase (114 nmol min^{-1} mg^{-1}) and *cis,cis*-muconate-lactonizing enzyme (250 nmol min^{-1} mg^{-1}), whereas catechol-2,3-oxygenase activity was undetectable (<0.1 nmol min^{-1} mg^{-1}). These results indicate that this strain is defective in catechol-2,3-oxygenase and catabolizes toluene via the alternative chromosomally-encoded *ortho* pathway.

Oxidation of 3,4-dimethylcatechol and 3-ethylcatechol by cell extracts. When cell extracts from toluene- or pseudocumene-grown cells were heat-treated at 55°C for 10 min to destroy catechol-1,2-oxygenase and then tested for their ability to oxidize catechol and its homologs, high rates of attack were observed towards each substrate (Table 5). Furthermore, in each case oxidation was accompanied by consumption of 1 mol of O_2 per mol of substrate. The highest activities were observed with catechol and 4-methylcatechol. 3-Methylcatechol, 3-ethylcatechol, and 3,4-dimethylcatechol were oxidized at about equal rates, representing approximately 70% of the rate observed with catechol (Table 5). Cell extracts obtained from succinate-grown cells contained approximately 10% of the activity seen in extracts of cells grown with toluene or pseudocumene.

When cell extracts were incubated with 3,4-dimethylcatechol and formation of the reaction product followed spectrophotometrically at pH 7.5, the results shown in Fig. 1 were obtained. A yellow compound with an absorption maximum at 322 nm (ϵ_{322} = 13,200 M^{-1}) and an additional absorption band at 390 nm (ϵ_{390} = 7,100 M^{-1}) was formed transiently. Upon acidification of the reaction mixture (pH 2.5) a single band was obtained with an absorption maximum of 322 nm, whereas in alkaline solution (pH 11.0) a single absorption band with λ_{max} of 390 nm was observed. When a similar experiment was performed with 3-ethylcatechol at pH 7.5, a yellow product was also formed (Fig. 2) with an absorption maximum of 390 nm (ϵ_{390} = 10,900 M^{-1}) and a second absorption band at 320 nm (ϵ_{320} = 7,400 M^{-1}). In acid (2.5) or base (11.0) pH, solutions possessed maxima only at 320 and 390, respectively.

Metabolism of ring fission products by cell extracts. When heat-treated *P. putida* mt-2 cell extracts were incubated with 3,4-dimethylcatechol or 3-ethylcatechol, each respective ring fission product was shown to accumulate (Fig. 3). These compounds disappeared on addition of unheated cell extract obtained from toluene- or pseudocumene-grown cells. The rates at which 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate, the ring fission product derived from 3,4-dimethylcatechol, and 2-hydroxy-6-oxo-2,4-octadienoate, the ring fission product of 3-ethylcatechol, were metabolized were shown to be independent of NAD^+. The relative rates at which the ring fission products derived from catechol, 3-methyl-, 4-methyl-, 3,4-dimethyl-, and 3-ethylcatechol were degraded are tabulated in Table 6. 2-Hydroxy-6-oxo-2,4-heptadienoate, the ring fission product of 3-methylcatechol, disappeared most rapidly as previously shown by

TABLE 4. *Induction of meta and ortho fission enzymes in P. putida mt-2*

Enzyme assayed	Sp act (nmol/min per mg) after growth with:		
	Toluene	Pseudocumene	Succinate
Meta pathway			
Catechol-2,3-dioxygenase	2,830	2,370	28
2-Hydroxy-6-oxo-2,4-heptadienoate hydrolase	670	304	7
2-Hydroxymuconic semialdehyde dehydrogenase	141	46	<0.1
Ortho pathway			
Catechol-1,2-dioxygenase	114	0.7	0.3
cis,cis-Muconate lactonizing enzyme	283	1	3

TABLE 5. *Oxidation of catechols by heat-treated P. putida mt-2 cell extracts*[a]

Assay substrate	Rates of oxygen consumption after growth with[b]:		
	Toluene	Pseudocumene	Succinate
Catechol	2,830	2,370	28
3-Methylcatechol	2,100	1,760	14
4-Methylcatechol	2,670	2,710	20
3,4-Dimethylcatechol	1,600	1,390	8
3-Ethylcatechol	1,700	1,590	13

[a] Oxygen consumption was measured polarographically with an oxygen electrode.

