JOURNAL OF BACTERIOLOGY, June 1981, p. 952-964
0021-9193/81/060952-13$02.00/0

Vol. 146, No. 3

Isolation and Characterization of Spontaneously Occurring TOL Plasmid Mutants of *Pseudomonas putida* HS1

DANIEL A. KUNZ†* AND PETER J. CHAPMAN

Departments of Microbiology and Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Received 14 November 1980/Accepted 19 March 1981

A strain of *Pseudomonas* (*P. putida* HS1) was found to resemble *P. putida* (*arvilla*) mt-2 in its ability to degrade toluene, *m*- and *p*-xylene, 1,2,4-trimethylbenzene (pseudocumene), and 3-ethyltoluene via oxidation of a methyl substituent and reactions of the *meta*-fission pathway. The ability to degrade these substrates by *P. putida* HS1 (PpC1) was shown to be encoded by a TOL (pDK1) plasmid as evidenced by: (i) spontaneous loss of the TOL-related phenotype after growth with benzoate, (ii) transfer of the TOL character from the wild type into cured recipients by conjugation, and (iii) isolation of a plasmid of identical molecular weight (120×10^6) from both the wild type and an exconjugant obtained by mating the wild type with a putative cured recipient. In addition to the isolation of apparent cured strains having lost the entire TOL-related phenotype, two additional mutant classes were observed after growth on benzoate. One class, represented by PpCT1, was unable to utilize the alkyl-substituted aromatic compounds but retained the ability to grow with toluene and benzyl alcohol. Analysis of PpCT1 revealed that it was unable to synthesize the TOL-encoded toluate oxidase and enzymes of the *meta* pathway but retained the ability to elaborate activities for toluene hydroxylase, benzyl alcohol, and benzaldehyde dehydrogenase, thereby mediating initial oxidation of toluene to benzoate, which was then further metabolized via enzymes of the chromosomally encoded *ortho*-fission pathway. A second class of mutants had lost the ability to utilize the hydrocarbons but could still grow with *m*-toluate but not *p*-toluate, 3,4-dimethylbenzoate, or 3-ethylbenzoate, intermediates in the oxidation of the corresponding hydrocarbons. One such mutant, PpCM1, could no longer synthesize enzymes required for initial oxidation of the hydrocarbons, but was able to produce the toluate oxidase and enzymes of the *meta* pathway, thereby facilitating degradation of *m*-toluate. Neither PpCT1, PpCM1, nor a putative cured strain, PpCC1, reverted at detectable frequencies ($<10^{-9}$). Analysis of each strain for plasmid deoxyribonucleic acid revealed the presence of a single plasmid in each strain with the following molecular weights: PpCM1, 100×10^6 (pDKM1); PpCT1, 80×10^6 (pDKT1); PpCC1, 20×10^6 (pDKC1). The results suggest that the TOL (pDK1) plasmid has undergone deletions giving rise to smaller replicons which either encode for only a fraction of the wild-type catabolic functions (pDKM1, pDKT1) or have lost all catabolic activities (pDKC1).

A number of studies have revealed that genes coding for the catabolism of a variety of compounds in the genus *Pseudomonas* are plasmid encoded (2). One such plasmid, designated TOL as originally described for *Pseudomonas putida* (*arvilla*) mt-2 (23, 25, 27), codes for catabolic sequences which lead to the dissimilation of toluene and *m*- and *p*-xylene via initial reactions forming benzoate and *m*- and *p*-toluate, respectively, followed by the *meta*-fission pathway (Fig. 1). A number of strains of *Pseudomonas* capable of utilizing toluene and *m*- and *p*-xylene have been isolated from soil, and in each of these, the genes coding for the degradative enzymes have been shown to be plasmid encoded (7, 24). Though these plasmids, termed the TOL plasmids (24), are isofunctionally identical, they differ in several respects. These include transmissibility (7, 24), the ability to form spontaneous deletion mutants in cells growing on benzoate (24, 28), the capacity to facilitate the rapid degradation of the *p*-methyl-substituted compounds (24), and molecular structure as evidenced by molecular weights and fragmentation patterns of restriction endonuclease digests (5).

† Present address: Central Research and Development Department, E.I. duPont de Nemours & Co., Inc., Wilmington, DE 19898.

Strains having lost the plasmid functions retain the ability to grow with benzoate since benzoate can also be degraded by a second route, the *ortho*-fission (β-ketoadipate) pathway, presumed to be chromosomally encoded (23). In strains carrying the plasmid, benzoate is metabolized via the *meta* pathway since benzoate serves as the inducer of catechol-2,3-oxygenase and the *meta* pathway (6, 23), whereas induction of the *ortho* pathway requires the initial conversion of benzoate to catechol, which must then first be converted by basal levels of catechol-1,2-oxygenase to *cis,cis*-muconic acid, its product inducer (17). Thus the ability to obtain strains which have been cured of their TOL plasmids after growth with benzoate can be explained by the presence of the second pathway, the *ortho* pathway, which promotes the dissimilation of benzoate.

During the course of an investigation on the ability of bacteria to utilize aromatic hydrocarbons, we discovered an organism designated *P. putida* HS1, which was phenotypically similar to *P. putida* mt-2 (23, 27) and other pseudomonads (7, 24) in its ability to utilize toluene and the xylenes and, as reported for *P. putida* mt-2 (11), in its ability to growth with 1,2,4-trimethylbenzene (pseudocumene) and 3-ethyltoluene. This communication shows that the pathways by which these hydrocarbons are degraded by *P. putida* HS1 are similar to those described for *P. putida* mt-2 and other pseudomonads (Fig. 1) (3, 4, 11, 15, 16, 23, 24, 27) and, as shown for *P. putida* mt-2, provides evidence that the genes coding for the catabolism of these compounds are also plasmid encoded. In the course of obtaining cured strains of *P. putida* HS1 after growth on benzoate, two additional classes of mutants were isolated having catabolic phenotypes intermediate between the wild type and apparent cured strains. This paper further describes the novel biochemical and genetic properties of these derivatives. The results suggest that these mutants arise by the spontaneous segregation of a new TOL plasmid. We have previously reported preliminary evidence for a TOL plasmid in this strain (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. Strains of *P. putida* HS1 used in this investigation are listed in Table 1. *P. putida* HS1 (PpC1) was isolated by Placida Venegas from soil in St. Paul, Minn. by enrichment with 0.05% *m*-toluate as the sole carbon source. This organism was identified

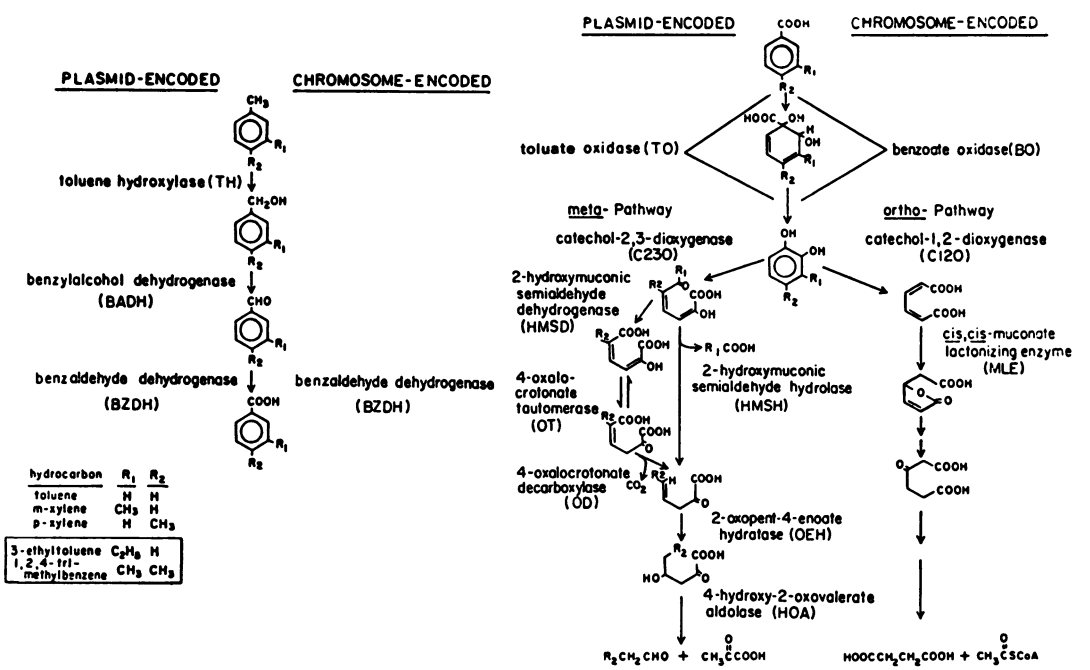

FIG. 1. *Enzymes of aromatic hydrocarbon and aromatic acid metabolism encoded by TOL plasmids. Toluene or benzoate may be metabolized via either the plasmid-encoded meta pathway or the chromosome-encoded ortho pathway. Alkyl-substituted toluenes and benzoates are metabolized exclusively via the plasmid-encoded meta pathway.*

