A Restriction Map of Naphthalene Catabolic Plasmid pWW60-1 and the Location of Some of Its Catabolic Genes

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A restriction map for the 87 kbp IncP9 naphthalene catabolic plasmid pWW60-1 is presented. Transposon mutants were obtained using direct Tn5 insertion or using an indirect method involving the intermediate formation of unstable cointegrates between RP4 and pWW60-1. Insertions which affected expression of the early enzymes of the pathway (naphthalene to salicylate) were separated from inserts which affected expression of salicylate hydroxylase (*nahG*) or catechol 2,3-oxygenase (*nahH*). *nahABC* were cloned on a contiguous region of the plasmid on either a 6.9 kbp *Hind*III fragment (HE) or a 5.7 kbp *Xho*I fragment (XD). *nahGH* were cloned on a region situated about 30 kbp from *nahABC* on an *Xho*I fragment (XC), but *nahH* was only expressed on the corresponding but smaller *Xho*I fragments from two derivative plasmids of pWW60-1 with a deletion in that region. The detailed restriction map of *nahH* shows no similarities with the restriction maps of the genes for catechol 2,3-oxygenases from TOL plasmids, although the cloned gene did hybridize with genes for catechol 2,3-oxygenase from two different TOL plasmids. The organization of the catabolic genes on pWW60-1 suggests a separation into two operons as also described for the naphthalene catabolic plasmid NAH7 (alternatively called pIG7), but the relative directions of their transcription differs from that in NAH7.

INTRODUCTION

In *Pseudomonas* strains which have been isolated by selective enrichment on naphthalene as sole carbon source, the genes coding for the breakdown of naphthalene are usually carried on plasmids (Dunn & Gunsalus, 1973; Boronin *et al.*, 1977, 1980; Cane & Williams, 1982). *Pseudomonas* sp. NCIB9816, also called *Pseudomonas* P_G (Williams *et al.*, 1975), is the strain in which the main features of the naphthalene catabolic pathway were first elucidated by Davies & Evans (1964). They showed that naphthalene was converted to salicylate along with the release of pyruvate: catechol formed from salicylate was then catabolized to central metabolites by the *meta* pathway, involving cleavage of the ring by catechol 2,3-oxygenase (C23O) (Fig. 1) (Davies & Evans, 1964; Murray *et al.*, 1972). Subsequently, there was some disagreement in the literature concerning the route of catechol metabolism when it was reported that the *ortho* or β -ketoadipate pathway was used (Fig. 1) (Barnsley, 1976), and it became apparent that a number of variants of NCIB9816 were in circulation (Barnsley, 1976; Cane & Williams, 1982).

A partial explanation for these variants was obtained by a study of the genetics of naphthalene catabolism by NCIB9816. This strain was found to contain two plasmids, one of which carried the naphthalene genes (Cane & Williams, 1982; Connors & Barnsley, 1982). This latter plasmid (87kbp; IncP9) was transferred into a plasmid-free *Pseudomonas putida* strain PaW340 to give a single transconjugant, PaW701 (Cane & Williams, 1982). The plasmid in PaW701, pWW60-1, was responsible for the conversion of naphthalene only as far as catechol, which was then further

Abbreviations: C23O, catechol 2,3-oxygenase; SH, salicylate hydroxylase.

Fig. 1. Pathway for the catabolism of naphthalene. Enzymes (genes) shown are: 1, naphthalene dioxygenase (*nahA*); 2, naphthalene *cis*-dihydrodiol dehydrogenase (*nahB*); 3, 1,2-dihydroxynaphthalene dioxygenase (*nahC*); 4, 2-hydroxychromene-2-carboxylate isomerase (*nahD*); 5, 2-hydroxybenzalpyruvate aldolase (*nahE*); 6, salicylaldehyde dehydrogenase (*nahF*); 7, salicylate hydroxylase (*nahG*); 8, catechol 2,3-oxygenase (*nahH*); 9, 2-hydroxymuconic semialdehyde dehydrogenase (*nahI*); 10, 4-oxalocrotonate tautomerase (*nahJ*); 11, 4-oxalocrotonate decarboxylase (*nahK*); 12, 2-oxo-4-pentenoate hydratase (*nahL*); 13, 2-oxo-4-hydroxypentanoate aldolase (*nahM*); 14, catechol 1,2-oxygenase (*catA*); 15, *cis,cis*-muconate lactonizing enzyme (*catB*).

