JOURNAL OF BACTERIOLOGY, Sept. 1981, p. 836-843
0021-9193/81/090836-08$02.00/0

Vol. 147, No. 3

Plasmid- and Chromosome-Mediated Dissimilation of Naphthalene and Salicylate in *Pseudomonas putida* PMD-1

MARTHA C. ZUNIGA,[1] DON R. DURHAM,[2]†* AND ROD A. WELCH[2]‡

Department of Biology, Yale University, New Haven, Connecticut 06520,[1] and Department of Microbiology, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298[2]

Received 3 March 1981/Accepted 10 June 1981

Pseudomonas putida PMD-1 dissimilates naphthalene (Nah), salicylate (Sal), and benzoate (Ben) via catechol which is metabolized through the *meta* (or α-keto acid) pathway. The ability to utilize salicylate but not naphthalene was transferred from *P. putida* PMD-1 to several *Pseudomonas* species. Agarose gel electrophoresis of deoxyribonucleic acid (DNA) from PMD-1 and Sal^+ exconjugants indicated that a plasmid (pMWD-1) of 110 megadaltons is correlated with the Sal^+ phenotype; restriction enzyme analysis of DNA from Sal^+ exconjugants indicated that plasmid pMWD-1 was transmitted intact. Enzyme analysis of Sal^+ exconjugants demonstrated that the enzymes required to oxidize naphthalene to salicylate are absent, but salicylate hydroxylase and enzymes of the *meta* pathway are present. Thus, naphthalene conversion to salicylate requires chromosomal genes, whereas salicylate degradation is plasmid encoded. Comparison of restriction digests of plasmid pMWD-1 indicated that it differs considerably from the naphthalene and salicylate degradative plasmids previously described in *P. putida*.

The biochemical pathways for the dissimilation of numerous aromatic compounds in bacteria have been elucidated (9, 28). The catabolism of many of these compounds proceeds through catechol or substituted catechols, which can be further oxidized by two distinct sets of enzymes (Fig. 1): those of the *ortho*-cleavage pathway (β-ketoadipate pathway; 25) and those of the *meta*-cleavage pathway (α-keto acid pathway; 3, 22, 25). Genetic analysis of strains of *Pseudomonas putida* has revealed that for certain aromatic compounds, particularly those which are dissimilated through the *meta* pathway, the genes encoding some, if not all, of the enzymes for a given degradative pathway are plasmid borne (7). Conjugative extrachromosomal elements conferring metabolic specificity for the degradation of salicylic acid (SAL), naphthalene (NAH), and xylenes and toluenes (TOL) have been isolated and studied (6, 10, 32, 33). Nonconjugative degradative plasmids have also been described, including one specific for xylenes and toluenes (XYL; 13).

Some conjugative plasmids have been reported to be transferred interspecifically (5, 6). Furthermore, recent evidence suggests that certain degradative plasmid gene clusters are transposable (8, 17, 23). Thus it is possible that the nutritional versatility of bacteria can be achieved by transposition or by plasmid transfer similar to R factor-determined drug resistance in several genera of bacteria or by both (4). An added feature is introduced in the case of degradative pathways since catabolism of polyaromatic compounds involves several enzymatic reactions and generates intermediates which are potential substrates for chromosomally encoded enzymes (25). There is evidence for some catabolic pathways that host chromosome and plasmid both contribute genetic information specifying enzymes in a degradative sequence (7, 34).

In this communication, a *Pseudomonas* strain capable of degrading naphthalene and salicylic acid via *meta*-cleavage of catechol is described. It is shown that the ability to utilize these compounds is associated with the presence of a plasmid which is transmissible to other strains of *Pseudomonas*. Genetic and biochemical evidence is presented which indicates that the conversion of naphthalene to salicylate requires chromosomal functions, whereas salicylate catabolism through the *meta* pathway is plasmid encoded. Restriction enzyme analysis of plasmid DNA is presented which reveals that this plasmid differs significantly from the NAH and SAL plasmids described by others (6, 10).

† Present address: Genex Corporation Laboratories, Rockville, MD 20852.

‡ Present address: Department of Microbiology and Immunology, University of Washington, Seattle, WA 98195.

