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The Plasmid-coded Metabolism of Naphthalene and 2-Methylnaphthalene in *Pseudomonas* Strains: Phenotypic Changes Correlated with Structural Modification of the Plasmid pWW60-1

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Pseudomonas sp. NCIB 9816 contains two plasmids: pWW60, an IncP9 plasmid of 87 kb encoding genes for the catabolism of naphthalene, and pWW61, a cryptic plasmid of about 65 kb. The ability to degrade naphthalene was transferred at low frequency by conjugation from strain NCIB 9816 into a plasmid-free strain of *Pseudomonas putida*, PaW340. A transconjugant, PaW701, containing the naphthalene plasmid pWW60-1, metabolized naphthalene and salicylate via the *ortho* pathway. 2-Methylnaphthalene was not a growth substrate but was partly metabolized with accumulation of a brown compound in the medium (λ_{max} = 440 nm). Spontaneous mutants of PaW701 with the ability to grow on 2-methylnaphthalene arose at a frequency of about 10^{-5}. These fell into two groups. Group A mutants had no detectable salicylate hydroxylase activity and accumulated salicylate from naphthalene in culture supernatants: they appeared to grow on the pyruvate released from oxidation of the first ring of both substrates. Their plasmids all contained a 16·7 kb insert in different sites within a small, limited region of the plasmid. Group B mutants used a *meta* pathway for catabolism of naphthalene and 2-methylnaphthalene. Their plasmids had undergone a small deletion of from 1·2 to 1·6 kb in a region of the plasmid close to the sites of the insertions in the group A mutants.

INTRODUCTION

The biochemistry and regulation of naphthalene metabolism in *Pseudomonas* sp. NCIB 9816 has been the subject of conflicting reports. Davies & Evans (1964), who first isolated the strain as *Pseudomonas* P_G and submitted it to the National Collection of Industrial Bacteria (NCIB), showed that naphthalene was converted through salicylate to catechol (see Fig. 1) and surmised that the catechol was further metabolized by the *meta* pathway because of the presence of its first enzyme, catechol 2,3-dioxygenase (EC 1.13.11.2). Williams *et al.* (1975) confirmed this pathway with some more detailed enzymological data, and also showed that 2-methylnaphthalene was metabolized by the same sequence of reactions. However, Barnsley (1976), using a fresh culture obtained from NCIB, showed that the catechol resulting from naphthalene catabolism was assimilated by the *ortho* or *β*-ketoadipate pathway, with catechol 1,2-dioxygenase (EC 1.13.11.1) as the first enzyme, and that the catechol 2,3-dioxygenase activity was present only at low constitutive levels. Barnsley (1976) found that there were differences in the biochemistry of naphthalene catabolism between this clone and that supplied by P. A. Williams, although his results with the latter differed significantly from those previously obtained by Williams *et al.* (1975). In both laboratories it was assumed that there were at least two variants of this strain in existence.

Abbreviations: C12O, catechol 1,2-oxygenase; C23O, catechol 2,3-oxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; MLE, *cis,cis*-muconate lactonizing enzyme; SH, salicylate hydrolase.