metabolized by the enzymes of the chromosomally-coded *ortho* pathway. However, it was possible to select spontaneous mutants from PaW701 cultures which used a plasmid-coded *meta* pathway. Examination of the plasmids in some of these mutants showed a deletion of 1.2 to 1.5 kbp between genes for salicylate hydroxylase (SH) and C23O which apparently allowed expression of previously silent *meta* pathway genes.

This paper describes further studies on pWW60-1. A restriction endonuclease map of the plasmid has been derived and some of the structural and regulatory genes have been cloned and mapped.

METHODS

Bacterial strains and plasmids. Details of all relevant parent bacterial strains and their plasmid complements are listed in Table 1. In Table 2, a list is presented of all the derivative strains into whose plasmids has been inserted a transposon or other transposable DNA: all these are in a PaW85 or PaW340 background. Table 3 is a list of all the plasmids carrying cloned fragments from pWW60-1 or its derivatives; these are in *Escherichia coli* PM191.

Preparation of plasmid DNA. Large plasmids (>40 kbp) were extracted by the method of Wheatcroft & Williams (1981). Small plasmids were screened according to Holmes & Quigley (1981), and prepared in quantity by CsCl density gradient centrifugation of cleared lysates (Guerre *et al.*, 1973).

Table 1. Bacterial strains used in this study

Strain	Plasmid	Relevant phenotype*	Notes and references
<i>Pseudomonas</i>			
NCIB9816	pWW60, pWW61	Nah ⁺ 2MeNah ⁺ Sal ⁻ 4MeSal ⁻	Wild-type
AC34	RP4	Ade ⁻ Ap ^r Km ^r Tc ^r	Franklin & Williams (1980)
PaW85	—	Nah ⁻ Sal ⁻ Str ^r	Bayley <i>et al.</i> (1977)
PaW130	—	Rif ^r	Keil <i>et al.</i> (1985b)
PaW340	—	Nah ⁻ Sal ⁻ Trp ⁻ Str ^r	Cane & Williams (1982)
PaW701	pWW60-1	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻	Cane & Williams (1982)
PaW705	pWW60-14	Nah ⁺ 2MeNah ⁺ Sal ⁺ 4MeSal ⁺ }	Spontaneous deletion mutants of PaW701; Cane & Williams (1982)
PaW707	pWW60-2		
PaW719	pWW60-22		
PaW740	pWW60-1, RP4		
PaW741	pWW60-40	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻ Trp ⁻ Str ^r Ap ^r Km ^r Tc ^r	Conjugation AC34 × PaW701; this paper
PaW742	pWW60-40, RP4	Nah ⁻ Sal ⁺ 4MeSal ⁺ Trp ⁻ Str ^r	Spontaneous Tn(TOL) insertion mutant of PaW707; this paper
		Nah ⁻ 2MeNah ⁻ Sal ⁺ 4MeSal ⁺ Trp ⁻ Str ^r Ap ^r Km ^r Tc ^r	Conjugation AC34 × PaW741; this paper
<i>E. coli</i>			
WG3110	pLG221	Km ^r	F.C.H. Franklin; unpublished
pM191	—	Thr ⁻ Leu ⁻ Thi ⁻ RecA ⁻	<i>recA</i> derivative of C600; Bachman (1972)
	pKT230	Km ^r Str ^r	Bagdasarjan <i>et al.</i> (1981)
	pBR322	Tc ^r Ap ^r	Bolivar <i>et al.</i> (1977)
	pNJ5000	Tc ^r	Derivative of RP4; Grinter (1983)

* Nah⁺, 2MeNah⁺, Sal⁺ and 4MeSal⁺ denote ability to grow on naphthalene, 2-methylnaphthalene, salicylate and 4-methylsalicylate, respectively, as sole carbon sources. Ade⁻, Trp⁻, Thr⁻, Leu⁻ and Thi⁻ denote requirements for adenine, tryptophan, threonine, leucine and thiamin respectively. Rif^r, Str^r, Km^r, Ap^r and Tc^r denote resistance to rifampicin, streptomycin, kanamycin, ampicillin and tetracycline respectively.