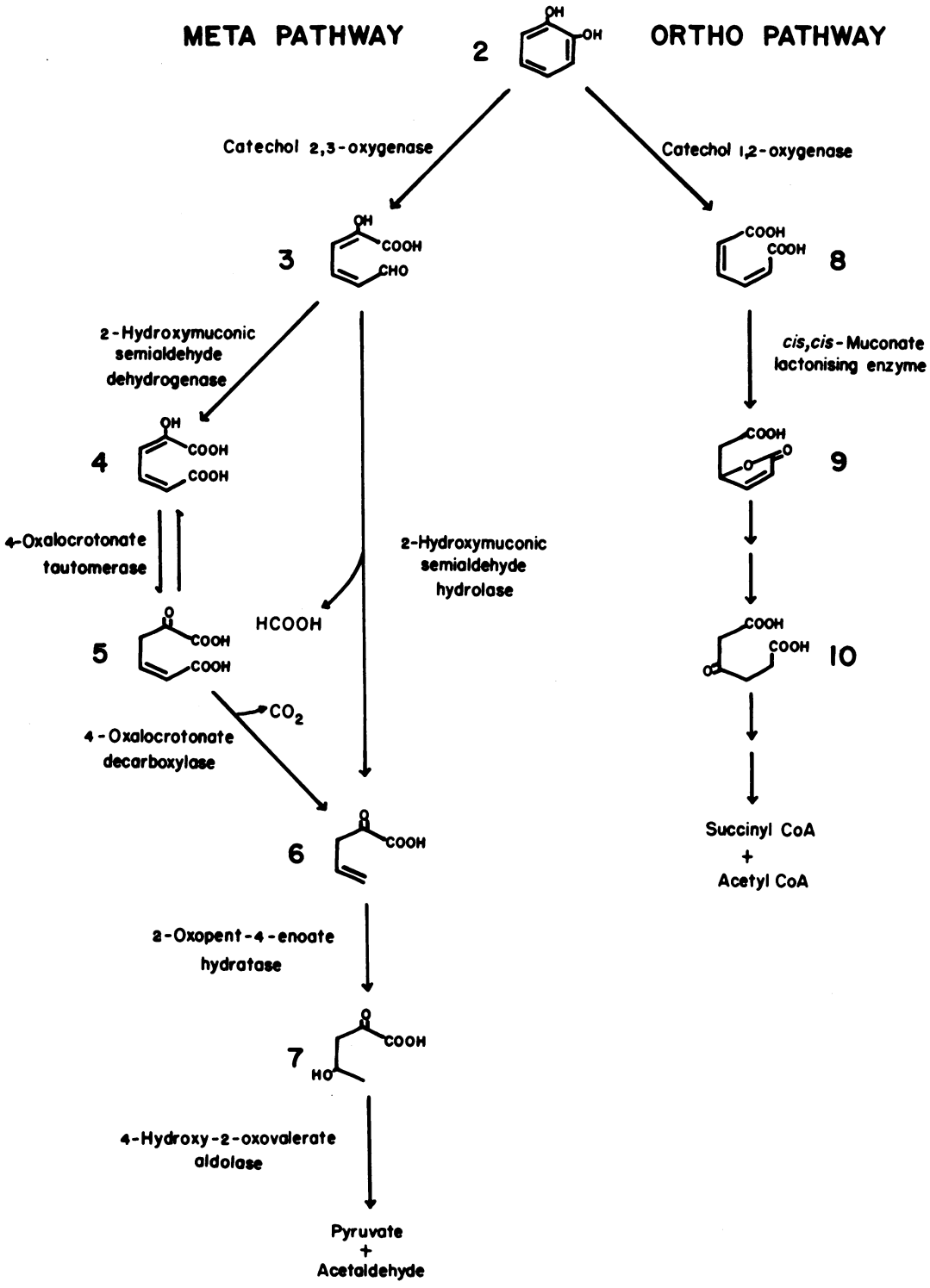

FIG. 1. *Ortho (β-ketoadipate) and meta (α-keto acid) pathways utilized by microorganisms for the catabolism of catechol.*

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *P. putida* strain PMD-1 (formerly NP), obtained from D. T. Gibson (The University of Texas, Austin), was originally isolated from soil by enrichment culture with naphthalene as a sole source of carbon and energy. Strain PMD-14, a leucine auxotroph, was selected by six cycles of growth in the presence of 0.02% Casamino Acids (vitamin-free), followed by amino acid starvation and penicillin-carbenicillin enrichment on 5 mM salicylate.

Media and growth conditions. Bacterial strains were cultured in 1-liter Erlenmeyer flasks containing 250 ml of basal salts medium (32). Hydrocarbons were added separately to the medium to a concentration of 5 mM; other substrates were added to 10 mM. Naphthalene was provided in crystalline form at a concentration of 0.46% (wt/vol). For growth of amino acid auxotrophs, the medium was supplemented with the appropriate amino acid at concentrations of 20 μg/ml. Unless otherwise stated, all liquid cultures were maintained at 30°C with aeration until late exponential growth was attained. Cells were harvested by centrifugation, washed with 50 mM sodium-potassium phosphate buffer (pH 7.0) and stored at −20°C until use.

Minimal agar plates were solidified with 1% Oxoid agar, and substrates were added at concentrations described above. Naphthalene was provided in the vapor phase by adding crystals to petri dish lids at concentrations equivalent to 0.46% (wt/vol) of the plate volume.

Preparation of cell extracts and enzyme assays. Cell pastes were suspended in 2 volumes of 50 mM sodium-potassium phosphate buffer (pH 7.0) containing 10% (wt/vol) ethanol, 10% (wt/vol) glycerol, and 1 mM glutathione and disrupted at 18,000 lb/in^2 using a French pressure cell. Cellular debris was removed by centrifugation at 28,000 × g for 20 min at 4°C. The supernatant fractions were clarified by ultracentrifugation at 105,000 × g for 90 min and were used as a source of crude extracts.