TABLE 1. *Strains of P. putida*

Strain designation	Phenotype[a]	Genotype[b]	Parent strain	Method of production[c] or reference
PpCl (*P. putida* HSl wild type)	Ben$^+$ Mtol$^+$ Ptol$^+$ Tln$^+$ Mxy$^+$ Pxy$^+$	wt/TOL (pDKl)		*m*-Toluate enrichment
PpCCl	Ben$^+$ Mtol$^-$ Ptol$^-$ Tln$^-$ Mxy$^-$ Pxy$^-$	TOL^{del}	PpC1	Benzoate curing
PpCTl	Ben$^+$ Mtol$^-$ Ptol$^-$ Tln$^+$ Mxy$^-$ Pxy$^-$	TOL^{del}	PpC1	Benzoate curing
PpCM1	Ben$^+$ Mtol$^+$ Ptol$^-$ Tln$^-$ Mxy$^-$ Pxy$^-$	TOL^{del}	PpC1	Benzoate curing
PpCl4	Ben$^+$ Mtol$^+$ Ptol$^+$ Tln$^+$ Mxy$^+$ Pxy$^+$ Leu$^-$ Ileu$^-$ Val$^-$ His$^-$	*leu-1 ileu-1 val-1 his-1*/TOL(pDKl)	PpC1	NG
PpCCll	Ben$^+$ Mtol$^-$ Ptol$^-$ Tln$^-$ Mxy$^-$ Pxy$^-$ Str^r	*str-1*/Tol^{del}	PpCC1	SM
PaM1 (*P. putida* mt-2)	Ben$^+$ Mtol$^+$ Ptol$^+$ Tln$^+$ Mxy$^+$ Pxy$^+$	wt/TOL(pWWO)		(11)
PaM3	Ben$^+$ Mtol$^-$ Ptol$^-$ Tln$^-$ Mxy$^-$ Pxy$^-$	TOL^{del}	PaM1	(11)
PaW15	Ben$^+$ Mtol$^+$ Ptol$^+$ Tln$^+$ Mxy$^+$ Pxy$^+$ Leu$^-$	*leu*-1/TOL(pWWO)	PaM1	(23)
PpC214	Ben$^+$ Mtol$^+$ Ptol$^+$ Tln$^+$ Mxy$^+$ Pxy$^+$ Str^r	*str-1*/TOL	PpC14 × PpCC11	Conjugation

[a] Phenotype abbreviations: Ben, benzoate; Mtol, *m*-toluate; Ptol, *p*-toluate; Tln, toluene; Mxy, *m*-xylene; Pxy, *p*-xylene; Leu, leucine; Ileu, isoleucine; Val, valine; His, histidine; Str^r, streptomycin resistance.

[b] Genotype abbreviations: wt, wild type; TOL^{del}, TOL plasmid deletion; pDK1, TOL plasmid from *P. putida* HS1; pWWO, TOL plasmid from *P. putida* mt-2.

[c] NG, Nitrosoguanidine mutagenesis; SM, spontaneous streptomycin-resistant mutant (100 μg/ml).

as *Pseudomonas putida* by the nutritional and biochemical criteria described by Stanier et al. (22). Derivatives of PpC1 were obtained as described in Table 1. *P. putida* (*arvilla*) mt-2 (PaM1) was kindly provided by G. D. Hegeman, Department of Microbiology, Indiana University, Bloomington. An auxotroph of this organism, PaW15, was given to us by P. A. Williams, University College of North Wales, U.K. Bacterial strains providing plasmid molecular weight standards were graciously provided by L. L. McKay, Department of Food Science, University of Minnesota, St. Paul, and by R. H. Olsen, Department of Microbiology, University of Michigan, Ann Arbor, and were as follows: *Escherichia coli* C600 (RSF1010, 5.5×10^6), *E. coli* J53 (Sa, 25×10^6), *E. coli* J5 (RP4, 34×10^6), *E. coli* DT78 (TP116, 143×10^6), *E. coli* DT41 (R27, 112×10^6), and *Salmonella typhimurium* LT2 (cryptic plasmid, 60×10^6) (9, 12).

Media and culture conditions. Bacterial strains were maintained on nutrient agar slants at 5 or −16°C in a 0.1 M sodium-potassium phosphate buffer (pH 7.0)-glycerol (1:1, vol/vol) mixture. In cases where the stability of strains was questionable, they were maintained on the medium from which they were isolated before storage in a buffer-glycerol mixture at −16°C. The minimal medium used and the procedures for growth tests were described previously (11).

Cells used for oxidation studies and enzyme assays were grown in 1-liter batches of minimal medium with water-soluble substrates supplied at 0.05 to 0.1% and incubated at 30°C on a rotary shaker for 16 to 20 h before harvesting. To obtain cells grown on aromatic hydrocarbons, cells were grown on agar plates with the substrate hydrocarbon supplied as a vapor as described by Gibson (8). These cultures were harvested after 24 to 36 h of incubation at 30°C by washing cells from the agar surface with 0.05 M sodium-potassium phosphate buffer (pH 7.0). When cells were not used immediately for enzyme assays they were stored at −16°C before use.

Cells used for the isolation of plasmid DNA were obtained from single clones. When it was necessary to ensure retention of the plasmid-encoded phenotype, strains were precultured in minimal medium supplied with an appropriate carbon source known to be degraded by plasmid-specified enzymes. For instance, cells of the wild type, PpC1, and PpCM1 were grown to stationary phase in 50 ml of minimal medium supplied 0.05% *m*-toluate. PpCT1, on the other hand, was precultured in 50 ml of minimal medium supplied with toluene vapor as described by Gibson (8). Such cultures were inoculated (0.25%) into 1-liter batches of L-broth and grown to 2×10^9 cells per ml (absorbancy at 660 nm of 2) before plasmid DNA was isolated by the dye-buoyant density centrifugation method of Johnston and Gunsalus (10). For the detection of plasmid DNA in crude cell lysates, cells were inoculated (0.5%) into 40 ml of L-broth and grown to approximately 2×10^8 cells per ml (50 to 75 Klett units, green filter) before being subjected to the lysis procedure of Hansen and Olsen (9). Bacteria used to supply plasmid molecular weight standards were grown in L-broth as previously described (9, 12). All strains were grown on a rotary shaker at either 30°C for *P. putida* or 37°C

for *E. coli* and *S. typhimurium*.

Isolation of cured strains and segregants. Cured strains as well as segregants of *P. putida* HS1 were isolated as spontaneous mutants after growth with 0.05% benzoate. Single colonies from a nutrient agar plate were grown overnight in 50 ml of nutrient broth at 30°C before transfer of 0.5 ml of cell suspension to 50 ml of minimal medium containing 0.05% benzoate as a sole carbon source. After incubation for 48 h at 30°C on a rotary shaker, 0.5 ml of cell suspension was diluted and plated to minimal agar plates containing 0.05% *m*-toluate plus 0.005% succinate; an additional 0.5 ml was simultaneously transferred to fresh benzoate medium. This procedure was repeated from days 2 to 24, with each culture plated and transferred every 48 h. Incubation on *m*-toluate–succinate agar plates allowed the differentiation of apparent wild-type and cured strains since wild-type colonies grew well on this medium, whereas putative cured strains grew poorly forming colonies surrounded by a brown halo presumed to result from the formation of 3-methylcatechol from *m*-toluate. Single colonies were picked from *m*-toluate–succinate agar plates and purified before replicating to plates with aromatic substrates to establish their phenotype. This procedure led to the isolation of putative cured strains and to two other classes of organisms with intermediate phenotypes.