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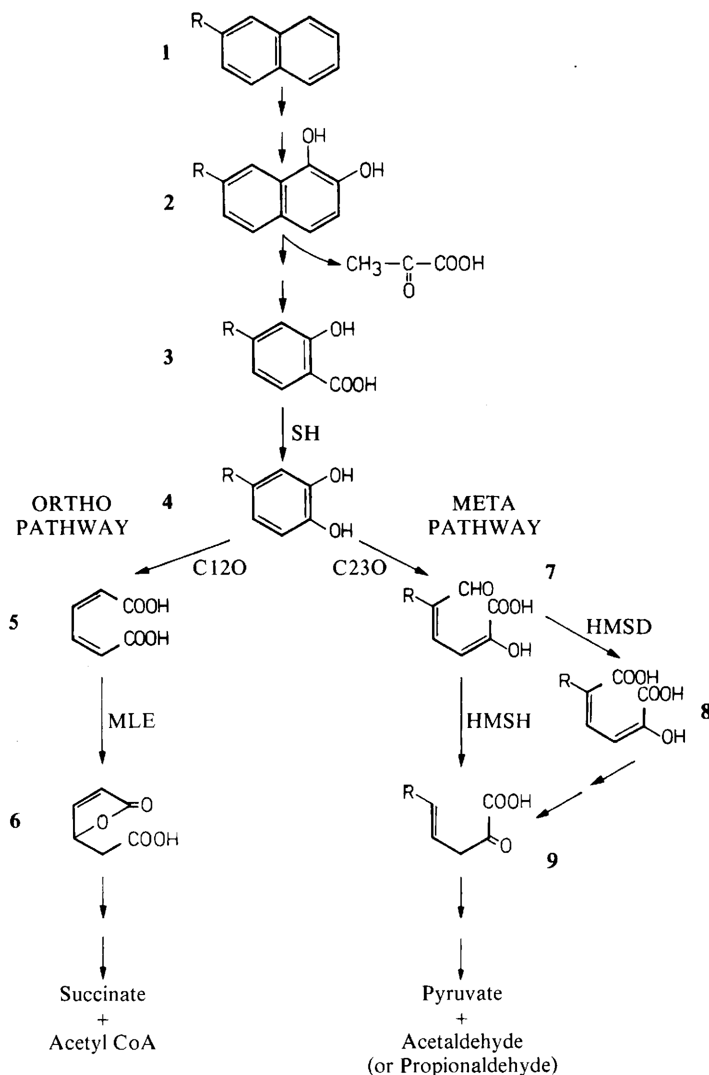

Fig. 1. The pathways for metabolism of naphthalene (R = H) and 2-methylnaphthalene ($R = CH_3$). Compounds shown are (2-methylnaphthalene metabolites in brackets): **1**, naphthalene (2-methylnaphthalene); **2**, 1,2-dihydroxynaphthalene (1,2-dihydroxy-7-methylnaphthalene); **3**, salicylate (4-methylsalicylate); **4**, catechol (4-methylcatechol); **5**, *cis,cis*-muconate; **6**, muconolactone; **7**, 2-hydroxymuconic semialdehyde (2-hydroxy-5-methylmuconic semialdehyde); **8**, 4-oxalocrotonate (2-methyl-4-oxalocrotonate); **9**, 2-oxopent-4-enoate (2-oxohex-4-enoate). Acetaldehyde and propionaldehyde are (with pyruvate) the final products of naphthalene and 2-methylnaphthalene respectively. The enzymes assayed are denoted by their abbreviations.

We have been prompted to reinvestigate naphthalene assimilation in *Pseudomonas* sp. NCIB 9816 for two reasons. Firstly, a number of reports have indicated that plasmids are invariably responsible for naphthalene catabolism in *Pseudomonas* spp. (Dunn & Gunsalus, 1973; Boronin *et al.*, 1977; Boronin *et al.*, 1980). Secondly, we have found a number of instances where the phenotype of strains carrying catabolic plasmids can alter spontaneously as a result of modifications in the plasmid DNA structure (Bayley *et al.*, 1977; Jeenes *et al.*, 1982; Pickup & Williams, 1982). This paper presents results on strains of *Pseudomonas putida* containing a catabolic plasmid, pWW60-1, derived by conjugation from strain NCIB 9816. Selection for

growth on 2-methylnaphthalene results in two classes of mutants in which both the biochemistry of naphthalene utilization and the plasmid structures have altered.

METHODS

Bacterial strains. Details of all relevant bacterial strains, and their plasmid complements, are shown in Table 1.

Conjugation experiments. Plate matings were the only consistently successful method of transferring the plasmids used in this study. Samples (0·1 ml) of overnight nutrient broth cultures of both donor and recipient were mixed directly on the selective agar plates. Streptomycin (1 mg ml^{-1}) and tryptophan (50 µg ml^{-1}) were incorporated into selective plates for all derivatives of PaW340.

DNA extraction and digestion. Plasmid DNA was extracted by the preparative method of Wheatcroft & Williams (1981). Restriction enzymes were purchased from Uniscience (Cambridge, U.K.) and used according to the suppliers' instructions. Digests of plasmid DNA were electrophoresed in 0·7% (w/v) agarose gels made in running buffer containing 89 mM-Tris, 2·5 mM-Na_2EDTA and 89 mM-H_3BO_3, pH 8·2, containing 0·5 µg ethidium bromide ml^{-1}. Samples were run into the gel from wells at 100 V for 15 min, after which they were completely submerged in running buffer (without ethidium bromide) and electrophoresed for 20 h at 160 V and 35 mA. Gels were photographed as previously described (Wheatcroft & Williams, 1981).