Restriction endonuclease digestion. All digestions and subsequent electrophoreses were done as described previously (Wheatcroft & Williams, 1981).

Extraction of DNA from agarose gels. This was done by the method of McDonnell *et al.* (1977).

Conjugation. Samples (10 µl) of overnight broth cultures of donor and recipient strains were pipetted onto sterile Millipore filters on nutrient agar plates and incubated for 15–24 h. The filters were then washed in 1 ml 100 mM-phosphate buffer (pH 7.5) and the washings spread, with dilutions as necessary, on agar plates of appropriate selective media.

Formation of RP4::pWW60-1 cointegrate plasmids. Donor strain *Pseudomonas aeruginosa* AC34(RP4) was mated with PaW701 and PaW741 selecting for transconjugants resistant to kanamycin (100 µg ml⁻¹). PaW740 and PaW742 were maintained as typical transconjugants from each mating. These were then mated with plasmid-free strain PaW85, selecting for simultaneous transfer of ability to grow on salicylate and resistance to kanamycin. Transconjugants were subsequently screened for loss of ability to grow on naphthalene (for strains derived from PaW701) or on 4-methylsalicylate (for strains derived from PaW741).

Insertion of Tn5 into naphthalene plasmids. *E. coli* WG3110 carrying the Tn5 suicide vector plasmid pLG221 was mated with PaW705 as recipient selecting for transconjugants able to grow on benzoate in the presence of 100 µg kanamycin ml⁻¹. The plasmid DNA of transconjugants was screened for insertion of Tn5, but none was found, even though there was no trace of pLG221: it was therefore assumed that Tn5 had inserted into the chromosome. One of the strains produced was then used as donor in matings with PaW85, selecting for transfer of kanamycin resistance, and relying on the kanamycin resistance gene being mobilized only if it had transposed into the plasmid. This occurred at only a very low frequency.

Cloning experiments. DNA from the vector plasmid (pKT230 or pBR322) and from the appropriate naphthalene plasmid were mixed and digested with the relevant restriction enzyme according to the suppliers protocol. Sodium acetate (pH 5.6) was added to a final concentration of 200 mM and the DNA precipitated with ethanol. After two washes with 70% (v/v) aqueous ethanol, the DNA was resuspended in ligation buffer and incubated with T4 ligase as specified by the supplier for 20 h at 20 °C. The ligated DNA was transformed into *E. coli* pM191 by standard procedures (Cohen *et al.*, 1972).

Detection of clones expressing naphthalene catabolic activities. Cultures of *E. coli* expressing naphthalene dioxygenase (*nahA*) turned blue when grown in well-aerated culture in nutrient broth due to the production

of indigo (Ensley *et al.*, 1983). When gene products of both *nahA* and *nahB* (naphthalene *cis*-dihydrodiol dehydrogenase) were present in the same strain, a brown colour developed around colonies grown on nutrient agar in the presence of naphthalene due to the auto-oxidation of accumulated 1,2-dihydroxynaphthalene. *nahC* (for 1,2-dihydroxynaphthalene dioxygenase) could be detected by spraying colonies with freshly prepared 100 mM-3-methylcatechol (but not catechol), which was rapidly converted to a bright yellow ring fission product. When 2.5 mM-salicylate was incorporated into isosensitest agar plates, the presence of SH (*nahG* gene product) alone produced a black colour in colonies, caused by catechol accumulation and auto-oxidation, whilst the joint presence of SH and C23O (*nahH*) resulted in the production of bright yellow 2-hydroxymuconic semialdehyde. By spraying colonies with 100 mM-catechol, *nahH*⁺ clones could be detected by their almost immediate conversion to a yellow colour.

Mobilization of recombinant plasmids into *P. putida*. Recombinant plasmids derived from pKT230 were mobilized into plasmid-free PaW130 by an RP4 derivative, pNJ5000 (Grinter, 1983), as previously described (Keil *et al.*, 1985b).