Manometric experiments. Oxidation rates were measured on freshly harvested cells as previously described, with the assay substrate supplied at 1 mM (11).

Cell-free extracts and enzyme assays. Cell extracts were prepared as described previously (11). Catechol-1,2-oxygenase (EC 1.13.11.1), *cis,cis*-muconate lactonizing enzyme (EC 5.5.1.1), catechol-2,3-oxygenase (EC 1.13.11.2), and 2-hydroxymuconic semialdehyde dehydrogenase were assayed by published methods (11). 2-Hydroxymuconic semialdehyde hydrolase was assayed by measuring the rate of disappearance of 2-hydroxy-6-oxo-2,4-heptadienoate, the *meta* ring-fission product of 3-methylcatechol, which represents the substrate most rapidly hydrolyzed by the enzyme and which is not attacked by 2-hydroxymuconic semialdehyde dehydrogenase, the other ring-fission product-metabolizing enzyme (13, 21, 23). Therefore, though we refer to the enzyme activity as 2-hydroxymuconic semialdehyde hydrolase, it should be noted that the substrate for the activity being measured was not 2-hydroxymuconic semialdehyde but rather 2-hydroxy-6-oxo-2,4-heptadienoate. Benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH, EC 1.2.1.28) were assayed spectrophotometrically by following the rate of NAD^+ reduction at 340 nm as described by Worsey and Williams (27), except that initial velocity was determined within the first 15 to 60 s rather than between 3 and 6 min.

Detection and isolation of aromatic acids produced by strain PpCT1. Strain PpCT1 was grown on agar plates with toluene as the sole carbon source for 36 to 48 h at 30°C, after which the cells were harvested and washed in 0.05 M sodium-potassium phosphate buffer (pH 7.0). Cells were then incubated in 2-liter flasks containing: 20 mM phosphate buffer (pH 7.0) containing 50 μg of chloramphenicol per ml (200 ml); cell suspension (200 ml, or 4 g wet weight); and water to 560 ml, with the aromatic hydrocarbon to be oxidized supplied as a vapor as described by Gibson (8). After 8 h of incubation at 30°C on a rotary shaker, the cells were removed by centrifugation, and the supernatant was acidified with 6 N H_2SO_4 and extracted with diethyl ether. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo at 30°C. The residue was then analyzed by chromatography on thin-layer silica gel plates (Eastman Chromatogram Sheets, type 130R, silica gel with fluorescent indicator). The solvent used for chromatography was petroleum ether-acetone-acetic acid (75:25:1). Compounds were located on chromatograms by viewing with a UV lamp and by spraying with neutralized 0.1% bromocresol green in 95% ethanol. Where sufficient quantities were available, they were recrystallized from ethanol-water and further analyzed by melting-point determination, gas chromatography-mass spectrometry, and infrared spectroscopy as previously described (11).

Production of mutant strains. Auxotrophic mutants of *P. putida* HS1 were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and enriched by counterselection of the wild type in the presence of penicillin G (Sigma Chemical Co.) and D-cycloserine (ICN Nutritional Biochemicals) (18). Selection for auxotrophs was achieved by screening for clones able to grow with 0.05% *m*-toluate supplemented with 0.02% Casamino Acids but unable to grow on 0.05% *m*-toluate or 0.05% succinate minimal medium. In many cases auxotrophs could be detected directly on selection media containing 0.05% *m*-toluate plus 0.005% Casamino Acids since they produced small colonies which, when replica plated to the same medium supplemented with 0.02% Casamino Acids, grew much more profusely.

Streptomycin-resistant mutants were isolated by plating 10^8 to 10^9 cells per ml to nutrient agar containing streptomycin (Sigma Chemical Co.) at 100 μg/ml.

Conjugation experiments. Genetic crosses between strains of *P. putida* HS1, and between *P. putida* HS1 and *P. putida* mt-2, were performed as described previously (11). Selection for exconjugants was achieved by selecting against an auxotrophic donor by omitting its auxotrophic requirement from the medium, or by using streptomycin-resistant recipients and plating to media containing streptomycin (100 μg/ml), thereby preventing growth of the donor. Exconjugants were further selected for by using either *m*-toluate or toluene as sole carbon sources in selection plates.

Isolation of plasmid DNA. Plasmid DNA was purified from cell lysates of strains of *P. putida* HS1 by the cesium chloride-ethidium bromide density gradient method of Johnston and Gunsalus (10). The detection of plasmid DNA in crude cell lysates was accomplished by the method of Hansen and Olsen (9).

Large plasmid molecular weight standards [e.g., TP116, R27, and LT2(cryptic)] from *E. coli* and *S. typhimurium* were isolated by the method of Hansen and Olsen (9). Plasmids designated RP4, Sa, and RSF 1010, which also served as molecular weight markers, were isolated by the method of Meyers et al. (12).

Agarose-gel electrophoresis. Samples of 10 to 30 μl of purified or crude plasmid DNA preparations were subjected to electrophoresis by the method of Meyers et al. (12) in a vertical Lucite EC slab gel apparatus (Thomas Scientific Apparatus, Philadelphia, Pa.) at 100 V (constant voltage) for 3 or 6 h in 0.7% agarose (Seakem EM; FMC Corp., Marine Colloids Div., Rockland, Me.) using Tris-borate buffer. The dimensions of the agarose gel were 13.8 by 17.2 by 0.3 cm. The gel was staind for 30 min in 0.4 μg of ethidium bromide per ml and photographed with a Polaroid MP-3 Land camera equipped with a Wratten K2 (yellow) and 25 (red) filter (Tiffin Optical Co.) using Polaroid (Speed 3000) film (Polaroid no. 117). The gels were illuminated during photography with a shortwave UV light or transilluminator available from Ultraviolet Light Products Inc. The error (~10%) in determining molecular weight by agarose-gel electrophoresis prevented us from assigning precise molecular weight values to plasmids. Nevertheless, this approach did allow relative and approximate size estimations since a linear curve was observed consistently for plasmids with molecular weights (as determined by contour length measurements [9]) ranging between 20×10^6 and 140×10^6 from which molecular weights of unknown plasmids could be determined.

Chemicals. All chemicals were obtained as previously reported (11) or otherwise were purchased from commercial sources.

RESULTS

Isolation, production, and comparative growth studies of strains of *P. putida* HS1. *P. putida* HS1 strain PpC1 resembled *P. putida* mt-2 and other pseudomonads (24, 26) in its ability to utilize toluene and *m*- and *p*-xylene for growth (Table 2). This organism was also able to grow with pseudocumene (1,2,4-trimethylbenzene), 3-ethyltoluene, and the corresponding oxidation products, properties shared with *P. putida* mt-2 and other strains (11). Growth of PpC1 on benzoate led to the formation of spontaneous putative cured strains at frequencies which increased from 5 to 99% with an increasing number of subcultures in benzoate medium. At least three transfers made at 48-h intervals were usually required before any apparent cured strains could be detected.

In addition to the isolation of apparent cured strains after growth with benzoate, two additional derivatives were detected with catabolic phenotypes intermediate between the wild type and cured organisms. One class of mutants, represented by PpCT1, was unable to utilize the xylenes, pseudocumene, and 3-ethyltoluene or the corresponding alcohols, aldehydes, and acids, but was still capable of growth with toluene and benzyl alcohol (Table 2). A second group of variants, represented by PpCM1, was unable to utilize any aromatic hydrocarbons but could still grow with *m*-toluate (Table 2). *p*-Toluate, 3,4-dimethylbenzoate, and 3-ethylbenzoate, however, did not support growth of this class of mutants. Again the frequencies with which these mutants occurred varied depending upon the number of subcultures in benzoate. Strains representative of the PpCM1 type were observed at frequencies as high as 10^{-2}, whereas those of the PpCT1 variety could be isolated at frequencies as high as 10^{-1}. We further observed that mutants with the phenotype of PpCT1 occurred most frequently in benzoate-grown cultures where the amount of curing had not exceeded 30%, whereas in cultures where the frequency of cured strains was high (i.e., >50%) strains having a phenotype similar to PpCT1 were no longer detectable. In contrast, strains of the PpCM1 variety were not detectable until a significant degree of apparent curing had taken place (i.e., ~80%).