The following enzymes were assayed spectrophotometrically by published procedures: catechol 1,2-oxygenase (EC 1.13.11; 12, 15), catechol 2,3-oxygenase (EC 1.13.12; 12, 15), 2-hydroxymuconic semialdehyde hydrolase (24), salicylaldehyde dehydrogenase (27), salicylate hydroxylase (31), and *cis*-naphthalene dihydrodiol dehydrogenase (26). Protein concentrations were determined by the method of Lowry et al. (20) or by the method of Kalb and Bernlohr (18). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of product per min. Specific activities of all enzymes are expressed as units per milligram of protein.

Conjugations. Donor and recipient cells were grown at 30°C in 50-ml Erlenmeyer flasks containing 10 ml of L-broth. Cells were grown to late exponential phase, harvested by centrifugation, and suspended in basal salts medium. Equal volumes (0.1 ml) of donor and recipient cells were spread onto selective media, and conjugation frequencies were determined after the titration of donor cells on agar plates containing salicylate and the required amino acid.

Single-colony plasmid isolation. Donor, recipient, and exconjugant colonies were examined for plasmid DNA by lysis of single colonies and subsequent electrophoresis in vertical 0.7% agarose slab gels. Using

TABLE 1. *Bacterial strains*

Strain	Laboratory designation	Relevant phenotype[a]	Relevant genotype (plasmid)[b]	Comments, references, or source
P. putida	PMD-1	Nah$^+$ Sal$^+$ Ben$^+$	Wild type (pMWD-1)	D. T. Gibson (26)
	PMD-14	Nah$^+$ Sal$^+$ Ben$^+$ Leu$^-$	*leu-3* (pMWD-1)	Amino acid starvation of PMD-1
	PRS2000	Nah$^-$ Sal$^-$ Ben$^+$ Mdl$^+$	Wild type	L. N. Ornston
	PMD-16	Nah$^-$ Sal$^+$ Ben$^+$ Mdl$^+$	Wild type (pMWD-1)	Conjugation of PRS2000 with PMD-14
	AC545	Nah$^-$ Sal$^+$ Ben$^+$ Met$^-$	*met* (SAL)	A. C. Chakrabarty
	PpG1064	Nah$^+$ Sal$^+$ Ben$^+$ Trp$^-$	*trp* (NAH)	I. C. Gunsalus
	PaW-1	M-tol†	Wild type (TOL)	P. A. Williams (32)
	B98	Nah$^-$ Sal$^-$ Tru$^+$	Wild type	L. N. Ornston
P. aeruginosa	PAO403	Nah$^-$ Sal$^-$ Ben$^+$ Trp$^-$ Res$^-$ Mod$^-$	*trp res mod*	A. C. Chakrabarty
	PMD-15	Nah$^-$ Sal$^+$ Ben$^+$ Trp$^-$	*trp* (pMWD-1)	Conjugation of PAO403 with PMD-14
	PAO2178	Nah$^-$ Sal$^-$Ben$^-$ Met$^-$	*met-9020 catA*	H. Matsumoto
P. stutzeri	226	Nah$^-$ Sal$^-$ Ben$^+$ Suu$^+$	Wild type	L. N. Ornston
P. fluorescens	C217	Nah$^-$ Sal$^-$ Ben$^+$ Gal$^+$	Wild type	L. N. Ornston

[a] Phenotype abbreviations: Nah, naphthalene utilization; Sal, salicylate utilization; Ben, benzoate utilization; Mdl, D-mandelate utilization; M-tol, *m*-toluate utilization; Tru, L-tryptophan utilization; Suu, sucrose utilization; Gal, galactose utilization; Leu$^-$, requires leucine; Trp$^-$, requires tryptophan; Met, requires methionine; Res, restrictionless; Mod, modificationless.

[b] *catA*, Catechol 1,2-oxygenase negative; pMWD-1 and SAL specify salicylate degradation; NAH specifies naphthalene degradation; TOL specifies degradation of toluenes and xylenes.

a toothpick, we suspended a small portion of colony from an L-agar plate in 50 μl of 25% (wt/vol) sucrose solution (50 mM Tris-hydrochloride, pH 8). Two microliters of RNase (5 mg/ml in 250 mM Tris-hydrochloride, pH 8), 4 μl of 250 mM EDTA, and 6 μl of egg white lysozyme (10 mg/ml in 250 mM Tris-hydrochloride) were added to the cell suspension, followed by a 20-min incubation at room temperature. Four microliters of 20% (wt/vol) sodium dodecyl sulfate in 10 mM Tris-hydrochloride (pH 8.0) was added, and the suspension was incubated for 2 min at room temperature. The preparation was placed on ice for 1 h. After incubation, the lysate was equilibrated to room temperature and then mixed by drawing up and expelling five times with a 100-μl Eppendorf pipette. The contents of each individual lysate were loaded in the well slot (0.68 cm wide and 0.38 cm thick) of a vertical 0.7% agarose slab gel (Bethesda Research Laboratories, Rockville, Md.). Tris-borate (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.0) was used as the running buffer. Electrophoresis was conducted at 100 V for approximately 2 h, using bromphenol blue, (bromphenol blue, 0.07 mg/ml; sodium dodecyl sulfate, 0.07 g/ml in 33% [vol/vol] glycerol aqueous solution) as the tracking dye.