Southern blotting. Southern blots (Southern, 1975) and hybridizations were done as previously described (Keil & Williams, 1985).

Enzyme assays. SH and C23O were assayed as previously described (Cane & Williams, 1982).

RESULTS

Restriction map of pWW60-1

The sizes of the restriction fragments of pWW60-1 produced by digestion with *Hind*III and *Xho*I have been published previously (Cane & Williams, 1982). *Bam*HI digestion gave six fragments: A, 23–25 kbp; B, 22–24 kbp; C, 19 kbp; D, 10.1 kbp; E, 5.1 kbp; F, 3.3 kbp. The map of the plasmid with respect to these enzymes is shown in Fig. 2: it is complete for the

Table 2. *Strains carrying plasmids with transposon insertions*

Strain	Original plasmid	Phenotype	Insert	Restriction fragments carrying the insert*
PaW711	pWW60-1	Nah ⁺ 2MeNah ⁺ Sal ⁻ 4MeSal ⁻	Tn(TOL)	BC, HH, XC
PaW741	pWW60-2	Nah ⁻ 2MeNah ⁻ Sal ⁺ 4MeSal ⁺		BB, HE, XD
PaW743	pWW60-22	Nah ⁺ 2MeNah ⁺ Sal ⁺ 4MeSal ⁺		BC, HA, XA
PaW744	pWW60-14			BF, HA, XA
PaW745	pWW60-1	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻		BC, HA, XA
PaW746	pWW60-14	Nah ⁺ 2MeNah ⁺ Sal ⁺ 4MeSal ⁺		BD, HF, XH
PaW927	pWW60-22			BF, HA, XA
PaW901	pWW60-1	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻	Tn/	BB, HG, XL
PaW902				BC, HD, XB
PaW903				BC, HA, XA
PaW907				BF, HA, XA
PaW908	pWW60-40	Nah ⁻ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻		BB, HO, XM
PaW909				BC, HC, XB
PaW911, PaW912, PaW913				BC, HQ, XC'
PaW914, PaW917	pWW60-1	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻		BC, HD, XC'
PaW918				BB, HJ, XD
PaW919, PaW920, PaW921				BB, XD, a†
PaW922	pWW60-1	Nah ⁺ 2MeNah ⁻ Sal ⁺ 4MeSal ⁻	Tn5	BF, HA, XA
PaW924				BC, HD, XB
PaW728, PaW729	pWW60-14	Nah ⁺ 2MeNah ⁺ Sal ⁺ 4MeSal ⁺		BA, HI, XA
PaW730				BB, HE, XD
PaW923	pWW60-22	Nah ⁻ 2MeNah ⁻ Sal ⁺ 4MeSal ⁺		BB, HJ, XD
PaW926				

* The first letter refers to the restriction enzyme (B, *Bam*HI; H, *Hind*III; X, *Xho*I), the second to the fragment (Fig. 2).

† XC''' is a 4.8 kbp fragment formed by a spontaneous deletion in XC found in parental pWW60-40; it is indistinguishable from XC' from pWW60-14.

‡ a refers to one or more small unidentified *Hind*III fragments (<1 kbp).