TABLE 2. *Growth characteristics of strains of P. putida HS1*

Growth substrate	Growth[a] of strain:				
	PpC1	PpCC1	PpCT1	PpCM1	PpC214
Toluene	+	−	+	−	+
Benzyl alcohol	+	−	+	−	+
Benzaldehyde	+	+	+	+	+
Benzoate	+	+	+	+	+
m-Xylene	+	−	−	−	+
m-Methylbenzyl 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	+	−	−	−	+
3-Ethylbenzoate	+	−	−	−	+

[a] Good growth; −, no growth; ±, poor growth.

Respiratory activities in strains of *P. putida* HS1. The wild-type strain, PpC1, extensively oxidized toluene, *m*-xylene, *p*-xylene, benzoate, *m*-toluate, and *p*-toluate without lag after growth with toluene (Fig. 2). In addition, pseudocumene, 3-ethyltoluene, 3,4-dimethylbenzoic acid, and 3-ethylbenzoic acid were also rapidly oxidized by similarly grown cells as illustrated in Fig. 2. *m*-Toluate-grown cells of PpC1 rapidly oxidized benzoate, *m*-toluate, *p*-toluate, catechol, and 3- and 4-methylcatechol, whereas the parent hydrocarbons were not oxidized under these conditions (Table 3). Succinate-grown cells did not oxidize appreciably any of the hydrocarbons and acids, whereas measurable activ-

ities towards catechol and 3- and 4-methylcatechol could be observed (Table 3). Since PpCM1 was incapable of growth with the aromatic hydrocarbons but was still capable of oxidizing the alkyl-substituted benzoates (e.g., *m*- and *p*-toluate), we tested whether this organism was defective in its inducibility by hydrocarbons. When PpCM1 was grown on succinate and supplied with toluene vapor and the cells were tested for their ability to oxidize toluene, the xylenes, and the corresponding acids and catechols, no oxidation beyond that observed for cells grown with succinate alone could be detected (Table 3). In contrast, the wild type, PpC1, grown similarly, was well induced for the oxidation of these compounds.

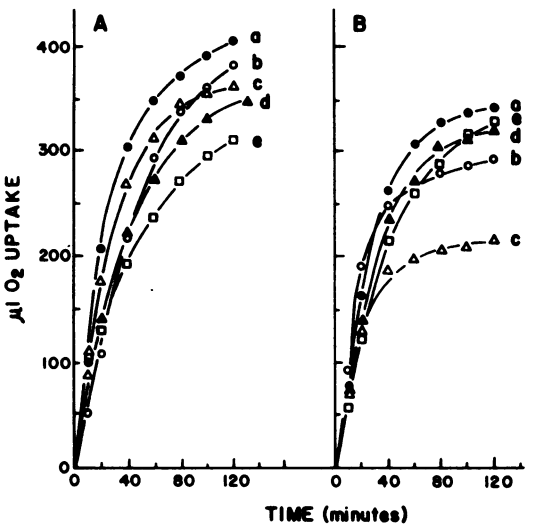

FIG. 2. *Oxidation of aromatic compounds by P. putida HS1 wild type (PpC1) grown on toluene. All results have been corrected for endogenous respiration (35 μl of O_2 consumed per h). (A) Oxidation of aromatic hydrocarbons: a, m-xylene; b, toluene; c, p-xylene; d, 1,2,4-trimethylbenzene; e, 3-ethyltoluene. (B) Oxidation of aromatic acids: a, m-toluic acid; b, benzoic acid; c, p-toluic acid; d, 3,4-dimethylbenzoic acid; e, 3-ethylbenzoic acid.*

When strain PpCT1 was grown on toluene and tested for its ability to oxidize aromatic compounds it was observed that *m*- and *p*-xylene were oxidized at about the same rate as that observed for toluene even though this organism was unable to utilize the xylenes for growth (Fig. 3). PpCT1 also oxidized pseudocumene; however, the extents of oxidation for pseudocumene and *m*- and *p*-xylene were significantly lower than that observed for toluene-grown cells of the wild type (Fig. 2).

Figure 4 illustrates the results obtained when PpC1 and its derivatives PpCM1, PpCT1, and PpCC1 were grown on benzoate and tested for their ability to oxidize the aromatic acids. The results show a similar pattern of oxidation for PpC1 and for PpCM1 (Fig. 4A and B). The relative rates of oxidation by PpC1 and PpCM1 were found to be similar for both strains, and each substrate was oxidized essentially to completion. Strains PpCT1 and PpCC1 exhibited a different pattern of oxidation. As seen in Fig. 4C and D, both PpCT1 and PpCC1 oxidized benzoate at higher initial rates than *m*- and *p*-toluate. Furthermore, the extents of oxidation for *m*-toluate were substantially higher than that observed for *p*-toluate (e.g., 2.5 mol of O_2 per mol of *m*-toluate versus 0.7 mol of O_2 per mol of *p*-toluate). In contrast, PpC1 and PpCM1 (Fig. 4A and B) oxidized benzoate and *m*- and *p*-toluate at about the same initial rates with extents of oxidation approaching completion. Other properties distinguished PpC1 and

TABLE 3. *Oxidation of aromatic compounds by strains of P. putida HS1*

Assay substrate	Rates of oxygen consumption after growth with[a]:					
	PpC1			PpCM1		
	m-Toluate	Toluene/ succinate	Succinate	*m*-Toluate	Toluene/ succinate	Succinate
Toluene	6	138	3	0	3	2
m-Xylene	3	206	1	5	0	3
p-Xylene	3	294	0	5	0	ND
Benzoate	178	189	3	229	2	1
m-Toluate	165	168	5	170	14	1
p-Toluate	117	176	2	175	7	2
Catechol	107	113	80	227	18	22
3-Methylcatechol	110	125	56	200	17	23
4-Methylcatechol	106	ND	20	152	2	0
Succinate	ND	128	96	ND	106	141

[a] Measured by Warburg respirometry and shown in microliters of O_2 consumed per hour per milligram of dry cell weight. Corrected for endogenous rates. ND, Not determined.

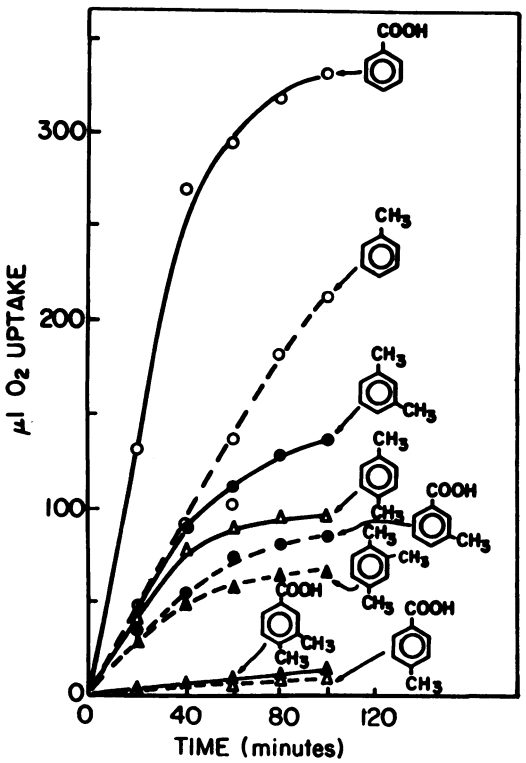

FIG. 3. *Oxidation of aromatic compounds by toluene-grown cells of PpCT1. The results have been corrected for endogenous respiration (47 μl of O_2 consumed per h).*

PpCM1 from PpCT1 and PpCC1, such as their ability to oxidize 3-ethyl- and 3,4-dimethylbenzoate. Both compounds were well oxidized by PpC1 and PpCM1, whereas PpCT1 and PpCC1 were capable of initiating only a limited attack on 3-ethylbenzoate and did not oxidize 3,4-dimethylbenzoate. We further observed that these strains could be distinguished on the basis of their ability to oxidize all three isomers of fluorobenzoate (unpublished data). For instance, benzoate-grown cells of PpC1 and PpCM1 were incapable of oxidizing the fluorobenzoates, whereas PpCT1 and PpCC1 rapidly oxidized all three isomers. On the basis of these results it was concluded that PpC1 and PpCM1 were capable of inducing a toluate oxidase (TO), whereas PpCT1 and PpCC1 were not, but retained the ability to elaborate a benzoate oxidase.