[b] Rates are expressed as nanomoles of O_2 consumed per minute per milligram of protein.

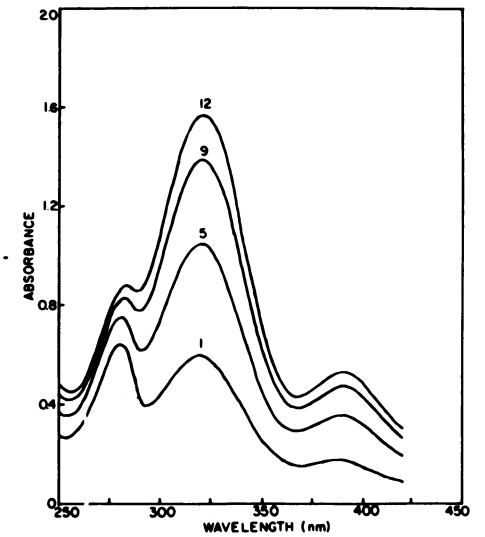

FIG. 1. *Spectral changes associated with the oxidation of 3,4-dimethylcatechol by cell extracts of P. putida mt-2 (arvilla). The cuvette contained 2.9 ml of 50 mM KH_2PO_4 buffer (pH 7.5), 100 μl of 10 mM 3,4-dimethylcatechol and 5 μl of cell extract that contained 0.06 mg of protein. Repeat scans were made at the intervals indicated (min). The band at 280 nm is that due to the substrate, 3,4-dimethylcatechol.*

Murray et al. (23) and as more recently demonstrated by Bayly and Di Berardino (4) with purified preparations of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from different *P. putida* strains. 2-Hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde, the ring fission products derived from catechol and 4-methylcatechol, respectively, served as poor substrates, with rates of hydrolysis representing only 7% of that observed for 2-hydroxy-6-oxo-2,4-heptadienoate. The ring fission products of 3,4-dimethyl- and 3-ethylcatechol were acted upon at similar rates, representing only 50% of the activity observed with 2-hydroxy-6-oxo-2,4-heptadienoate (Table 6). Ring fission products derived from 3-methyl-, 3,4-dimethyl- and 3-ethylcatechol were converted to pyruvate in amounts approaching 1:1 stoichiometry, depending upon which substrate was incubated with cell extract (Table 7).

Genetic evidence for TOL (pWWO) plasmid mediated catabolism of pseudocumene and 3-ethyltoluene. When spontaneous mutants putatively cured of the TOL (pWWO) plasmid were screened for their ability to utilize toluene, the xylenes, pseudocumene, 3-ethyltoluene, and the corresponding oxidation products, none were able to do so. When 25 independently isolated exconjugants, selected on the basis of their leucine$^+$, *m*-toluate$^+$ character after mating PaW15 with PaM3, were tested for their ability to grow with pseudocumene and 3-ethyltoluene, all were able to do so (e.g., PaM300, Table 2). These same exconjugants were also able to grow with 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoate, 3-ethylbenzoate, and with toluene, the xylenes and the corresponding alcohols, aldehydes, and acids.

DISCUSSION

The results presented here demonstrate that pseudocumene and 3-ethyltoluene are metabolized via routes similar to those described for toluene (27) and *m*- and *p*-xylene (9, 44) which involve single-step oxidations of a methyl group to form the corresponding benzoic acids (Fig. 4). Intact cells oxidized these substrates without lag

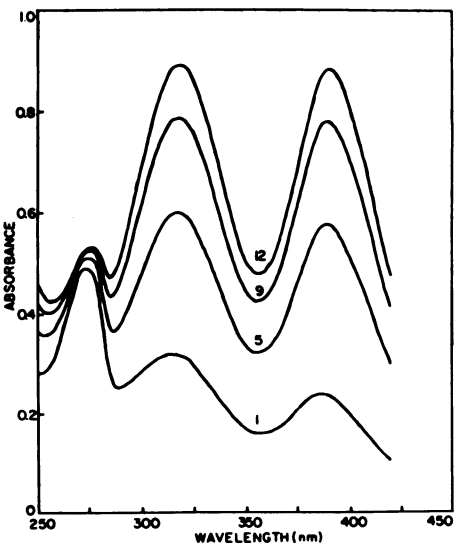

FIG. 2. *Spectral changes associated with the oxidation of 3-ethylcatechol by P. putida mt-2 (arvilla) cell extracts. The assay conditions were as described in Fig. 1. Repeat scans were made at the intervals indicated (min).*