Table 3. Recombinant plasmids carrying cloned pWW60-1 genes

Plasmid	NAH plasmid	Genes detected	Notes
pWW60-3002	pWW60-1	<i>nahA, nahB</i>	<i>Hind</i> III fragment HE in pKT230
pWW60-3017			As for pWW60-3002, but with HE in reversed orientation
pWW60-3021	pWW60-22	None	<i>Bam</i> HI fragment BD in pBR322
pWW60-3006		<i>nahC</i>	<i>Xho</i> I fragment XD in pKT230
pWW60-3008		None	As for pWW60-3006, but with XD in reversed orientation
pWW60-3001	pWW60-1	<i>nahG</i>	<i>Hind</i> III fragment HH in pKT230
pWW60-3003			<i>Xho</i> I fragment XC in pKT230
pWW60-3004	pWW60-14	<i>nahG, nahH</i>	<i>Xho</i> I fragment XC' in pKT230
pWW60-3005		<i>nahG, nahH, nahR</i>	<i>Xho</i> I fragment XC'' in pKT230
pWW60-3010*		<i>nahG</i>	<i>Xho</i> I- <i>Hind</i> III fragment of pWW60-3005 obtained by <i>Hind</i> III digestion and religation
pWW60-3011*	pWW60-22	None	<i>Xho</i> I- <i>Sma</i> I fragment of pWW60-3005 obtained by <i>Sma</i> I digestion and religation
pWW60-3012*		<i>nahH</i>	<i>Sma</i> I- <i>Xho</i> I fragment of pWW60-3005 obtained by <i>Sma</i> I digestion and ligation into new pKT230
pWW60-3013*			<i>Kpn</i> I- <i>Xho</i> I fragment of pWW60-3005 obtained by partial <i>Kpn</i> I digestion and religation
pWW60-3014*		None	<i>Kpn</i> I- <i>Xho</i> I fragment of pWW60-3005 obtained by <i>Kpn</i> I digestion and religation
pWW60-3016*		<i>nahG</i>	<i>Pst</i> I- <i>Pst</i> I fragment of pWW60-3005 obtained by <i>Pst</i> I digestion and ligation into pBR322

* Subclones of pWW60-3005 (see Fig. 4).

*Bam*HI fragments, and essentially complete for the other enzymes, apart from the order of some fragments, particularly those internal to *Bam*HI-B, and the location of some smaller fragments (<1 kbp). The map was derived from a variety of different experimental results which included the examination of spontaneous deletion plasmids and plasmids with inserts (Table 2), and the multiple restriction digestion of cloned fragments (Table 3), and of bands extracted from agarose gels.

Insertion mutants

Transposition of Tn5 into pWW60-14 or pWW60-22 occurred at very low frequency and only five such insertion strains were isolated (Table 2). However, experiments to form RP4::pWW60-1 or RP4::pWW60-40 cointegrates proved a much more successful, although indirect, way of obtaining insertion mutants. In the matings selecting for cotransfer of both plasmid phenotypes, cointegrates were formed in which the entire RP4 and naphthalene plasmids were linked; however these cointegrates were very unstable and dissociated, during subculture of strains, to give a complete naphthalene plasmid that contained a 5 kbp insert with an identical restriction pattern to Tn1, even though there was no selection for ampicillin resistance. This formation of cointegrates apparently involving Tn1 appears similar to that reported by McCormick *et al.* (1981).

In addition, a number of spontaneous insertions in pWW60-1 and its derivatives were found: PaW741 (Table 1) is a typical example of a spontaneous insertion mutant of this type. In these strains, the DNA inserted was about 17 kbp in size and had the characteristic digestion pattern previously found for the inserts which enabled some mutants of PaW701 to grow on 2-methylnaphthalene (Cane & Williams, 1982). In the original mutants it was shown by Southern blotting that this insert was derived from the TOL plasmid pWW0, of which the host strain PaW340 is a cured derivative. It appears that this element is present in the chromosome of PaW340 (Meulien & Broda, 1982) and acts as a transposon, able to insert spontaneously into plasmids resident in PaW340 (Williams *et al.*, 1983). This has occurred into other catabolic

Fig. 2. Restriction endonuclease map of pWW60-1. ▲, Insertions which have a catabolic effect; △, insertions without a catabolic effect, with the proviso that these insertions were made in plasmids which already have the natural insertion between *nahG* and *nahH*. The number of the insertion refers to the strain number given in Table 2. The deletion which has occurred in pWW60-22 and which removes the natural block to expression of the *meta* pathway genes (*nahHIJKLM*) is shown. Arrows show the direction of transcription of the genes. The major fragments cloned in this paper are hatched.

plasmids resident in PaW340 in our laboratory and we believe that the insert may well be a transposon, although we have not been able to test its independence of *recA* function: we propose that it be called Tn(TOL) as a temporary nomenclature until definitive experiments are done.