Enzyme activities in strains of *P. putida* HS1. As illutrated in Table 4, extracts of PpC1 contained high levels of enzymes of the *meta*-fission pathway when cells were grown with toluene, benzoate, or *m*-toluate, whereas the enzymes of the *ortho*-fission pathway were present at low levels under these conditions. The levels of BADH and BZDH were substantially higher in toluene-grown than in benzoate-, *m*-toluate-, or succinate-grown cells (Table 4). By contrast, strain PpCC1 exhibited undetectable levels of the *meta*-fission enzymes in benzoate-grown cells, whereas the activities of catechol-1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme were high. When cell extracts of PpCC1 were incubated with catechol, the formation of β-ketoadipate could be shown by the Rothera reaction (20). Whereas low but measurable levels of BADH and BZDH could be detected in either benzoate- or succinate-grown cells of the wild type, these activities were effectively absent in similarly grown cells of the putative cure, PpCC1 (Table 4). Similar results were also obtained for catechol-2,3-oxygenase, 2-hydroxymuconic semialdehyde hydrolase, and 2-hydroxymuconic semialdehyde dehydrogenase (Table 4).

From respiratory studies it was shown that PpCT1 resembled the cure, PpCC1, in its pattern of oxidation of benzoic acid and toluic acids, but differed in its ability to grow with and to

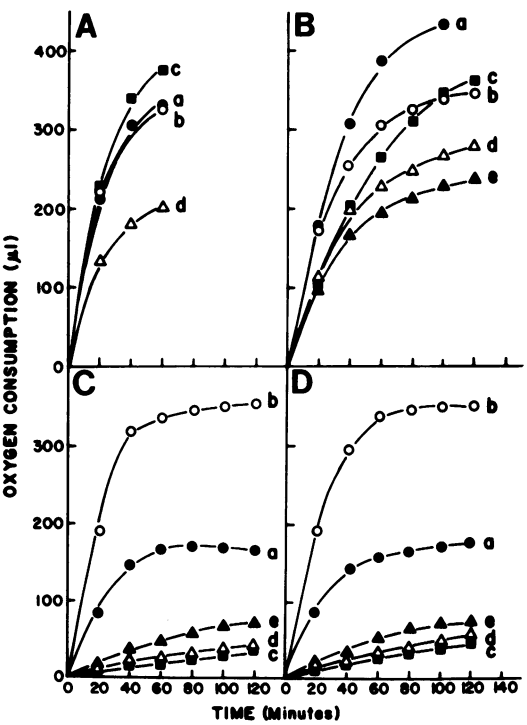

FIG. 4. *Oxidation of aromatic carboxylic acids by strains of P. putida HS1 grown on benzoic acid. All results were corrected for endogenous respiration (40 μl of O_2 consumed per h). (A) PpC1 (wild type), (B) PpCM1, (C) PpCT1, (D) PpCC1. (a) m-Toluic acid; (b) benzoic acid; (c) 3,4-dimethylbenzoic acid; (d) p-toluic acid; (e) 3-ethylbenzoic acid.*

TABLE 4. *Enzyme activities in strains of P. putida HS1*

Strain/growth conditions	Sp act (nmol/min per mg of protein)[a]						
	BADH	BZDH	C230	HMSH	HMSD	C120	MLE
PpC1 (wild type)							
Toluene	1,477	269	730	169	48	8	<0.05
Benzoate	28	45	1,100	590	102	17	100
m-Toluate	6	32	500	500	260	1	<0.05
Succinate	22	7	83	57	8	0.1	<0.05
PpCT1							
Toluene	923	262	<0.04	0.5	0.2	71	92
Benzoate	37	12	<0.04	0.1	<0.04	407	800
Succinate	17	7	<0.04	0.1	<0.04	0.05	0.08
PpCM1							
Benzoate	0.7	59	1,430	1,700	143	24	206
m-Toluate	1	17	283	371	28	0.2	0.3
Succinate	0.6	3	33	52	3	0.2	1
PpCC1							
Benzoate	0.7	1	<0.04	0.2	<0.04	240	624
Succinate	<0.1	3	<0.04	<0.06	<0.04	<0.05	<0.05

[a] Notations for enzymes assayed are as illustrated in Fig. 1.

oxidize toluene. When cell extracts of PpCT1 were examined, it was found that toluene-grown cells had high levels of BADH, BZDH, catechol-1,2-oxygenase, and *cis,cis*-muconate lactonizing enzyme, whereas activities of the *meta*-fission enzymes could not be detected (Table 4). These results suggested that PpCT1 was capable of metabolizing toluene exclusively via the *ortho* pathway. This hypothesis was supported by demonstrating the formation of β-ketoadipate by the Rothera reaction (20) when cell extracts of toluene-grown cells were incubated with catechol. Growth of PpCT1 on benzoate also resulted in the induction of the *ortho* enzymes and not the enzymes of the *meta* pathway (Table 4). Furthermore, cell extracts prepared from succinate-grown cells had undetectable levels of the *meta*-pathway enzymes in contrast to the low levels observed for similarly grown cells of the wild type (Table 4). BADH and BZDH activities in cell extracts of either benzoate- or succinate-grown cells of PpCT1 were similar to the basal levels of these activities as measured in the wild type when grown with the same substrates.

When enzyme activities were measured in strain PpCM1 it was found that benzoate- or *m*-toluate-grown cells were induced for the *meta*-fission enzymes (Table 4). Growth with benzoate also resulted in partial induction of enzymes of the *ortho*-fission pathway as evidenced by measurable activities recorded for catechol-1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme. Similar results were obtained with the wild type, PpC1, when grown on benzoate. Differences between the wild type and PpCM1 were noted, however, with respect to the levels of BADH when cells were grown on noninducing substrates (Table 4). Whereas negligible basal levels of BADH were observed in PpCM1 grown with benzoate, *m*-toluate, or succinate, in PpC1 this activity remained detectable in cells grown on the same substrates. An assessment of the levels of BZDH in this organism was more difficult since, as shown for *P. putida* mt-2 (27), two possible enzymes, one coded for by the plasmid and the other by the chromosome, could contribute to the overall activity. We were unable to conclude, therefore, whether the low but measurable activities of BZDH observed in uninduced cells of PpCM1 were a consequence of the action of one or more enzymes (Table 4).

Though the initial enzyme catalyzing the oxidation of toluene or the xylenes to the corresponding alcohols, which we refer to as toluene hydroxlyase, was not assayed in crude cell extracts, the inability of intact cells of PpCM1 to oxidize toluene or the xylenes after growth in the presence of toluene as an inducer (Table 3) suggests that this organism is also incapable of being induced for this activity.

Isolation of metabolic products produced by PpCT1. PpCT1 was able to utilize toluene but not *m*- and *p*-xylene for growth, and yet cells grown on toluene exhibited a significant oxidation of these hydrocarbons (Fig. 3). When toluene-grown cells were incubated with *p*-xylene in the presence of chloramphenicol, and the culture filtrate was extracted at acid pH (pH 2.0) and analyzed by thin-layer chromatography, a product (122 mg/g of wet cell weight) was shown to

accumulate. The product was shown to have an R_f value (R_f, 0.28) similar to that of *p*-toluic acid and gave an acidic reaction with bromocresol green spray reagent. Confirmation of its identity was provided by its melting point (m.p. 179°C), infrared spectrum, and gas chromatographic-mass spectral analysis, which gave a parent molecular ion with $m/e = 136$ and a fragmentation pattern identical with that of authentic *p*-toluic acid. In contrast with these results, the incubation medium from cells incubated with *m*-xylene contained no readily extractable acidic products. When analogous experiments were performed with pseudocumene, 3,4-dimethylbenzoate (6 mg/g of wet cell weight) was shown to accumulate; however, no acidic product was detected in incubations using 3-ethyltoluene.