The size of fragments was calculated by comparison of their mobilities with those of fragments of pWW0 of known size (Downing & Broda, 1980). Plasmid sizes were obtained by summation of the sizes of the fragments produced by single and/or double digestion.

Preparation of cell extracts. Cells were grown in batch culture (Williams *et al.*, 1975) and were harvested by centrifuging at 1000 *g* for 1 h. They were washed in 100 mM-phosphate buffer, pH 7·5, and stored as a pellet at −20 °C. When required the pellet was resuspended in the appropriate buffer and sonicated at 0 °C in an MSE 100 W sonic disintegrator in three 60 s bursts. For all enzymes, except the catechol dioxygenases, extracts were made in 100 mM-phosphate buffer, pH 7·5; in extracts for assay of the catechol dioxygenases, the same buffer included 10% (v/v) acetone.

Enzyme assays. Enzymes were assayed by the following published procedures: salicylate hydroxylase (SH), Yamamoto *et al.* (1965); catechol 1,2-dioxygenase (C12O, EC 1.13.11.1), Hayaishi *et al.* (1957); catechol 2,3-dioxygenase (C23O, EC 1.13.11.2), 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) and 2-hydroxymuconic semialdehyde hydrolase (HMSH), Sala-Trepat *et al.* (1972); *cis,cis*-muconate lactonizing enzyme (MLE), Ornston (1966). Protein was determined by the biuret procedure.

Identification of salicylates in culture media. Culture supernatants were shaken twice with 0·5 vol. diethyl ether, and the ether extract discarded. The supernatant was then acidified with HCl to 0·1 M and the ether extraction

Table 1. *Strains of Pseudomonas*

Strain	Relevant phenotype*	Plasmids	Method of formation or reference
PGB1 (*Pseudomonas* sp. NCIB 9816, wild-type)	Nah^+ $2MeNah^+$ Sal^+ $4MeSal^-$ $5MeSal^-$	pWW60, pWW61	–
PGB2	Nah^- $2MeNah^-$ Sal^- $Mtol^+$	pWW61, pWW0	PaW15 × PGB1†
PGB3	Nah^- $2MeNah^-$ Sal^- $Mtol^-$	pWW61	Benzoate curing of PGB2
PaW1 (*P. putida* mt-2, wild-type)	$Mtol^+$	pWW0	Williams & Murray (1974)
PaW11	$Mtol^-$ Ben^- Cat^-	–	PaW1, mutagenesis
PaW15	$Mtol^+$ Leu^-	pWW0	Williams & Murray (1974)
PaW85	$Mtol^-$ Ben^+ Cat^+	–	Bayley *et al.* (1977)
PaW340	$Mtol^-$ Ben^+ Cat^+ Trp^- Str^r	–	PaW1, mutagenesis
PaW701	Nah^+ $2MeNah^-$ Sal^+ $4MeSal^-$ $5MeSal^-$ Trp^- Str^r	pWW60-1	PGB1 × PaW340†
PaW711	Nah^+ $2MeNah^+$ Sal^- $4MeSal^-$ $5MeSal^-$ Trp^- Str^r (group A)	pWW60-9	PaW701, selection for growth on 2-methylnaphthalene
PaW721		pWW60-16	
PaW722		pWW60-17	
PaW705	Nah^+ $2MeNah^+$ Sal^+ $4MeSal^+$ $5MeSal^+$ Trp^- Str^r (group B)	pWW60-14	
PaW719		pWW60-22	

* Phenotype designations: Nah^+, $2MeNah^+$, Sal^+, $4MeSal^+$, $5MeSal^+$, $Mtol^+$, Ben^+ and Cat^+ denote the ability to grow on naphthalene, 2-methylnaphthalene, salicylate, 4-methylsalicylate, 5-methylsalicylate, *m*-toluate, benzoate and catechol, respectively, as sole carbon sources. Trp^-, requirement for tryptophan; Str^r, resistance to streptomycin.