Purified plasmid DNA preparation. Lysates of cells used for cesium chloride-ethidium bromide preparative ultracentrifugation were prepared by the technique of Hansen and Olsen (14). All other aspects of the cesium chloride-ethidium bromide ultracentrifugation technique have been described elsewhere (21).

Electron microscopy. Plasmid DNA purified by the cesium chloride-ethidium bromide technique was kept for 2 weeks at 4°C in 1.5-ml polypropylene tubes and then examined for the presence of open circular forms in the electron microscope (Hitachi HS-7). The plasmid DNA preparation was spread and stained by the technique of Kleinschmidt et al. (19). The open circular forms of plasmid pBF4 (molecular size equal to 27.2×10^6 daltons; 29) were used as an internal reference in the contour length determination of pMWD-1. Contour lengths were measured by use of a numonics projection device integrated with a numonics electronic graphics calculator.

Restriction endonuclease digestion. Digestions were carried out in buffers and under conditions recommended by the suppliers of the enzymes (Boehringer-Mannheim Biochemicals). Digests were subjected to electrophoresis in vertical 0.7% agarose slab gels as described above.

RESULTS

Nutritional characteristics of *P. putida* strain PMD-1. *P. putida* strain PMD-1 was tested for its ability to utilize aromatic compounds which are dissimilated through catechol. Naphthalene, salicylate, benzoate, and *m*-toluate were utilized by PMD-1 as sole sources of carbon and energy, whereas mandelate, *o*-toluate, *p*-toluate, toluene, *m*-xylene, and phenol were unable to support growth. In addition, *ortho*-cleavage intermediates of catechol, namely, *cis, cis*-muconate, and β-ketoadipate, were not utilized.

Biochemical analysis of crude extracts prepared from cells grown on naphthalene, salicylate, *m*-toluate, and benzoate indicated that these substrates were metabolized through the *meta* (α-keto acid) pathway (Fig. 1). Extracts of strain PMD-1 exhibited catechol 2,3-oxygenase at levels which were elevated relative to the basal levels observed for glutamate-grown cells (Table 2). Undetectable or very low levels of catechol 1,2-oxygenase were present in these extracts (Table 2). Strain PMD-1 grew poorly on *m*-toluate and accumulated a brown compound in the medium. However, cell extracts from these cultures had levels of catechol 2,3-oxygenase comparable to those found in salicylate- and naphthalene-grown cells. Further analysis revealed that oxygenation of 3-methylcatechol by PMD-1 catechol 2,3-oxygenase occurred at low levels (specific activity of 0.32). In contrast, parallel assays with extracts of *P. putida* PAW-1 (32), an organism which readily metabolizes *m*-toluate, yielded a specific activity of 1.44 for this isofunctional enzyme with 3-methylcatechol as a substrate. Thus, the brown discoloration of the medium apparent during growth on *m*-toluate is presumably due to the accumulation and autooxidation of 3-methylcatechol.

Conjugal transfer of the Sal$^+$ phenotype. The genetic basis for the ability of PMD-1 to utilize aromatic compounds through the *meta*-cleavage pathway was examined by demonstrating the transmission of the Nah$^+$ Sal$^+$ phenotype to other pseudomonads which express only the *ortho*-cleavage pathway. These experiments showed that the Sal$^+$ phenotype can be transferred to two other strains of *P. putida* and to other representatives of the genus *Pseudomonas* (Table 3). Surprisingly, we were unable to directly select exconjugants on naphthalene and

TABLE 2. *Specific activities of catechol 2,3-oxygenase (meta-cleavage) and catechol 1,2-oxygenase (ortho-cleavage) in crude extracts of P. putida PMD-1*[a]

Grown on:	Sp act of[b]:	
	Catechol 2,3-oxygenase	Catechol 1,2-oxygenase[c]
Naphthalene	4.00	0.003
Salicylate	3.60	0.002
Benzoate	0.23	0.040
m-Toluate	3.43	0.007
Glutamate	0.04	0.002

[a] Growth of cells and preparation of cell extracts were as described in the text.

[b] Specific activities are expressed as micromoles of product per minute per milligram of protein.

[c] Assays were performed after inhibiting catechol 2,3-oxygenase with H_2O_2.