Conjugative transfer of the TOL phenotype in *P. putida* HS1. *P. putida* HS1 had the ability to degrade toluene, *m*- and *p*-xylene, pseudocumene, and 3-ethyltoluene which was lost in an apparent cured strain as represented by PpCC1. This ability could be transferred into cured recipients of *P. putida* HS1 or into a cured recipient of *P. putida* mt-2 (Table 5). Genetic transfer of the TOL-related phenotype was accomplished by mating the wild type, PpC1, with a streptomycin-resistant derivative of the apparent cured strain, PpCC11, or alternatively by mating an auxotrophic derivative of PpC1 (e.g., PpC14) with PpCC1 or PpCC11. Exconjugants were selected for their ability to utilize *m*-toluate or toluene. In cases where an auxotroph was used as the donor, the transfer frequency was approximately 1,000-fold greater than when the parent strain was used (Table 5). Furthermore, the ability of PpC14, an auxotrophic derivative of PpC1, to transfer its properties into a cured recipient did not appear to be markedly affected by the presence of streptomycin in the medium, as indicated from matings performed between PpC14 and PpCC1 and between PpC14 and PpCC11 (Table 5). The substantially lower transfer frequency noted for PpC1 as compared with its auxotrophic derivative, PpC14, cannot be explained at this time. However, previous reports have shown that differences in transfer frequencies for the TOL (pWWO) plasmid may occur between prototrophic and auxotrophic donors of *P. putida* mt-2 (14, 24).

TABLE 5. *Conjugative transfer of catabolic functions in strains of P. putida*

Donor	Recipient[a]	Selection	Frequency of transfer[b]
PpC1[c]	PpCC11	*m*-Toluate	$1 \times 10^{-6} - 1 \times 10^{-5}$[d]
PpC1[c]	PpCC11	Toluene	5×10^{-6}[d]
PpC14[e]	PpCC1	*m*-Toluate	5×10^{-3}
PpC14[e]	PpCC11	*m*-Toluate	5×10^{-3}[d]
PpC14[e]	PaM3	*m*-Toluate	1×10^{-4}
PaW15[f]	PpCC1	*m*-Toluate	7×10^{-3}

[a] Reversion frequency to *m*-toluate$^+$ was undetectable ($<10^{-9}$).

[b] Expressed as the number of exconjugants per donor cell.

[c] Did not spontaneously mutate to streptomycin resistance at a frequency of $>10^{-9}$.

[d] Streptomycin added to selection agar at 100 μg/ml.

[e] Reversion frequency to *leu*$^+$ *ileu*$^+$ *val*$^+$ *his*$^+$ ($<10^{-9}$).

[f] Reversion frequency to *leu*$^+$ ($<10^{-9}$).

When 150 exconjugants obtained from matings between strains of *P. putida* HS1 were tested and scored for the ability to utilize toluene, the xylenes, pseudocumene, and 3-ethyltoluene, as well as the corresponding alcohols, aldehydes, and acids, all were able to do so. The TOL-related phenotype from *P. putida* HS1 could also be transferred to a cured recipient of *P. putida* mt-2 with about the same transfer frequency as that observed with an apparent cured recipient of *P. putida* HS1 (PpCC1) (Table 5). We further observed that the same cured strain of *P. putida* HS1 could also serve as a recipient in crosses where *P. putida* mt-2 served as a donor of the TOL (pWWO) plasmid. Exconjugants obtained from these matings regained the ability to utilize all of the aromatic substrates capable of supporting growth of the wild-type donor strains.

Isolation and size estimation of plasmid DNA. Plasmid DNA from strains of *P. putida* HS1 was isolated by the dye-buoyant density method of Johnston and Gunsalus (10). By this method, plasmid DNA could be isolated from cell lysates of PpC1, PpCM1, PpCT1, and PpCC1, as revealed by distinct satellite DNA bands visible in gradients upon illumination with UV light. Examination of the DNA from PpC1 by agarose-gel electrophoresis revealed a single plasmid species, as evidenced by only one DNA-containing band in agarose gels (Fig. 5). Figure 6 shows that a similar migrating plasmid could also be detected when crude cell lysates of the wild type and of an exconjugant (PpC214) obtained by mating PpC1 with the putative cured strain, PpCC1, were analyzed by electrophoresis.

Examination of plasmid DNA isolated from PpCC1 also showed it to contain a single plasmid species (Fig. 5). This plasmid migrated considerably faster than that obtained from the wild type (PpC1), and estimations of molecular weights by comparison with the migration pattern of standard plasmids gave size estimates of 120×10^6 for the wild-type plasmid (pDK1) and 20×10^6 for the plasmid isolated from PpCC1 (pDKC1) (Table 6). Efforts to detect the small 20×10^6-molecular-weight plasmid in crude cell

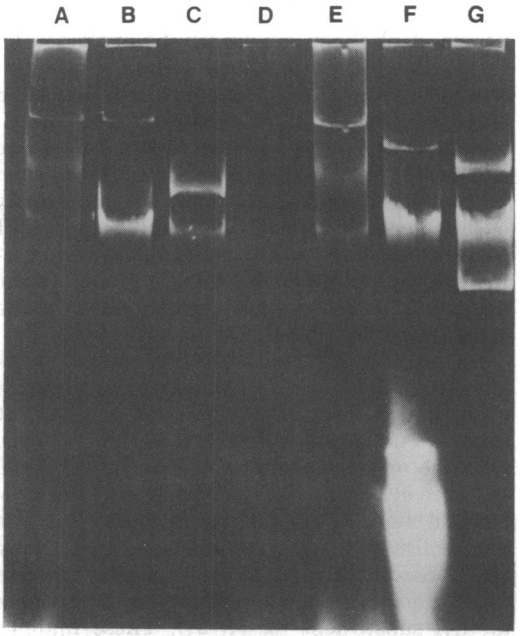

FIG. 5. *Comparison of electrophoretic mobility of plasmid DNA (pDK1) isolated from PpC1 by (A) the crude-lysate isolation procedure of Hansen and Olsen (9) or (B) the dye-equilibrium density-gradient centrifugation method of Johnston and Gunsalus (10). The top bands shown in lanes B and C represent plasmid DNA, and the bottom band is due to chromosomal fragments not removed in the isolation procedure. (C) Plasmid DNA from PpCC1(pDKC1) isolated by the method of Johnston and Gunsalus (10). Plasmids from strains (D) DT78 (TP116); (E) DT41 (R27); (F) LT2 (cryptic); (G) J5 (RP4) (top band), C600 (RSF1010) (bottom band), middle band due to chromosomal DNA fragments. The time of electrophoresis was 3 h.*

lysates of PpCC1 prepared by the method of Hansen and Olsen (9) were unsuccessful. Similarly, when the exconjugant, PpC214, was analyzed for plasmid DNA by the same method, a large plasmid presumed to harbor the TOL degradative genes (pDK1) was detected, whereas the smaller 20 × 10^6-molecular-weight species (pDKC1) was again undetectable (Fig. 6). We concluded from these findings that the small pDKC1 plasmid was not extractable by the Hansen and Olsen (9) method. Further physical studies on the plasmid content of strain PpC214 are currently in progress.

Analysis of plasmid DNA from PpCM1 and PpCT1 by electrophoresis showed each strain to contain a single plasmid species with molecular weights estimated at 100 × 10^6 and 80 × 10^6, respectively (Table 6). Plasmids identical to those isolated by density-gradient centrifugation were detected in PpCM1 and PpCT1 when crude cell lysates were subjected to electrophoresis (Fig. 6).