Insertions into the catabolic genes can be monitored by testing the host strains for growth on naphthalene, salicylate and 4-methylsalicylate. Growth on naphthalene, albeit poorly, can occur even with the pathway blocked after salicylaldehyde, since pyruvate released from oxidation of the first ring can serve as a growth substrate. Salicylate, but not 4-methylsalicylate, can be metabolized via the chromosomal β -ketoadipate if the plasmid *meta* pathway is blocked below SH. Several classes of structural gene mutants are therefore possible: (i) with the pathway blocked between *nahA* and *nahF*, strains would be unable to grow on naphthalene but able to grow on salicylate or 4-methylsalicylate; (ii) with the pathway blocked in *nahG*, strains would be able to grow poorly on naphthalene but not on salicylate; (iii) with the pathway blocked in *nahH*, strains would be able to grow on naphthalene and salicylate but not on their methylated derivatives; (iv) with the pathway blocked between *nahI* and *nahM*, strains would be expected to grow only poorly or not at all on naphthalene and salicylate due to accumulation of potentially toxic *meta* fission products. There are also possible regulatory mutants with different phenotypes depending on how the pathway is regulated.

All mutants of class (i) had insertions in fragments XD, BB, and HE or HJ or in a small unmapped *HindIII* fragment of <1 kbp (Table 2, Fig. 2). All class (ii) mutants had inserts in fragments XC, BC and HH confirming the location of *nahG* in the region previously described (Cane & Williams, 1982). The mutants obtained which were of class (iii) all lacked C23O activity, as shown by the catechol spray test, and therefore had inserts directly in *nahH* or in a gene necessary for *nahH* expression, and all mapped in fragments XC, BC and HD or HQ. No mutants of class (iv) were obtained.

Fig. 3. Restriction map of the region overlapping *Hind*III fragment E (HE), *Bam*HI fragment D (BD) and *Xho*I fragment D (XD) of pWW60-1. Abbreviations used for restriction endonucleases are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I.

Between the insertions of class (i) and classes (ii) and (iii), there was a region of 15–20 kbp into which several inserts were found which had no apparent effect on the catabolic phenotype (PaW901, 902, 908, 909). However, since these strains were derived from PaW701, which has a cryptic *meta* pathway and uses the chromosomal *ortho* pathway for catechol metabolism, it is possible that this region, or part of it, encodes the *meta* pathway genes *nahI* to *nahM* (Fig. 1).

Cloning of *nahABC*

A list of recombinant plasmids carrying cloned fragments is shown in Table 3. Two fragments, HE and XD, shown by transposition mutagenesis to be involved in the conversion of naphthalene to salicylate were cloned into *E. coli* on pKT230. Recombinant plasmid pWW60-3002 (HE) expressed *nahA* and *nahB*, while pWW60-3006 (XD) expressed *nahC*, presumably under the influence of the kanamycin promoter of pKT230. When HE was inserted into pKT230 in the opposite orientation (pWW60-3017), *nahA* continued to be expressed, as shown by the production of indigo, although qualitatively this appeared to be at a lower level than with pWW60-3002. This would suggest the presence of an operator-promoter region on HE upstream of *nahAB* which allows its weak expression. In contrast, pWW60-3008, carrying XD in the opposite orientation, no longer expressed *nahC*.

The *Bam*HI fragment BD, which overlapped HE (Fig. 2), was also cloned into pBR322, but the *E. coli* host showed no accumulation of indigo: this indicates that the *Bam*HI site between BD and BB lies within or upstream of *nahAB*.

The restriction endonuclease map of the HE/XD region of pWW60-1 is shown in Fig. 3.

Cloning of *nahGH*

Recombinant plasmids carrying XC (pWW60-3003) and HH (pWW60-3001) from pWW60-1 expressed SH (*nahG*) but not C23O (*nahH*). However, the plasmids in derivatives of PaW701, namely PaW705 and 719, selected for their ability to grow on 2-methylnaphthalene, have deletions of 1.2 and 1.5 kbp respectively in XC (XC', XC''); when these fragments were cloned into pKT230, in pWW60-3004 and pWW60-3005, both SH and C23O were expressed. A map of XC and its deleted derivatives is shown in Fig. 4. The recombinant plasmid pWW60-3005 was the subject of more detailed analysis by fine mapping and subcloning and these results are also shown in Fig. 4.