TABLE 3. *Conjugal transfer of Sal⁺ phenotype*[a]

Donor	Recipient	Selected for:	Frequency of transfer	Phenotype of exconjugants
P. putida PMD-14	*P. putida* PRS 2000	Sal⁺	1×10^{-5}	Mdl⁺ Nah⁻
	B98	Sal⁺	1×10^{-7}	Tru⁺ Nah⁻
P. putida PMD-14	*P. aeruginosa* PAO403	Sal⁺	5×10^{-6}	Mdl⁺ Trp⁻ Nah⁻
P. putida PMD-14	*P. fluorescens* C217	Sal⁺	4×10^{-4}	Gal⁺ Nah⁻
P. putida PMD-14	*P. stutzeri* 226	Sal⁺	4×10^{-8}	Suu⁺ Nah⁻
P. aeruginosa PMD-15	*P. putida* PRS2000	Sal⁺	6×10^{-6}	Trp⁺
P. aeruginosa PMD-15	*P. aeruginosa* PAO2178	Sal⁺	6×10^{-7}	Ben⁺ Met⁻
		Ben⁺	9×10^{-7}	Sal⁺ Met⁻

[a] Matings were performed as described in the text. Abbreviations: Sal, salicylate utilization; Mdl, D-mandelate utilization; Nah, naphthalene utilization; Gal, D-galactose utilization; Tru, tryptophan utilization; Suu, sucrose utilization; Ben, benzoate utilization; Met, requires methionine; Trp, requires tryptophan.

m-toluate. Moreover, all Sal⁺ exconjugants were Nah⁻ and M-tol⁻.

Sequential transfer of the Sal⁺ phenotype was demonstrated by mating PMD-15 with Sal⁻ recipients. Strain PMD-15 was able to transfer the Sal⁺ marker to PRS2000 and PAO2178 (Table 3). The latter strain is a *catA* mutant (catechol 1,2-oxygenase deficient) and thus has a Ben⁻ phenotype. Direct selection either on benzoate or salicylate resulted in Ben⁺ Sal⁺ exconjugants, suggesting that the genetic information received during conjugation allows this strain to cleave catechol and thus to utilize benzoate. Addition of catechol (1 mM) to colonies of Ben⁺ Sal⁺ exconjugants on either salicylate or benzoate agar media resulted in the appearance of yellow colonies, indicative of the formation of 2-hydroxymuconic semialdehyde (Fig. 1), the ring cleavage product of catechol 2,3-oxygenase. These results are consistent with the existence of a transmissible plasmid which encodes enzymes for the degradation of salicylate via the *meta*-cleavage of catechol.

The biochemical basis for the Sal⁺ Nah⁻ phenotype of exconjugants was substantiated by enzymatic analysis of *P. putida* PMD-16 and *P. aeruginosa* PMD-15. PMD-15 and PMD-16 had specific activities of salicylate-catabolizing enzymes comparable to those observed in PMD-14 (Table 4). However, they lacked detectable levels of naphthalene dihydrodiol dehydrogenase and salicyaldehyde dehydrogenase, two enzymes involved in the conversion of naphthalene to salicylate. These results indicate that the enzymes responsible for the conversion of naphthalene to salicylate are chromosomally encoded. Alternatively, these enzymes might be encoded by plasmid-borne genes which are regulated by chromosomal elements.

TABLE 4. *Specific activities of enzymes for naphthalene and salicylate dissimilation in P. putida PMD-14 and in exconjugants P. aeruginosa PMD-15 and P. putida PMD-16*[a]

Sp act of:	PMD-14	PMD-15	PMD-16
Naphthalene dihydrodiol dehydrogenase	0.04[b]	<0.001	<0.001
Salicylaldehyde dehydrogenase	0.21	<0.001	<0.001
Salicylate hydroxylase[c]	0.10	0.11	0.16
Catechol 2,3-oxygenase	4.06	4.66	3.21
2-Hydroxymuconic semialdehyde hydrolase	0.14	0.15	0.15
Catechol 1,2-oxygenase[c]	0.09	0.002	0.03

[a] Cells were grown on salicylate-basal salts medium, and assays were performed as described in the text. The Sal⁻ background strains of PMD-15 (PAO403) and PMD-16 (PRS2000) were devoid of all enzyme activities listed in the table when grown on 20 mM lactate supplemented with 5 mM salicylate.

[b] Micromoles of product per minute per milligram of protein.

[c] Assayed after treatment of extracts with H_2O_2.

Physical evidence for the plasmid origin of the Sal⁺ phenotype. The Sal⁺ Nah⁻ phenotype of the exconjugants may be due to either incomplete transfer of the parental plasmid or segregation or deletion of a portion of the plasmid in the recipients, thus resulting in the loss of essential structural gene(s) required for the oxidation of naphthalene to salicylate. PMD-14 and two exconjugants, PMD-15 and PMD-16, were examined for plasmid DNA by DNA purification procedures appropriate for plasmid isolation. Analysis by agarose gel electrophoresis (Fig. 2) revealed that PMD-1 possesses extrachromosomal DNA. Moreover, all Sal⁺ exconjugants examined had plasmid DNA that comigrated with the PMD-1 plasmid on agarose gels (Fig. 2). In addition, restriction digestion patterns for plasmid DNA isolated from PMD-15