† Conjugation (donor × recipient).

repeated. The acid ether extracts were combined, dried with anhydrous Na_2SO_4, decanted and evaporated to dryness at room temperature. Portions of the residue were dissolved in 0·1 M-NaOH and their UV spectra compared with authentic salicylate (λ_{max} at 209 nm, 230 nm and 296 nm), 4-methylsalicylate (λ_{max} at 214 nm, 239 nm and 296 nm) and 5-methylsalicylate (λ_{max} at 215 nm, 230 nm and 305 nm). For TLC, portions of the residue were dissolved in ethanol, applied to silica gel plates and chromatographed in one of two solvents: (i) chloroform/acetic acid (9:1, v/v) and (ii) benzene/dioxan/acetic acid (90:25:4, by vol.). All three salicylates co-chromatographed in both solvents, with an R_F of 0·85 in solvent (i) and an R_F of 0·5 in solvent (ii). Trimethylsilyl derivatives for GLC were prepared as follows. The dry residue (2 mg) after acid ether extraction was dissolved in 0·2 ml pyridine, then 0·2 ml *N,O*-bis-(trimethylsilyl)trifluoroacetamide was added and the mixture heated at 60 °C for 30 min. Samples were separated on a 1·5 m × 4 mm internal diameter silanized glass column packed with 1% (w/w) OV-17 on Chromasorb G (100 to 120 mesh) in a Pye 104 gas chromatograph fitted with a flame ionization detector. The temperature of the column was increased at a rate of 16 °C min^{-1} from 100 °C to 300 °C.

RESULTS

Plasmids in Pseudomonas sp. NCIB 9816

The fragments obtained after *Hin*dIII digestion of plasmid DNA from PGB1 (*Pseudomonas* sp. NCIB 9816, wild-type) are shown in Table 2. These fragments were shown to derive from two plasmids. Transfer of the IncP9 TOL plasmid pWW0 into PGB1, selecting for growth on the TOL specific growth substrate *m*-toluate (Williams & Murray, 1974), resulted in transconjugants, all of which had lost the ability to grow on naphthalene. One such transconjugant, PGB2, was cured of the TOL plasmid by growth on benzoate (Williams & Murray, 1974) to give PGB3. The naphthalene plasmid from PGB1 was isolated by conjugation with a plasmid-free strain PaW340 (Trp^- Str^r) as recipient, selecting for growth on naphthalene: PaW701 was one of a very few transconjugants from this mating. Comparison of the *Hin*dIII digests of the plasmid DNA from PGB3 and PaW701 with PGB1 (Table 2), shows that the plasmid DNA of PGB1 was resolved as two plasmids: pWW61, an apparently cryptic plasmid of about 60–65 kb (in PGB3) and a naphthalene catabolic plasmid pWW60 (87 kb).

All further studies were carried out on PaW701, the plasmid of which we have called pWW60-1 to distinguish it from the parent plasmid in PGB1. Plasmid pWW60-1 appears to be identical to pWW60 on digestion with *Hin*dIII, *Bam*H1, *Xho*I and *Sal*I. However *Eco*RI digests are different. We can detect 11 *Eco*RI fragments of pWW60 whereas there are 15 fragments of pWW60-1; only seven fragments in each appear to correspond. We assume that a number of minor changes in structure, perhaps similar to modification, take place when pWW60 is transferred into PaW340 and that these are only detectable on *Eco*RI digestion.

The implication that the naphthalene catabolic plasmid is of the P9 incompatibility group from its exclusion by pWW0 was confirmed by the reverse transfer. Plasmid pWW60-1 was transferred from PaW701 into PaW1, selecting for transconjugants able to grow on naphthalene (Nah^+). All had lost the ability to grow on the pWW0 substrate *m*-toluate ($Mtol^-$). Examination of the plasmid content of one such transconjugant confirmed that it contained only pWW60-1 and that pWW0 had been eliminated.

Metabolic properties of PaW701

The activities of some of the relevant enzymes of naphthalene catabolism are shown in Table 3. Both C12O and MLE were induced by growth on salicylate and naphthalene whereas the *meta* pathway enzymes C23O, HMSH and HMSD were not significantly induced; C23O in particular maintained a constant low constitutive level of activity.