DISCUSSION

In a previous communication we reported that *P. putida* HS1 was capable of growth with toluene, *m*- and *p*-xylene, pseudocumene, 3-ethyltoluene, and the corresponding benzoic acids (11). In this paper we show that the metabolism of these compounds by *P. putida* HS1 is similar to that previously described for *P. putida* mt-2 (11, 13, 23, 27) and other pseudomonads (3, 4, 15, 16, 24), involving oxidation of a methyl substituent to form the corresponding benzoates followed by reactions of the *meta*-fission pathway. For example, whole cells oxidized the par-

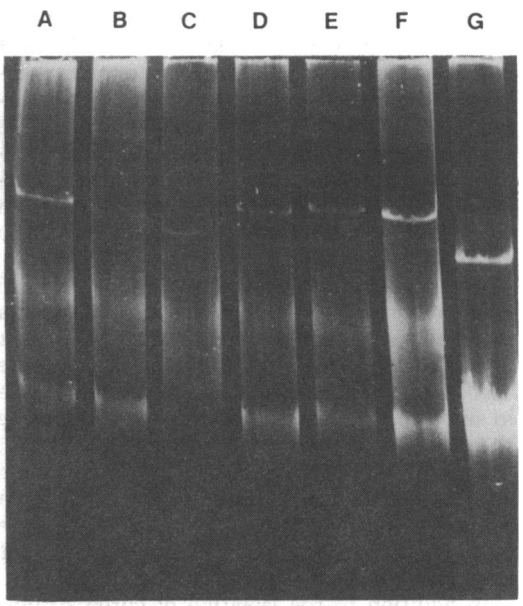

FIG. 6. *Agarose-gel electrophoresis of plasmid DNA from strains of P. putida HS1 isolated by the procedure of Hansen and Olsen (9). Samples on the gel are as follows: (A) PpC1(pDK1); (B) PpCM1-(pDKM1); (C) PpCT1(pDKT1); (D) PpCT214, exconjugant from a mating of PpC1 with PpCC1; (E) DT78 (TP116); (F) DT41(R27); (G) LT2(cryptic). The time of electrophoresis was 6 h.*

TABLE 6. *Size estimation of plasmids from P. putida HS1*[a]

Strain	Plasmid designation	Plasmid size (×10^6 ± standard error)
PpC1	pDK1	120 ± 12
PpCM1	pDKM1	100 ± 10
PpCT1	pDKT1	80 ± 8
PpCC1	pDKC1	20 ± 2

[a] By agarose-gel electrophoresis.

ent hydrocarbons and acids after growth on toluene (Fig. 2), and cell extracts prepared from toluene- or benzoate-grown cells were induced for enzymes of the *meta* pathway (Table 4).

Repeated cultivation of *P. putida* HS1 on benzoate led to the production of strains which lost the ability to utilize the hydrocarbons and corresponding oxidation products but were still capable of growth with benzaldehyde and benzoate (Table 2). Similar findings were reported for *P. putida* mt-2 (23) and other pseudomonads (24) capable of growth with toluene and the xylenes and are consistent with loss of a plasmid-encoded pathway for the degradation of these compounds. The finding that an apparent cured strain of *P. putida* HS1, PpCC1, was induced for the *ortho* enzymes after growth on benzoate whereas the *meta* enzymes were absent (Table 4) also provides evidence that this strain has lost a TOL-type plasmid coding for the *meta* pathway. Further evidence that *P. putida* HS1 carries a TOL plasmid was suggested by genetic experiments in which the ability to utilize toluene, the alkyl-substituted toluenes, and breakdown products thereof could be transferred from the wild type into a cured recipient and also into a cured derivative of *P. putida* mt-2 (Table 5). These findings are consistent with our physical evidence for a plasmid in *P. putida* HS1 with a molecular weight of 120×10^6, which is absent from a putative cured strain unable to elaborate the TOL functions (PpCC1) but is recoverable in an exconjugant (PpC214) obtained by mating the wild type (PpC1) with PpCC1 (Fig. 5 and 6). The ability of the cured strain (PpCC1) to serve as a recipient of the TOL (pWWO) plasmid from *P. putida* mt-2 indicates that either strain is capable of providing a suitable background for expression of the TOL-related phenotype.

In addition to the isolation of cured strains, two additional classes of mutants were selected during growth on benzoate. One class, represented by PpCT1, was still capable of growth with toluene and benzyl alcohol but had lost the ability to utilize alkyl-substituted toluenes and corresponding intermediates (Table 2). Analysis of PpCT1 revealed that it had lost the activities of a TOL-type TO and enzymes of the *meta* pathway but was still able to synthesize toluene hydroxylase, BADH, and BZDH. Further analysis revealed that growth of PpCT1 on toluene or benzoate resulted in the induction of catechol-1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme, and crude extracts from these cells catalyzed the degradation of catechol to β-ketoadipate (Table 4). These results are consistent with a catabolic pathway for toluene and benzoate degradation which proceeds via the *ortho* (β-ketoadipate) pathway. Thus the ability of PpCT1 and strains like it to utilize toluene and benzyl alcohol can apparently be explained by their ability to utilize a combination of plasmid- and chromosomal-encoded enzymes. That is, the plasmid-encoded toluene hydroxylase, BADH, and BZDH facilitate the conversion of toluene and benzyl alcohol to benzoate, which can then be completely degraded via the chromosomally encoded *ortho* pathway. The inability of PpCT1 to utilize the alkyl-substituted toluenes and corresponding benzoates is evidently related to the absence of a TO complex and enzymes of the *meta*-fission sequence.

Intact cells of PpCT1 oxidized *m*- and *p*-xylene, pseudocumene, and 3-ethyltoluene (Fig. 3) even though these compounds did not support growth. Furthermore, incubation of PpCT1 with *p*-xylene or pseudocumene led to the isolation, respectively, of *p*-toluic acid and 3,4-dimethylbenzoid acid. These findings indicate that the metabolism of these hydrocarbons is initiated by reactions similar to those previously reported in other organisms accomplishing oxidation of a methyl substituent (3, 11, 27). These findings also provide evidence that this strain retains the ability to elaborate a plasmid-encoded toluene hydroxylase and BADH, and presumably a plasmid-determined BZDH as well, all of which facilitate the oxidation of the hydrocarbons to the corresponding acids; however, because of the lack of a functional TO and subsequent *meta* enzymes, their further degradation cannot be effected. The inability to detect the formation of *m*-toluic acid from *m*-xylene or of 3-ethylbenzoic acid from 3-ethyltoluene is apparently due to the ability of these acids to serve as substrates for the chromosomal-specified benzoate oxidase, which can be shown to catalyze their further degradation (Fig. 4). *p*-Toluic acid and 3,4-dimethylbenzoic acid, on the other hand, do not appear to serve as substrates for the chromosomal-determined benzoate oxidase but are well oxidized by strains capable of inducing a TOL-type TO (Fig. 4). Thus the accumulation of *p*-toluate and 3,4-dimethylbenzoate by PpCT1 would imply that this strain cannot elaborate a plasmid-encoded TO. This is supported by oxygen uptake experiments which showed that benzoate-grown cells of PpCT1 or the cured strain, PpCC1, were unable to induce a plasmid-specified TO as evidenced by poor oxidation of the alkyl-substituted benzoates (Fig. 4). These results are consistent with previous reports demonstrating differences in the substrate specificity shown towards methyl-substituted benzoates by organisms capable of elaborating a TO or benzoate oxidase complex (11, 19, 28).

A second class of mutants selected after growth on benzoate lost the ability to utilize the

aromatic hydrocarbons but were still able to grow with *m*-toluate (Table 2). Cell extracts prepared from one such mutant, PpCM1, showed that growth with benzoate of *m*-toluate resulted in the induction of the *meta* pathway, whereas the *ortho* enzymes were present at low levels only (Table 4). Intact cells of PpCM1 grown on succinate in the presence of toluene as an inducer did not oxidize toluene, the xylenes, or the corresponding benzoates and catechols (Table 3). These observations indicate that the hydrocarbons do not induce either early enzymes responsible for oxidation of the hydrocarbons to the levels of the acids, or later enzymes, including those of the *meta* pathway, in this strain. Furthermore, cell extracts of benzoate-, *m*-toluate-, or succinate-grown cells contained basal levels of BADH which were significantly lower than the levels of this activity in the wild type grown under similar conditions (Table 4). These results also suggest that PpCM1 is incapable of synthesizing a TOL-type BADH. At this time we are unable to conclude whether this organism has also lost the ability to specify a plasmid-encoded BZDH because of complications with measurement of this activity caused by the presence of a second BZDH activity presumably encoded by the chromosome.

Observations with PpCM1 indicate that its inability to utilize hydrocarbons can be attributed to its inability to elaborate early enzymes necessary for their oxidation. PpCM1 is still capable, however, of growth with *m*-toluate since this substrate induces the *meta* pathway required for its degradation (Table 4). The lack of growth on *p*-toluate, 3,4-dimethylbenzoate, and 3-ethylbenzoate, even though this strain is capable of elaborating a functional *meta* pathway, is thought to be due to the inability of these compounds to serve as inducers of the plasmid-specified TO and of enzymes of the *meta* pathway. This hypothesis is supported by the finding that benzoate- or *m*-toluate-grown cells of PpCM1 rapidly oxidized all of the alkyl-substituted benzoates, indicating that the structural genes for TO are present (Fig. 4; Table 3). Thus the inability of PpCM1 to utilize *p*-toluic acid, 3,4-dimethylbenzoic acid, and 3-ethylbenzoic acid for growth cannot be due to the absence of a plasmid-specified TO but rather appears to be related to an altered pattern of regulation of synthesis of this system.