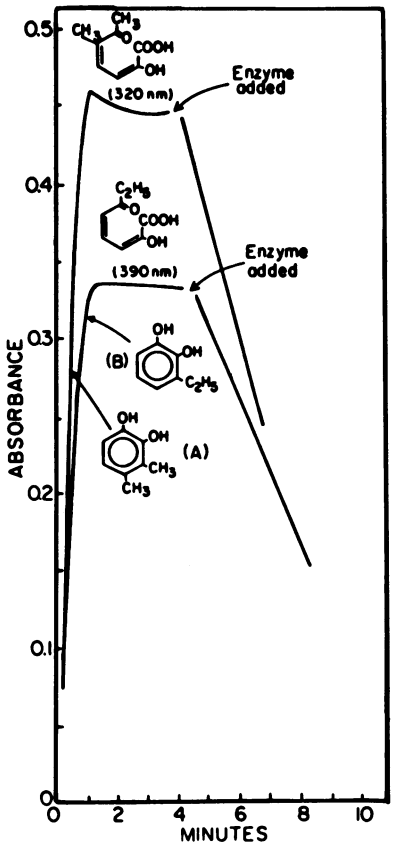

FIG. 3. *Formation and degradation of ring fission products from 3,4-dimethyl- (A) and 3-ethylcatechol (B) by P. putida mt-2 (arvilla) cell extracts. Cuvettes contained 3.0 ml of 50 mM KH_2PO_4 buffer (pH 7.5) and 0.10 μmol of the respective catechol. Each reaction was started by the addition of heat-treated extract (0.1 mg of protein) and at the times indicated untreated cell extract was added (0.1 mg of protein) to the reaction mixtures which contained the ring fission products of 3,4-dimethyl- (A, measured at 320 nm) and 3-ethylcatechol (B, measured at 390 nm).*

after growth with toluene or pseudocumene (Table 3). Growth of the mutant strain, PaM5, in the presence of pseudocumene resulted in the accumulation of both 3,4-dimethylbenzoic acid and 3,4-dimethylcatechol as products. These results agree with previous reports on the initial reactions in the oxidation of toluene and the xylenes (9, 10, 27, 30–32) and support earlier findings which demonstrated the formation of 3,4-dimethylbenzoate in the oxidation of pseudocumene (31). Accumulation of 3,4-dimethylcatechol suggests that the mutant is defective in catechol-2,3-oxygenase activity. This is supported by the identification of 3-ethylcatechol formed from 3-ethyltoluene and by enzyme assays which showed that this strain is incapable of synthesizing this activity. Failure of PaM5 to accumulate 3-ethylbenzoate when incubated with 3-ethyltoluene is analagous to the report of Davey and Gibson (9), who showed that 3-methylbenzoate did not accumulate when a mutant, *Pseudomonas* sp. strain Pxy-40, was incubated with *m*-xylene. This inability to detect a metabolite from 3-alkyl-substituted toluenes (i.e., *m*-xylene, 3-ethyltoluene) may be due to the ability of certain 3-alkylbenzoates to serve as substrates for a chromosomally-encoded benzoate oxidase complex and, therefore, are likely to be converted to the corresponding 3-alkyl-substituted catechols or later metabolites of the catechol *ortho* fission pathway. In contrast, 3,4-dimethylbenzoate, as shown in this paper, and *p*-toluate as demonstrated by Davey and Gibson (9), are accumulated by mutants defective in a plasmid-specified benzoate oxidase or later enzymes of the catechol *meta* fission pathway, since 4-methyl substituted benzoates do not appear to serve as substrates for the alternative chromosomal benzoate oxidase (unpublished data).