Strain PaW701 was unable to grow on 2-methylnaphthalene or on 4- or 5-methylsalicylate ($2MeNah^-$ $4MeSal^-$ $5MeSal^-$). When it was incubated in medium containing 2-methylnaphthalene, 4-methylsalicylate or 4-methylcatechol, there was little or no growth but the medium gradually changed colour with early accumulation of a yellow compound with a spectrum identical to that of 2-hydroxy-5-methylmuconic semialdehyde, the *meta* ring fission product of 4-methylcatechol (Murray *et al.*, 1972). The medium later turned brown, having an absorbance maximum at 440 nm which was enhanced in alkali but disappeared in acid. We have not been

Table 2. *Sizes (kb) of DNA fragments obtained from restriction endonuclease digestion of plasmid DNA from Pseudomonas strains*

*Hin*dIII digestion

Fragment	PGB1 (pWW60, pWW61)	PGB3 (pWW61)	PaW701 (pWW60–1)	PaW711 (pWW60–9)	PaW721 (pWW60–16)	PaW722 (pWW60–17)
HA*/HB*	(23–26)†	(23–26)†	—	—	—	—
HA	12·7	—	12·7	12·7	12·7	12·7
HB	11·2	—	11·2	11·2	11·2	11·2
HC	8·6	—	8·6	8·6	8·6	8·6
HD	7·4	—	7·4	7·4	7·4	7·4
HC*	7·2	7·2	—	—	—	—
				7·0‡	7·0‡	7·0‡
HE	6·9	—	6·9	6·9	6·9	6·9
HD*	6·5	6·5	—	—	—	—
HF	6·4	—	6·4	6·4	6·4	6·4
						5·8‡
				5·3‡		
HG	5·2	—	5·2	5·2	5·2	5·2
				5·0‡	5·0‡	5·0‡
					4·7‡	
					3·9‡	
HH	3·8	—	3·8	—	—	—
				3·3‡		
HI/HJ	3·0†	—	3·0†	3·0†	3·0†	3·0†
						2·9‡
HK	2·2	—	2·2	2·2	2·2	2·2
HL	2·1	—	2·1	2·1	2·1	2·1
HE*	2·05	2·05	—	—	—	—
HM	2·0	—	2·0	2·0	2·0	2·0
HN	1·9	—	1·9	1·9	1·9	1·9
HO	1·7	—	1·7	1·7	1·7	1·7
HP	1·5	—	1·5	1·5	1·5	1·5
HQ/HR	1·4†	—	1·4†	1·4†	1·4†	1·4†
HS/HT	1·0†	—	1·0†	1·0†	1·0†	1·0†
HU	0·9	—	0·9	0·9	0·9	0·9
HV	0·8	—	0·8	0·8	0·8	0·8

*Xho*I digestion

Fragment	PaW701 (pWW60–1)	PaW705 (pWW60–14)	PaW719 (pWW60–22)
XA	(35–40)	(35–40)	(35–40)
XB	10·2	10·2	10·2
XC	6·0	—	—
XD	5·7	5·7	5·7
		4·8‡	
XE	4·7	4·7	4·7
XF	4·5	4·5	4·5
			4·4‡
XG	2·3	2·3	2·3
XH	2·0	2·0	2·0
XI/XJ	1·9†	1·9†	1·9†
XK	1·8	1·8	1·8
XL	1·5	1·5	1·5
XM	1·0	1·0	1·0
XN	0·9	0·9	0·9
XO	0·8	0·8	0·8

* Bands exclusive to pWW61. † Double bands. ‡ Novel bands.

Table 3. *Enzyme activities in Pseudomonas strains*

Strain	Growth substrate	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]					
		SH	C12O	MLE	C23O	HMSD	HMSH
PaW701	Acetate	<5	<5	<5	20	3	<1
	Salicylate	125	200	115	37	3	<1
	Naphthalene	11	54	85	28	2	8
PaW711	Acetate	<5	<5	<5	21	2	4
	Naphthalene	<5	21	<5	18	<1	4
	2-Methylnaphthalene	<5	<5	<5	13	<1	<1
PaW705	Acetate	<5	<5	ND	72	7	<1
	Salicylate	ND	<5	ND	1540	124	<1
	Naphthalene	ND	<5	ND	1430	25	5
	2-Methylnaphthalene	ND	<5	ND	1460	30	4
PaW719	Acetate	<5	<5	ND	160	7	<1
	Salicylate	ND	<5	ND	2560	180	12
	Naphthalene	ND	<5	ND	1460	28	3
	2-Methylnaphthalene	ND	<5	ND	1070	32	3

ND, Not determined.

able to identify the compound responsible but assume that it is formed from 2-hydroxy-5-methylmuconic semialdehyde but is not further metabolized.