The results obtained with the wild type, PpC1, indicate that growth with toluene leads to the induction of toluene hydroxylase, BADH, and BZDH, as well as enzymes of the *meta* pathway, whereas growth on benzoate or *m*-toluate leads to induction of the *meta* enzymes only (Table 4). These results agree with previous reports which implicated the involvement of at least two separate operons in the degradation of toluene and *m*- and *p*-xylene, of which one is inducible by the hydrocarbons (and alcohols) and the other is induced by the acids (and aldehydes) (26–28).

The segregation of mutant strains by *P. putida* HS1 during growth on benzoate parallels previous reports of similar results with *P. putida* mt-2 (23) and other pseudomonads (24) carrying TOL plasmids. The selection of strains with the catabolic phenotypes characteristic of PpCT1 and PpCM1 has, however, not previously been reported for other TOL-carrying organisms. Williams and Worsey (24) reported the isolation of a second class of mutants from several of their isolates, which were unable to utilize *m*-toluate but could still grow with toluene and *m*-xylene. These workers concluded from the analysis of one such mutant (*Pseudomonas* sp. strain MT 20-B3) that a regulatory mutation, probably a deletion, was responsible for blocking the induction of a plasmid-encoded TO and enzymes of the *meta* pathway by *m*-toluate and benzoate but not by toluene and *m*-xylene (28). Failure to observe the segregation of variants having this catabolic phenotype may be related to differences in molecular structure between the TOL plasmid in HS1 and that in *Pseudomonas* sp. strain MT 20.

Worsey and Williams (28) proposed that the selection of cured strains or derivatives having a phenotype similar to that of strain MT 20-B3 as described above was favored during growth on benzoate since these strains used the chromosomal *ortho* pathway for the dissimilation of benzoate and grew more rapidly than the parent organisms. Though we have not tested the growth rates of strains of *P. putida* HS1 on benzoate, the same argument could account for the occurrence of strains with the phenotypes of PpCC1 and PpCT1, both of which strains use the *ortho* pathway for the degradation of benzoate. The selection of mutants having the phenotype of PpCM1 is more difficult to explain since these mutants to not appear to metabolize benzoate exclusively via the chromosomal pathway.

The pleiotropic and nonrevertible nature of segregants obtained from *P. putida* HS1 after growth on benzoate, as represented by PpCM1, PpCT1, and PpCC1, indicates that these strains have suffered deletions in the TOL (pDK1) plasmid. The isolation of single plasmid DNA species with notably smaller molecular weights than the wild-type plasmid from these derivatives by two separate approaches supports this conclusion (Table 6). These findings help to explain why these strains are capable of expressing only a portion (e.g., PpCM1, PpCT1) or none (e.g.,

PpCC1) of the wild-type TOL-encoded degradative functions. Thus the results we have presented here suggest that the TOL (pDK1) plasmid has undergone deletions giving rise to smaller replicons as represented by pDKM1, pDKT1, and pDKC1. Spontaneous loss of all TOL functions in the putative cured strain, PpCC1, by the apparent deletion of 100×10^6 daltons from the native TOL (pDK1) plasmid is analogous to results obtained in *P. putida* mt-2, wherein a similar deletion of 27×10^6 daltons from the TOL (pWWO) plasmid resulted in loss of the TOL-related phenotype (1). Further studies on the physical nature of the genetic information present in derivatives of *P. putida* HS1 and the molecular mechanisms by which these variants arise will be communicated separately.

ACKNOWLEDGMENTS

This research was supported in part by a grant-in-aid from the University of Minnesota Graduate School. D.A.K. was supported by a Public Health Service predoctoral traineeship grant (1-T32-CA-09138) from the National Cancer Institute.

We are grateful to Larry McKay, Ron Olsen, and Jim Johnston for making strains available and for helpful discussions. We are also indebted to Tom Krick for assistance in the operation of the mass spectrometry facilities provided by the Minnesota Agricultural Experiment Station, and to Kimberly F. Potratz-Gul and Steve Kuhl for their expert technical assistance with certain aspects of this work.

LITERATURE CITED

1. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154**:203–304.
2. **Chakrabarty, A. M.** 1976. Plasmids in *Pseudomonas*. Annu. Rev. Genet. **10**:7–30.
3. **Davey, J. F., and D. T. Gibson.** 1974. Bacterial metabolism of *para*-and *meta*-xylene: oxidation of a methyl substituent. J. Bacteriol. **119**:923–929.
4. **Davis, R. S., F. E. Hossler, and R. W. Stone.** 1968. Metabolism of *p*- and *m*-xylene by species of *Pseudomonas*. Can. J. Microbiol. **14**:1005–1009.
5. **Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda.** 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. **130**:1274–1280.
6. **Feist, C. F., and G. D. Hegeman.** 1969. Phenol and benzoate metabolism in *Pseudomonas putida*: regulation of tangential pathways. J. Bacteriol. **100**:869–877.
7. **Friello, D. A., J. R. Mylroie, D. T. Gibson, J. E. Rogers, and A. M. Chakrabarty.** 1976. XYL, a nonconjugative xylene degradative plasmid in *Pseudomonas* Pxy. J. Bacteriol. **127**:1217–1224.
8. **Gibson, D. T.** 1976. Initial reactions in the bacterial degradation of aromatic hydrocarbons. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B **162**:157–168.
9. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
10. **Johnston, J. B., and I. C. Gunsalus.** 1977. Isolation of metabolic plasmid DNA from *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **75**:13–19.
11. **Kunz, D. A., and P. J. Chapman.** 1981. Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida* (*arvilla*) mt-2: evidence for new functions of the TOL (pWWO) plasmid. J. Bacteriol. **146**:179–191.
12. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
13. **Murray, K., C. J. Duggleby, J. M. Sala-Trepat, and P. A. Williams.** 1972. The metabolism of benzoate and methylbenzoates via the *meta*-cleavage pathway by *Pseudomonas arvilla* met-2. Eur. J. Biochem. **28**:301–310.
14. **Nakazawa, T., and T. Yokata.** 1977. Isolation of a mutant TOL plasmid with increased activity and transmissibility from *Pseudomonas putida* (*arvilla*) mt-2. J. Bacteriol. **129**:39–46.
15. **Omori, T., S. Horiguchi, and K. Yamada.** 1967. Studies on the utilization of hydrocarbons by microorganisms. X. Screening of aromatic hydrocarbon-assimilating microorganisms and *p*-toluic acid formation from *p*-xylene. Agric. Biol. Chem. **31**:1337–1342.
16. **Omori, T., and K. Yamada.** 1969. Studies on the utilization of hydrocarbons by microorganisms. XIII. Oxidation of *m*-xylene and pseudocumene by *Pseudomonas aeruginosa*. Agric. Biol. Chem. **33**:979–985.
17. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. **241**:3800–3810.
18. **Ornston, L. N., M. K. Ornston, and G. Chou.** 1969. Isolation of spontaneous mutant strains of *Pseudomonas putida*. Biochem. Biophys. Res. Commun. **36**:179–184.
19. **Reineke, W., and H. J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. Biochim. Biophys. Acta **542**:412–423.
20. **Rothera, A. C. H.** 1908. Note on the nitroprusside reaction for acetone. J. Physiol. **37**:491–494.
21. **Sala-Trepat, J. M., K. Murray, and P. A. Williams.** 1972. The metabolic divergence in the *meta*-cleavage of catechols by *Pseudomonas putida* NCIB 10015. Eur. J. Biochem. **28**:347–356.
22. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159–273.
23. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
24. **Williams, P. A., and M. J. Worsey.** 1976. Ubiquity of plasmids coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bacteriol. **125**:818–828.
25. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas putida* mt-2. Genet. Res. **23**: 227–232.
26. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL (pWWO) plasmid from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
27. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and the xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.
28. **Worsey, M. J., and P. A. Williams.** 1977. Characterization of a spontaneously occurring mutant of the TOL20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. J. Bacteriol. **130**:1149–1158.