The isolation of 3-ethylsalicylate as a metabolite of 3-ethyltoluene degradation by PaM5 agrees with earlier findings in which the accumulation of this product was demonstrated from the oxidation of 3-ethyltoluene (20). Davey and Gibson (9) observed a similar accumulation of 3-methylsalicylic acid from the oxidation of *m*-xylene. These workers (9) speculated that since whole cells of *Pseudomonas* sp. strain Pxy and *P. aeruginosa* S668B2 (31) did not oxidize 3-methylsalicylic acid after growth with *m*-xylene, that this compound might accumulate as a result of the acid catalyzed dehydration of its dihydrodiol precursor, 3-methylcyclohexa-3,5-diene-1,2-diol-1-carboxylic acid. By analogy, the isolation of 3-ethylsalicylic acid by Jigami et al. (20), and as reported here, may be accounted for by a

TABLE 6. *Metabolism of ring fission substrates by P. putida mt-2 cell extracts*[a]

Assay substrate	Specific activity (nmol/min per mg) after growth with:		
	Toluene	Pseudocumene	Succinate
2-Hydroxymuconic semialdehyde	44	23	2
2-Hydroxy-6-oxo-2,4-heptadienoate	670	304	7
2-Hydroxy-6-oxo-2,4-octadienoate	286	120	3
2-Hydroxy-5-methylmuconic semialdehyde	40	ND[b]	1
2-Hydroxy-5-methyl-6-oxo-2,4-heptadienoate	381	157	4

[a] Measured in the absence of NAD^+.
[b] ND, Not determined.

TABLE 7. *Stoichiometry of conversion of alkyl-substituted catechols to pyruvate by P. putida mt-2 cell extracts*

Growth substrate	Substrate supplied	Amt of substrate oxidized (nmol)[a]	Pyruvate formed (nmol)[b]	% conversion pyruvate/catechol oxidized
Toluene	3-Methylcatechol	42	29	69
	3,4-Dimethylcatechol	40	29	73
	3-Ethylcatechol	36	34	95
Pseudocumene	3-Methylcatechol	32	21	66
	3,4-Dimethylcatechol	43	26	60
	3-Ethylcatechol	36	31	86

[a] Measured by oxygen electrode.
[b] Measured by the oxidation of NADH with lactate dehydrogenase.

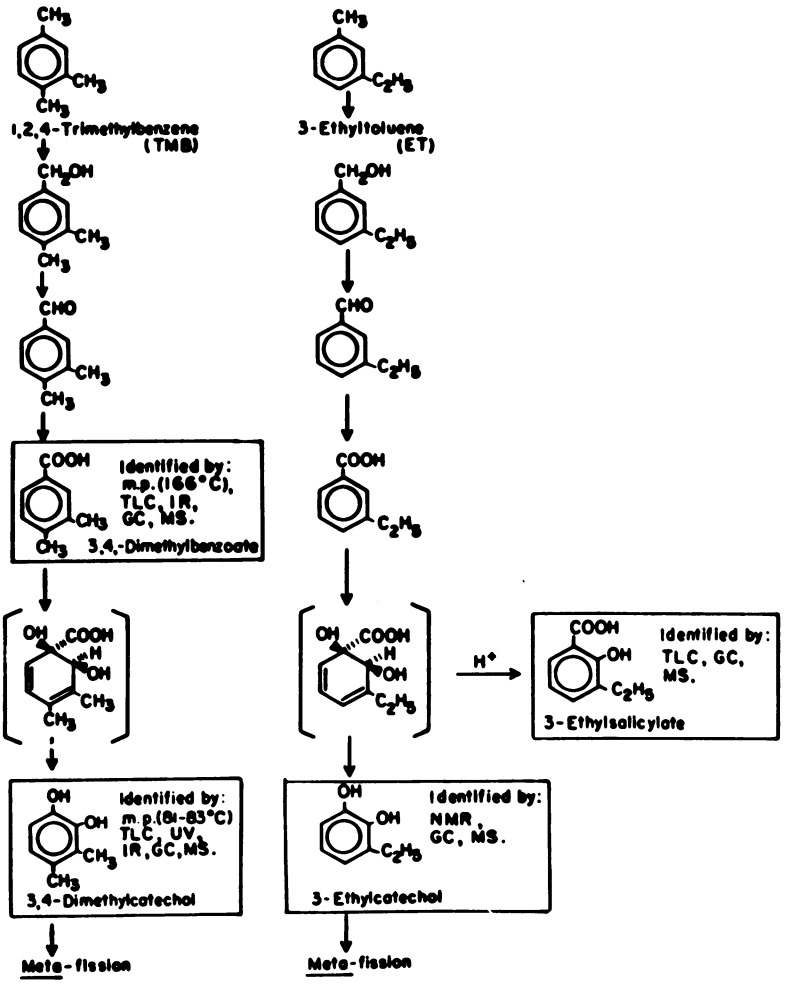

FIG. 4. *Proposed pathways for the metabolism of pseudocumene and 3-ethyltoluene by P. putida mt-2. Compounds shown in blocks were identified in this investigation.*