Mutants of PaW701 appeared spontaneously on 2-methylnaphthalene plates at a frequency of about 10^{-5}. Fifteen independently isolated mutants were purified and, on the basis of their growth phenotype, were found to fall into two groups. Thirteen mutants (group A) were Nah$^+$ 2MeNah$^+$ Sal$^-$ 4MeSal$^-$ 5MeSal$^-$ whereas two mutants (group B) were Nah$^+$ 2MeNah$^+$ Sal$^+$ 4MeSal$^+$ 5MeSal$^+$.

Metabolic properties of group A mutants

Group A mutants grew only weakly on either naphthalene or 2-methylnaphthalene, both on plates and in liquid culture; with limiting naphthalene only about 30–40% of the turbidity of the parent PaW701 was obtained. Enzyme assay of one group A mutant, PaW711 (Table 3) showed that neither the *ortho* pathway enzymes, C12O and MLE, nor the *meta* pathway enzymes, C23O, HMSH and HMSD, were induced to any significant degree by growth on naphthalene or 2-methylnaphthalene, and no SH activity could be detected under any growth conditions. These results were typical of all the other group A mutants tested.

Culture supernatants of group A strains growing on naphthalene accumulated a compound with a UV spectrum identical to that of salicylate. Acid ether extracts of the supernatant of naphthalene-grown PaW711 contained a UV fluorescent compound which co-chromatographed with authentic salicylic acid on TLC using two different solvents. Further confirmation of the identity of the compound as salicylic acid was obtained by preparing the trimethylsilyl derivative of the acid ether extractable material and running it on GLC with authentic trimethylsilylated salicylic acid. Quantitative estimates of the amount of salicylate present in supernatants, both by direct measurement of their absorbance and by the method of Trinder (1954), indicated that about 60% of the naphthalene present at the start of the culture was converted to salicylate. This is likely to be a minimum estimate because of the difficulty of estimating whether there had been incomplete metabolism of the naphthalene or whether there had been losses due to its volatilization during growth.

When PaW711 was grown on 2-methylnaphthalene, 4-methylsalicylate accumulated in the medium; this was distinguished from salicylate by GLC of its trimethylsilyl derivative, and from 5-methylsalicylate by its UV spectrum.

Metabolic properties of group B mutants

Both strains, PaW705 and PaW719, grew vigorously on naphthalene, 2-methylnaphthalene, salicylate and 4- and 5-methylsalicylate. Enzyme assay (Table 3) showed that the *meta* pathway

enzymes C23O and HMSD were induced to high levels, but that HMSH was not significantly induced. SH activity could not be assayed because the rapid production of 2-hydroxymuconic semialdehyde (from the action of the high levels of C23O on catechol, the product of SH) interfered with the spectrophotometric assay for SH. No C12O activity could be detected, although it is possible that it was present in low levels but that these could not be measured in the presence of the high specific activity of C23O competing for the same substrate.

Plasmid structure in group A mutants

The plasmid DNA of all group A mutants was isolated and digested with *Hin*dIII and *Xho*I restriction endonucleases; the fragments after *Hin*dIII digestion of three representative plasmids are shown in Table 2. Compared with the parental plasmid pWW60-1, all the mutant plasmids had lost a single band, HH (3·8 kb) in the *Hin*dIII digests, and XC (6·0 kb) in the *Xho*I digests. In their place all had acquired four new *Hin*dIII bands and three new *Xho*I bands; the total additional DNA in all plasmids was 16·7 kb. Each plasmid had in common two new *Hin*dIII bands of 7·0 and 5·0 kb, the other two novel bands being different for each. In the *Xho*I digests one novel band (2·7 kb) was common to all mutants and of the other two, one was always large (15·5–17·5 kb) and one small (2·0–4·0 kb) (results not shown).

These results are consistent with the insertion of a 17 kb DNA fragment containing three *Hin*dIII and two *Xho*I sites into the region of the plasmid common to fragments HH and XC. The exact site of insertion obviously differs from mutant to mutant since the flanking insert–plasmid hybrid fragments are of varying sizes.

Plasmid structure in group B mutants

The plasmids from both group B mutants were digested with *Xho*I (Table 2) and *Hin*dIII. Plasmid pWW60-22 had lost fragment XC (6·0 kb) and acquired a novel 4·4 kb fragment, with net loss of 1·6 kb. From the *Hin*dIII digest it had lost fragment HH (3·8 kb) and one element from each of the double bands HQ/HR (1·4 kb) and HS/HT (1·0 kb), and acquired a novel band of 4·5 kb, with net loss of 1·7 kb. The other plasmid, pWW60-14 had also lost fragment XC which was replaced by a 4·8 kb band, with net loss of 1·2 kb. From the *Hin*dIII digest it had lost fragment HH and one element from the double HS/HT band and acquired a novel 3·5 kb band, with net loss of 1·3 kb.