pWW60-3016 (Table 3) expressed SH but not C23O indicating that the start of *nahG* is downstream of the *Pst*I site at the coordinate of 1 kbp (Fig. 4). pWW60-3013 does not express SH indicating that the *Kpn*I site at 1.8 kbp is within the gene. Neither pWW60-3011 nor pWW60-3012 expressed SH, and we have shown that transposon insertion either side of the *Sma*I site inactivated *nahG* (data not shown), also indicating that this site is within the gene. However, *nahG* cannot extend more than 0.2 kbp downstream of this *Sma*I site since the *Hind*III

Fig. 4. Restriction map of the region overlapping *Hind*III fragment H (HH) and *Xho*I fragment C (XC) of pWW60-1, together with the clones and subclones derived from this region of pWW60-1 and from the same region of the deleted derivative plasmids pWW60-22 and pWW60-14, which express *meta* pathway genes. Abbreviations for restriction endonucleases are given in Fig. 3, except for: A, *Ava*I; Pv, *Pvu*II. Arrows show the direction of transcription from the vector promoters. [stippled], Cloned catabolic plasmid DNA in the recombinant plasmids; ---, deletions produced in the subcloning; Δ , deletions in the parental plasmids.

fragment carried by pWW60-3001, which does express SH, goes no further than this. Thus *nahG* must lie between points at 1 kbp and 2.8 kbp on the parent fragment.

Subclone pWW60-3012 expressed C23O indicating that *nahH* is downstream of *nahG*. The starting point of this gene must be downstream of the boundary of the deletion in pWW60-3005 in which it is also expressed (Fig. 4). Subclones pWW60-3010 and pWW60-3014 did not express *nahH* indicating that there are *Hind*III and *Kpn*I sites within the gene. The restriction

Fig. 5. Hybridization analysis of cloned fragments carrying catechol 2,3-oxygenase genes. (a) Agarose gel of restriction digests of plasmids and (b) an autoradiogram of same gel after Southern blotting and hybridization to the *Sma*I fragment of pWW60-3012, which was extracted from agarose gels prior to being radiolabelled with 32 P by nick-translation. Track 1, control containing plasmid pWW60-3012 (Fig. 4) cut with *Sma*I. The upper band is the vector, pKT230, and the lower band is the 2.3 kbp *Sma*I fragment carrying *nahH*. Track 2, pWW0-3004 cut with *Xho*I. This plasmid contains the 2.3 kbp *Xho*I fragment I (XI), from TOL plasmid pWW0, which carries the *xylE* gene (for catechol 2,3-oxygenase). The vector is pKT230 (upper band), which, as in track 1, hybridizes against the probe. Track 3, pWW15-3151 cut with *Xho*I. This plasmid is a recombinant between pKT230 and the 2.1 kbp *Xho*I fragment h (Xh) from TOL plasmid pWW15. The insert carries the gene for C23OI, previously shown to be homologous with *xylE* from pWW0 (Track 2) (Keil *et al.*, 1985a). Track 4, pWW15-3171 cut with *Hind*III and *Xba*I. This plasmid is a recombinant between vector pUC12 (lower band) and a 3.4 kbp *Xba*I–*Hind*III insert, from TOL plasmid pWW15 (upper band), which carries the gene for C23OII. This second gene has been shown to share no apparent homology with the C23OI gene from pWW15 or with *xylE* from pWW0 (Keil *et al.*, 1985a). It should be noted that the fragment used as probe contains about 0.25 kbp of pKT230 (see Fig. 4), which might account for the relatively weaker hybridization of the vector band in tracks 1 and 2.

endonuclease sites in the vicinity of this C23O gene showed no similarities with those of the C23O genes from TOL plasmids (Keil *et al.*, 1985a; L. K. Chatfield & P. A. Williams, unpublished results). However, when pWW60-3012 was used as a probe in Southern blots against recombinant plasmids carrying either the C23O gene (*xylE*) from archetypal TOL plasmids pWW0 or the homologous gene for C23OI from TOL plasmid pWW15 (Keil *et al.*, 1985a), strong hybridization was seen (Fig. 5); there was no hybridization with the cloned gene for the second C23O (C23OII) from pWW15. In addition, there was no apparent hybridization with the pWW60-1 XD fragment carrying the *nahC* gene for 1,2-dihydroxynaphthalene dioxygenase, an enzyme functionally similar to C23O.