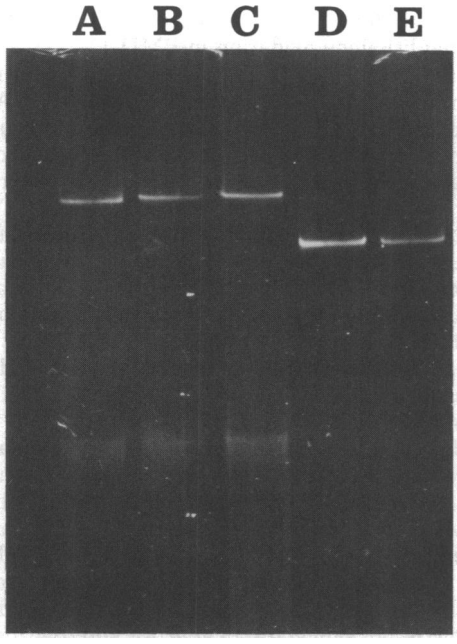

FIG. 2. *Agarose gel electrophoretic analysis of plasmid DNA purified from the parental strain and Sal⁺ exconjugants. Plasmid DNA isolated from PMD-14 (A); Sal⁺ exconjugants P. aeruginosa PMD-15 (B) and P. putida PMD-16 (C); P. putida AC545 (SAL) (D); and P. putida PpG1064 (NAH) (E). Plasmid isolation procedures and agarose gel electrophoresis were done as described in the text.*

were identical to the restriction pattern for the plasmid isolated from PMD-14 (Fig. 3). These findings favor the conclusion that the Sal^+ phenotype is plasmid encoded and that this plasmid (pMWD-1) is transmitted intact from PMD-14 to recipients. Therefore, the Nah^- nature of the exconjugants is not due to a detectable deletion of the plasmid in the new hosts. Hence, the molecular data support the notion of chromosomally encoded regulatory or structural gene(s) for naphthalene catabolism.

Molecular comparison of pMWD with NAH and SAH. It was of interest to compare pMWD-1 with other plasmids encoding the same degradative pathway, since molecular analysis of NAH and SAL has revealed that these plasmids are very similar (2, 11, 16). The relative mobility of pMWD-1 in agarose gels was considerably less than that observed for NAH or SAL (Fig. 2). Contour length measurements of pMWD-1 gave an estimate of 1.1×10^8 for the monomeric molecular weight of this plasmid, roughly twice that of NAH and SAL. It is possible that pMWD-1 arose by a simple duplication of SAL or NAH. If this were the case, then restriction enzyme digestion patterns of pMWD-1 and NAH or SAL should be similar, if not indistinguishable. Comparative restriction digestion analysis of SAL and pMWD-1 revealed that these plasmids differ markedly in restriction digestion patterns (Fig. 4). These data led to the conclusion that pMWD-1 either arose independently of NAH and SAL or that its sequence has diverged considerably.

DISCUSSION

Genetic basis of *P. putida* strain PMD-1's nutritional properties. The biochemical, genetic, and molecular data presented in this communication show that the ability of PMD-1 to use naphthalene and salicylic acid as sole carbon sources is due to the presence of a plasmid of 110 Mdal. Enzymatic analysis of exconjugants suggests that salicylate dissimilation via *meta*-cleavage of catechol is entirely plasmid encoded (Table 4). Naphthalene dissimilation, which proceeds through salicylate, requires some chromosomal gene(s), since all exconjugants of

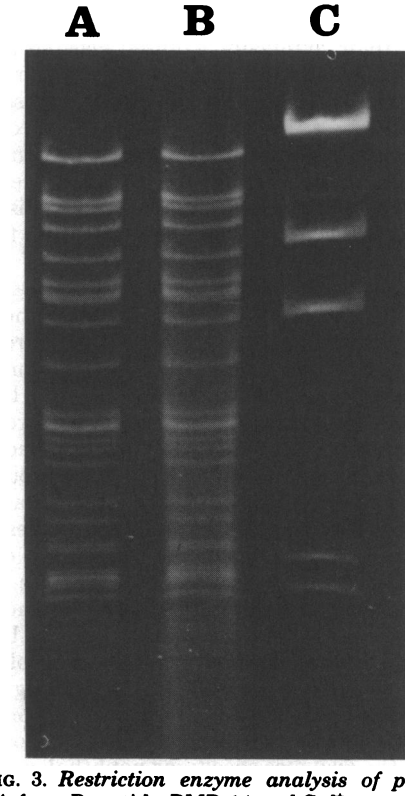

FIG. 3. *Restriction enzyme analysis of plasmid DNA from P. putida PMD-14 and Sal⁺ exconjugant P. aeruginosa PMD-15. Cesium chloride-ethidium bromide purified plasmid DNA from PMD-14 and PMD-15 was digested with BstEII and electrophoresed in 0.7% agarose gels. (A) PMD-14, (B) PMD-15, and (C) HindIII digest of λ.*

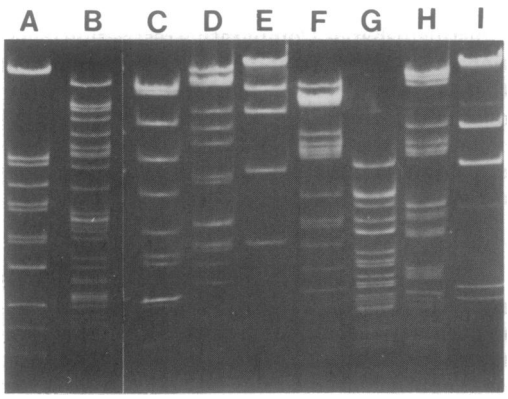

FIG. 4. *Comparative restriction analysis of plasmids pMWD and SAL. (A) BstEII digest of SAL, (B) BstEII digest of pMWD, (C) HindIII digest of SAL, (D) HindIII digest of pMWD, (E) SmaI digest of SAL, (F) SmaI digest of pMWD, (G) SalI digest of SAL, (H) SalI digest of pMWD, and (I) HindIII digest of* λ.