The plasmids in these two mutants each appear to have suffered a deletion of DNA (about 1·6 kb in pWW60-22 and about 1·2 kb in pWW60-14) in the region corresponding to fragment XC and the end of fragment HH running into HQ and HS.

Location of the ortho and meta pathway genes

It is possible that the two catechol-degrading pathways, the *ortho* pathway in PaW701, and the *meta* pathway in PaW719, a group B mutant, were determined either by plasmids in those strains or by the host chromosomes. In order to distinguish between these possibilities, both strains were used as donors in matings with two plasmid-free recipients, selecting for Nah$^+$. Strain PaW85 is derived from PaW1 by loss of pWW0 (Bayley *et al.*, 1977) and has a fully functional chromosomal *ortho* pathway. Strain PaW11 is a plasmid-free mutant also derived from PaW1, which cannot grow on benzoate or catechol and therefore has a non-functional *ortho* pathway.

Strain PaW719 transferred its plasmid (pWW60-22) to both PaW85 and PaW11 to produce transconjugants with the same catabolic phenotype as itself. The plasmids in the transconjugants were identical to that in the donor. Thus, the *meta* pathway is plasmid coded and a functional chromosomal *ortho* pathway is not required.

Strain PaW701 transferred pWW60-1 to PaW85, to produce transconjugants with identical catabolic phenotype and plasmid content as the donor. However, only pinprick transconjugant colonies were found when PaW11 was the recipient, although analysis of their plasmid DNA showed it to be identical to pWW60-1. We therefore conclude that full expression of the Nah$^+$ phenotype in PaW701 and its transconjugants requires a functional chromosomal *ortho* pathway in addition to the plasmid genes for conversion of the naphthalene to catechol. The ability of

transconjugants of PaW11 containing pWW60-1 to produce even minute colonies on naphthalene must result from the pyruvate released from oxidation of the first ring. This idea is given added weight by their total inability to grow on salicylate.

DISCUSSION

Strain PaW701 contains an IncP9 plasmid, pWW60-1, derived by conjugation from PGB1 (NCIB 9816) which carries genes for the catabolism of naphthalene. In PaW701 the naphthalene appears to be metabolized through salicylate, catechol and the *ortho* pathway. The evidence for this is as follows. (a) C12O and MLE, the first two enzymes of the *ortho* sequence, are induced by growth on naphthalene whereas the corresponding *meta* pathway enzymes, C23O, HMSH and HMSD, although detectable, are at low levels and are not significantly induced. (b) PaW701 does not grow on 2-methylnaphthalene, contrary to what might be expected were it a strain with a functional *meta* pathway, given the well documented non-specificity of that pathway (Bayly & Dagley, 1969; Murray *et al.*, 1972): 2-methylnaphthalene and 4-methylsalicylate are only partially metabolized by PaW701, through 4-methylcatechol and its *meta* ring fission product to an unidentified brown product which is not metabolized further, indicating that the *meta* pathway is not completely functional. Strain PaW701 can however acquire the ability to grow on 2-methylnaphthalene by mutation in one of two ways.

In all the 13 independently isolated group A mutants, a large insertion of about 17 kb appears in the same region of the plasmid (HH and XC). The evidence from the enzyme assays, the almost stoichiometric accumulation of salicylate in the medium and the loss of the mutants' ability to grow on salicylate, shows that their SH is not active, presumably as a direct result of the insertion. Their ability to grow on naphthalene must be due to their utilization of the three carbon atoms released as pyruvate, resulting from cleavage of the first ring (Davies & Evans, 1964); this accounts for their poorer growth yield. We cannot say whether the loss of SH activity is due to insertional inactivation of its structural gene or of a regulatory gene essential for its expression.