Cloning of a possible regulatory gene

pWW60-3005 was mobilized into *P. putida* PaW130 to give strain PaW936 for enzyme analysis (see Methods). Addition of 5 mM-anthranilate, a gratuitous inducer of both upper and lower pathways (Shamsuzzaman & Barnsley, 1974), to a nutrient broth culture of PaW936 resulted in an increase in C23O activity from 1 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ in uninduced cells to 26 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ in induced cells. This suggests the presence of a positive regulator gene on this fragment, similar to *nahR* of pIG7 (Grund & Gunsalus, 1983; Yen & Gunsalus, 1985).

DISCUSSION

The naphthalene catabolic plasmid pWW60-1 has a number of interesting features. The first of these is that there appears to be a sequence of DNA between *nahG* and *nahH* which

terminates transcription after salicylate hydroxylase, thus channelling the catechol formed through the chromosomal β -ketoadipate pathway (Cane & Williams, 1982). However, selection for growth on 2-methylnaphthalene or 4-methylsalicylate, which are converted by SH to methylcatechols that can only be processed by the *meta* pathway, can result in a loss of a minimum of 1.2 kbp between *nahG* and *nahH*, which permits complete transcription of the *meta* pathway. If such an apparently innocuous procedure as growth on 2-methylnaphthalene can result, through a deletion of a segment of DNA, in a complete change in the route by which naphthalene is assimilated, then it is not surprising that different laboratories have obtained contradictory results with clones of the same strain. One variant of NCIB9816, *Pseudomonas* P_G, has an inducible SH but a constitutive C23O (Barnsley, 1976); the plasmid in this strain has an identical restriction pattern to pWW60-1. However, the original parent strain of *Pseudomonas* P_G, from which all the other strains have ultimately been derived but which appears to be no longer extant, apparently had a fully inducible *meta* pathway (Catterall *et al.*, 1971), similar to that shown by PaW705 and 719 (Cane & Williams, 1982). These results suggest that the original wild-type may have had separate and independent regulation of *nahG* and the *meta* pathway genes, unlike the situation in NAH7 where the entire pathway from SH onwards is within one operon (Yen & Gunsalus, 1982). Point mutations could then switch off the *meta* pathway, as in PaW701, or make it constitutive, as in *Pseudomonas* P_G, or, after a large deletion, allow the *meta* pathway to come under the same control as SH, as in PaW705 and 719.

One feature of this plasmid is that the upper and lower operons appear to be transcribed in opposite directions towards each other. This is in contrast to the situation reported for NAH7, where the two operons are transcribed in the same direction from the upper operon through to the lower operon with the regulatory gene *nahR* in between, although the *nahR*-like gene of pWW60-1 also appears to lie immediately before *nahG*. We have no evidence as to whether the *nah* genes on the two plasmids share any homology but, even if they do, the different relative orientations of the two operons exclude the possibility of a simple recombinant event leading to the acquisition of essentially the same piece of DNA by the two plasmids. However, the separation of the operons on both plasmids and of the two operons on the TOL plasmid pWW0 (Worsey *et al.*, 1978; Nakazawa *et al.*, 1980) suggests an attractive hypothesis for their evolution. It is possible that the pathway was formed by the combination on a single replicon of pre-evolved metabolic 'modules', one responsible for enzymes for converting naphthalene to salicylate, one for SH, and a third element encoding the *meta* pathway enzymes. This would account for the apparent homology between the C23O genes of pWW60-1 and TOL plasmids, indicating that TOL and pWW60-1 may share related *meta* pathway DNA, and would also suggest that the presence of a separate operon for SH may be necessary in the initial evolution of a naphthalene catabolic plasmid. Such a hypothesis of related *meta* pathways would obviously require subsequent refinement to take account of some differences in the enzymology such as, for example, the absence of a hydrolytic branch (2-hydroxymuconic semialdehyde hydrolase) from the naphthalene *meta* pathway (Catterall *et al.*, 1971). However, conclusive evidence on the evolution of these long catabolic pathways and their plasmid carriers will only come from a detailed comparative investigation of their molecular biology.

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