similar acid-catalyzed dehydration of the analogous ethyl-substituted dihydrodiol. A similar explanation might account for the accumulation of 3,4-dimethylphenol during growth of *P. aeruginosa* S668B2 on pseudocumene or 3,4-dimethylbenzoate as reported by Omori and Yamada (31). Support for this hypothesis lies in the observation that *P. aeruginosa* S668B2 cells were unable to oxidize 3,4-dimethylphenol after growth with pseudocumene (32). The results

reported here are consistent with the known instability of alkyl-substituted-3,5-cyclohexadiene-1,2-dihydrodiols (13, 14) and are in agreement with previous findings which demonstrated the involvement of *cis*-3,5-cyclohexadiene-1,2-diol-1-carboxylic acids in the bacterial metabolism of benzoate (37, 38), *m*-toluate (36, 38), and 3-substituted fluoro-, chloro-, and bromobenzoates (36).

Whole cells and cell extracts from *P. putida* mt-2 grown with toluene or pseudocumene oxidized 3,4-dimethylcatechol and 3-ethylcatechol. The identification of 3,4-dimethylcatechol as an intermediate of pseudocumene oxidation by PaM5 provides strong evidence for its role as the ring fission substrate in the metabolism of pseudocumene. This is also supported by experiments which show that 3,4-dimethylcatechol is oxidized by cell extracts to give a ring fission product consistent with aromatic ring cleavage by a catechol *meta* fission mechanism (Table 5; Fig. 1) (6). Analogous experiments show that 3-ethylcatechol is an intermediate in the metabolism of 3-ethyltoluene by *P. putida* mt-2 (Fig. 2).

The further metabolism of ring fission intermediates derived from 3,4-dimethyl- and 3-ethylcatechol to pyruvate is also consistent with sequences for their degradation proceeding via the *meta* pathway. In addition, the inability of NAD^+ to stimulate the rate of disappearance of the ring fission product of 3-ethylcatechol by cell extracts implies that the metabolism of this intermediate occurs exclusively via the hydrolytic branch (3, 7) and not by the so-called 4-oxalocrotonate or oxidative route (26, 40) of the *meta* pathway. This finding also provides evidence that the ring fission product of 3-ethylcatechol is 2-hydroxy-6-oxo-2,4-octadienoate, a ketonic acid in structure (Fig. 4). In contrast, *meta* cleavage of 4-ethylcatechol would be expected to yield an aldehydo-acid, the further metabolism of which might occur via either the hydrolytic or the more predominant NAD^+-dependent oxidative route. The inability to observe a stimulation by NAD^+ at the rate in which the ring fission intermediate of 3-ethyltoluene metabolism was degraded and the inability to obtain chemical evidence for 4-ethylcatechol as the ring fission substrate support the conclusion that 3-ethyltoluene is catabolized via 3-ethylcatechol to 2-hydroxy-6-oxo-2,4-octadienoate, which is then further metabolized by the hydrolytic route of the *meta* pathway to pyruvate (Fig. 4).

Our results show that catechol-2,3-oxygenase activity in *P. putida* mt-2 cell extracts catalyzes ring cleavage of 3-ethyl- and 3,4-dimethylcatechol (Table 3) in addition to catechol, 3-methyl-, and 4-methylcatechol as previously reported (29). Furthermore, the specific activities of catechol-2,3-oxygenase with respect to the above-mentioned catechols were almost identical in extracts of cells obtained after growth with toluene or pseudocumene (Table 4). 2-Hydroxy-6-oxo-2,4-heptadienoate hydrolase, the next enzyme in the *meta* pathway, utilized the respective ring fission products derived from each of the five catechols as substrates (Table 6), with relative activities which were again similar regardless of the growth substrate (Table 4). These results agree with previous studies which showed that the enzymes of the *meta* fission pathway in *P. putida* mt-2 are induced to the same extent regardless of whether cells are grown on aromatic hydrocarbons, alcohols, or acids (23, 44) and thus provide further evidence that for each of the activities of the *meta* fission pathway there may be a single enzyme. The pleiotropic effect of a mutation in catechol-2,3-oxygenase as observed with strain PaM5 (Table 2) would be consistent with this hypothesis.