Depending on which carbon atoms are attacked by the initial oxygenase, it is possible to envisage a number of different metabolites of 2-methylnaphthalene at the level of salicylate. However, the accumulation of 4-methylsalicylate from 2-methylnaphthalene in almost stoichiometric quantities by the group A mutants shows that this initial attack must be on carbon atoms 7 and 8, as described by Rogoff & Wender (1959). The mutants must also use the pyruvate from oxidation of the unsubstituted ring of 2-methylnaphthalene as carbon for growth. We assume that the reason why the parent strain PaW701 does not grow on the same pyruvate when it appears to be able to metabolize 2-methylnaphthalene past the 4-methylsalicylate stage is because some product of its partial metabolism, probably the brown unidentified product accumulated in the medium, is toxic; only mutational loss of the SH activity will stop its formation and thus allow utilization of the three available carbons.

The 17 kb fragment of DNA, which is found as an insert in the plasmids of the group A mutants, behaves as a transposon. In other derivatives of PaW701 we have found it present in different fragments of pWW60-1 without any effect on the host phenotype, and we have also found it as an insertion in other catabolic and R plasmids which have been transferred into PaW340. Preliminary evidence from Southern blot hybridization and from mapping the cutting sites of a number of restriction enzymes has led us to identify tentatively the 17 kb region as a residue of DNA in the chromosome of PaW340 left from the TOL plasmid pWW0 which was originally present in *P. putida* mt-2 (PaW1), the parental strain from which PaW340 was derived. The same 17 kb has also been described as a residue of pWW0 after its transfer and subsequent structural alteration in a chlorobenzoate-degrading *Pseudomonas* sp. (Jeenes & Williams, 1982). However, it should be stressed that none of the TOL catabolic genes appear to be located on this region (Jeenes & Williams, 1982), and that the changes in catabolic phenotype found in PaW701 and its derivatives therefore cannot be attributed to the introduction of novel catabolic genes originally present on another plasmid. We believe that the effect of the 17 kb transposon in the group A mutants is solely that of insertional mutagenesis.

In the group B mutants, a deletion in a closely linked region of pWW60-1 DNA allows high levels of induction of the *meta* pathway enzymes. Not only is good growth maintained on naphthalene and salicylate, but these mutants have acquired the ability to grow on 2-methylnaphthalene and both 4- and 5-methylsalicylates. This is due to the relaxed specificity of the *meta* pathway enzymes allowing complete metabolism of methylcatechols, as compared with the specificity of the *ortho* pathway, capable of metabolizing only catechol itself (Feist & Hegeman, 1969). The low levels of HMSH even in PaW719, where C23O and HMSD are induced, are consistent with the observation that the hydrolase branch of the *meta* pathway is of no metabolic significance in strain NCIB 9816 (Catterall *et al.*, 1971).

A curious feature of pWW60-1 is that it apparently carries the *meta* pathway genes in a form normally unexpressed, and that it requires a functional chromosomal *ortho* pathway for complete metabolism of naphthalene. Only when it acquires a small deletion, as in the group B mutants, is the full catabolic potential of the plasmid determined genes made available to the host bacterium by allowing the *meta* pathway to be utilized. It is possible that pWW60-1 is itself derived from a plasmid which has acquired an insertion sequence blocking expression of its *meta* pathway enzymes. Normally this might be expected to destroy any selective advantage conferred on the host by the plasmid, and thus counterselect against its continued maintenance in the population. However, the nature of the block is such that pWW60-1 can still function as a naphthalene plasmid in hosts carrying a functional *ortho* pathway, a virtually ubiquitous feature of *P. putida* strains.

The ease with which changes in plasmid structure which have a drastic effect on the catabolic phenotype occur in these strains may well explain the conflicting reports on the naphthalene catabolism of *Pseudomonas* NCIB 9816. Strain PaW701 behaves very similarly to the clone of this strain described by Barnsley (1976) in appearing to use the *ortho* pathway, and yet expressing low constitutive levels of C23O. On the other hand, the group B mutants resemble the clone of NCIB 9816 (*Pseudomonas* P_G) as described by Williams *et al.* (1975) which used the *meta* pathway and grew on 2-methylnaphthalene. It is easy to see how biochemical and genetic variants of a single original clone can arise in different laboratories if they are subjected to selective pressures which so readily result in irreversible changes in genotype.

Note added in proof. After preparation of this manuscript, a paper by M. A. Connors & E. A. Barnsley [*Journal of Bacteriology* (1982), **149**, 1096–1101] has confirmed the presence of two plasmids in NCIB 9816, and the involvement of the larger in naphthalene catabolism.

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