PMD-1 are Sal^+ but Nah^- and lack early enzyme activities for naphthalene degradation even though their conjugally acquired plasmid is apparently identical to pMWD-1 (Fig. 3). Our data do not identify the nature of the chromosomal contribution: either structural genes encoding degradative enzymes or regulatory elements or both might be provided by the host cell to achieve catabolism of naphthalene. Wheelis (30) suggested that degradative plasmids arose fairly recently by recombinational excision of clustered chromosomal genes. If this is the case, then either a genetic segment encoding the entire pathway must be excised or some chromosome-plasmid cooperation is required for the expression of the excised genes. In the latter situation, the complete pathway is not expressed if the plasmid is transmitted to a host lacking the appropriate chromosomal "background." Plasmid pMWD-1 may, in fact, be an example of such a situation. When this plasmid resided in PMD-1, the bacterial cell was able to dissimilate either naphthalene or salicylate, but when pMWD-1 was transmitted to another pseudomonad of different chromosomal background, the new host was capable of catabolizing salicylate but not naphthalene, suggesting that it cannot provide the necessary enzymes or regulatory elements to achieve naphthalene oxidation. If the enzymes specifying naphthalene degradation are plasmid encoded but require chromosomal regulation, then transmission of pMWD-1 to a host lacking appropriate regulatory elements would render them "silent." We would greatly advance our understanding of chromosome-plasmid interactions if we could directly identify the chromosomal contribution to naphthalene oxidation in PMD-1.

The plasmid pMWD-1 bore no molecular resemblance to the degradative plasmids NAH and SAL. pMWD-1 had restriction enzyme digestion patterns which differed strikingly from those of the plasmid SAL (Fig. 4). This was true for all seven restriction enzymes tested. The extent of base sequence homology (or lack thereof) could not be ascertained from these data. Heteroduplex or hybridization analyses are necessary to measure the extent of homology between these two DNAs. Nevertheless, it is clear that pMWD-1 is a unique plasmid and is dissimilar to NAH and SAL both in size and in restriction enzyme target sites.

Regulation of the *ortho*- and *meta*-cleavage pathways in *P. putida* PMD-1. Results of biochemical analysis demonstrated that PMD-1 catabolizes naphthalene, salicylate, and benzoate via *meta*-cleavage of catechol (Table 2). Similarly, *P. aeruginosa* PMD-15 preferentially expressed *meta*-cleavage enzymes during growth on salicylate (Table 4) and benzoate (data not shown). *P. putida* PMD-16 had elevated levels of both catechol 2,3-oxygenase and of catechol 1,2-oxygenase during growth on benzoate (data not shown). Thus, the presence of the *meta*-cleavage pathway does not necessarily preclude the induction of the *ortho*-cleavage enzymes. Nevertheless, the *meta*-cleavage of catechol was the sole route used for the dissimilation of naphthalene and salicylate by PMD-1, and its Sal^+ exconjugants. Previous studies by Barnsley (1) demonstrated that naphthalene dissimilation occurs via the *ortho*-cleavage pathway in some strains and via the *meta*-cleavage pathway in others. In some strains, enzymes of both pathways are induced during growth on naphthalene. The genetic basis of naphthalene utilization in all of the strains examined by Barnsley has not been identified. Others (7) have noted that the *meta*-cleavage pathway tends to prevail in strains harboring degradative plasmids, whereas *ortho*-cleavage of catechol during the dissimilation of aromatic compounds occurs in strains lacking extrachromosomal pathways. The adaptive significance of this correlation is not understood, and its bearing on the evolution of catabolic plasmids remains obscure.

ACKNOWLEDGMENTS

Portions of this research were performed in the laboratories of L. N. Ornston, Yale University, and F. L. Macrina and P. V. Phibbs, Jr., Medical College of Virginia, which we gratefully acknowledge. We also thank Carroll Carter and J. Smith for assistance with manuscript preparation and R. Roehl for critical review of the manuscript.