It is noteworthy that, whereas growth with pseudocumene led only to the induction of the *meta* enzymes, growth with toluene resulted in the induction of both the *meta* pathway enzymes as well as catechol-1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme, representative enzymes of the catechol *ortho* fission pathway (Table 4). These results would suggest that catechol, an intermediate in toluene metabolism, can be converted to *cis,cis*-muconate by low levels of catechol-1,2-oxygenase, which in turn can serve as an inducer for catechol-1,2-oxygenase and enzymes of the *ortho* fission pathway. This interpretation is consistent with the mechanism of synthesis control established for the *ortho* enzymes in *P. putida* (34). These results also agree with the finding that the mutant, PaM5, defective in catechol-2,3-oxygenase, remains able to grow with toluene by utilizing enzymes of the *ortho* pathway. The lack of induction of the *ortho* pathway enzymes after growth of the wild type with pseudocumene could thus be interpreted as a failure of either low levels of catechol-1,2-oxygenase to act on 3,4-dimethylcatechol, an intermediate in pseudocumene metabolism, or the inability of an α,β-dimethyl-substituted *cis,cis*-muconate, formed by the action of catechol-1,2-oxygenase on 3,4-dimethylcatechol, to serve as an inducer for enzymes of the *ortho* pathway. We have not attempted to distinguish between these possibilities. It is known, however, that purified catechol-1,2-oxygenase from *P. putida* C-1 (21) will attack 3-methyl- and 4-methylcatechol as will crude cell extracts of *P. putida* NCIB 10015 grown on benzoate (24). Even so, growth of the latter organism in the presence of these catechols does not lead to induction of catechol-1,2-

oxygenase and the remaining enzymes of the *ortho* pathway (24). These findings have been interpreted to mean that the methyl-substituted *cis,cis*-muconic acids derived from 3- and 4-methylcatechol cannot serve as inducers of catechol-1,2-oxygenase (24).

The *meta* enzymes in *P. putida* mt-2 appear to be induced from the top by either the parent hydrocarbons, alcohols, or acids (23, 44). Similar observations have been made with phenol and the cresols which serve as growth substrates for *P. putida* NCIB 10015 (2) and *P. aeruginosa* T1 (39). Furthermore in all of these organisms, which utilize the *meta* pathway enzymes for degradation of the growth substrate, induction is nonspecific in that several substrate analogs can serve as inducers. The results presented in this paper provide an extension of this example of the broad specificity of the *meta* pathway with regards to its induction and enzymic function. As a result of this breadth of specificity, *P. putida* mt-2 is able to utilize no fewer than 20 different substrates, including five aromatic hydrocarbons as well as the corresponding alcohols, aldehydes, and acids, by employing what is apparently a single set of enzymes. Since most of the products of aromatic ring fission derived from the above compounds are ketonic rather than aldehydo-acids, it is also apparent that it is the hydrolytic branch of the *meta* pathway which plays a major role in broadening the nutritional capabilities of this organism.

In previous studies, it has been demonstrated in *P. putida* mt-2 that the genes responsible for the catabolism of toluene and the xylenes are plasmid-encoded (42, 44, 46). The evidence presented in this paper shows that the ability to utilize pseudocumene and 3-ethyltoluene is both cotransferred and simultaneously lost with the ability to utilize toluene and the xylenes. We therefore conclude that the metabolism of pseudocumene and 3-ethyltoluene is facilitated by the TOL (pWWO) plasmid. Utilization of toluene via the *ortho* pathway in a strain blocked in the *meta* pathway (PaM5) shows that this organism can use a combination of plasmid- (enzymes necessary for initial hydrocarbon oxidation) and chromosome-specified (*ortho*) functions to facilitate the degradation of this hydrocarbon. Further, it has been shown in several additional *P. putida* strains, including *Pseudomonas* sp. strain Pxy, a strain known to carry a nonconjugative TOL-type plasmid of 10×10^6 molecular weight (5), that the ability to utilize pseudocumene and 3-ethyltoluene is paralleled by the ability to grow with toluene and the xylenes. These findings suggest that the ability to grow with these additional hydrocarbons may represent a common trait of TOL plasmid-carrying strains.

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