This work was supported by Public Health Service grant GM-25487 from the National Institutes of Health and by

National Research Service Award GM-7414-01 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Barnsley, E. A.** 1976. Role and regulation of the *ortho*- and *meta*-pathways of catechol metabolism in pseudomonads metabolizing naphthalene and salicylate. J. Bacteriol. **125**:404–408.
2. **Bayley, S. A., D. W. Morris, and P. Broda.** 1979. The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group. Nature (London) **280**:338–339.
3. **Bayly, R. C., and G. J. Wigmore.** 1973. Metabolism of phenol and cresols by mutants of *Pseudomonas putida*. J. Bacteriol. **113**:1112–1120.
4. **Bennett, P. M., and M. H. Richmond.** 1978. Plasmids and their possible influence on bacterial evolution. The Bacteria **6**:1–69.
5. **Benson, S., and J. Shapiro.** 1978. TOL is a broad host range plasmid. J. Bacteriol. **135**:278–280.
6. **Chakrabarty, A. M.** 1972. Genetic basis of the biodegradation of salicylate in *Pseudomonas*. J. Bacteriol. **112**:815–823.
7. **Chakrabarty, A. M.** 1976. Plasmids in *Pseudomonas*. Annu. Rev. Genet. **10**:7–30.
8. **Chakrabarty, A. M., D. A. Friello, and L. H. Bopp.** 1978. Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. Proc. Natl. Acad. Sci. U.S.A. **75**:3109–3112.
9. **Dagley, S.** 1979. Pathways for the utilization of organic growth substrates. The Bacteria **6**:305–388.
10. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114**:974–979.
11. **Farrell, R., I. C. Gunsalus, I. P. Crawford, J. B. Johnston, and J. Ito.** 1978. Restriction endonuclease sites and aromatic plasmid structure. Biochem. Biophys. Res. Commun. **82**:411–416.
12. **Feist, C. F., and G. D. Hegeman.** 1969. Regulation of the *meta* cleavage pathway for benzoate oxidation by *Pseudomonas putida*. J. Bacteriol. **100**:1121–1123.
13. **Friello, D. A., J. K. 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.
14. **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.
15. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of the enzymes of the wild type. J. Bacteriol. **91**: 1140–1154.
16. **Heinaru, A. L., C. J. Duggleby, and P. Broda.** 1978. Molecular relationships of degradative plasmids determined by *in situ* hybridization of their endonuclease-generated fragments. Mol. Gen. Genet. **160**:347–351.
17. **Jacoby, G. A., J. E. Rogers, A. E. Jacoby, and R. W. Hedges.** 1978. Transposition of *Pseudomonas* toluene-degrading genes and expression in *Echerichia coli*. Nature (London) **274**:179–180.
18. **Kalb, V. F., and R. W. Bernlohr.** 1977. A new spectrophotometric assay for proteins in cell extracts. Anal. Biochem. **82**:362–371.
19. **Kleinschmidt, A. K., D. Lang, D. Jacherts, and R. K. Zahn.** 1962. Preparation and length measurements of the total deoxyribonucleic acid content of T_2 bacteriophages. Biochim. Biophys. Acta **61**:857–864.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. **Macrina, F. L., J. L. Rieder, S. S. Virgili, and D. J. Kopecko.** 1977. Survey of the extrachromosomal gene pool of *Streptococcus mutans*. Infect. Immun. **17**:215–226.
22. **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* mt-2. Eur. J. Biochem. **28**:301–310.
23. **Nakazawa, T., E. Hayashi, T. Yokota, Y. Ebina, and A. Nakazawa.** 1978. Isolation of TOL and RP4 recombinants by integrative suppression. J. Bacteriol. **134**: 270–277.
24. **Nakazawa, T., and T. Yokota.** 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: demonstration of two benzoate pathways. J. Bacteriol. **115**:262–267.
25. **Ornston, L. N., and D. Parke.** 1976. The evolution of induction mechanisms in bacteria: insights derived from the study of the β-ketoadipate pathway. Curr. Top. Cell. Regul. **12**:209–262.
26. **Patel, T. R., and D. T. Gibson.** 1974. Purification and properties of (+) cis-naphthalene dihydrodiol dehydrogenase of *Pseudomonas putida*. J. Bacteriol. **119**:879–888.
27. **Shamsuzzaman, K. M., and E. A. Barnsley.** 1974. The regulation of naphthalene metabolism in *Pseudomonas*. Biochem. Biophys. Res. Commun. **60**:582–589.
28. **Stanier, R. Y., and L. N. Ornston.** 1973. The β-ketoadipate pathway. Adv. Microbial. Physiol. **9**:89–151.
29. **Welch, R. A., K. R. Jones, and F. L. Macrina.** 1979. Transferable lincosamide-macrolide resistance in *Bacteroides*. Plasmid **2**:261–268.
30. **Wheelis, M.** 1975. The genetics of dissimilatory pathways in *Pseudomonas*. Annu. Rev. Microbiol. **29**:505–524.
31. **White-Stevens, R. H., and H. Kamin.** 1972. Studies of a flavoprotein, salicylate hydroxylase. I. Preparation, properties, and the uncoupling of oxygen reduction from hydroxylation. J. Biol. Chem. **247**:2358–2370.
32. **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.
33. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and m-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. **23**: 227–232.
34. **Wong, C. L., and N. W. Dunn.** 1976. Combined chromosomal and plasmid encoded control for the degradation of phenol in *Pseudomonas putida*. Genet. Res. **27**:405–412.