Journal of General Microbiology (1988), **134**, 2769–2778. *Printed in Great Britain*

Comparison of the *meta* Pathway Operons on NAH Plasmid pWW60-22 and TOL Plasmid pWW53-4 and Its Evolutionary Significance

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(*Received 14 March 1988; revised 27 June 1988*)

The regulated *meta* pathway operon for the catabolism of salicylate on the naphthalene plasmid pWW60-22 was cloned into the broad-host-range vector pKT230 on a 17·5 kbp *Bam*HI fragment. The recombinant plasmid conferred the ability to grow on salicylate when mobilized into plasmid-free *Pseudomonas putida* PaW130. A detailed restriction map of the insert was derived and the locations of some of the genes were determined by subcloning and assaying for their gene products in *Escherichia coli* and *P. putida* hosts. The existence of a regulatory gene was demonstrated by the induction of enzyme activities in the presence of salicylate. DNA–DNA hybridization indicated a high degree of structural homology between the pWW60-22 operon and the analogous *meta* pathway operon on TOL plasmid pWW53-4. The data are consistent with the structural genes being arranged in an identical linear array and suggest an evolutionary link between the two catabolic systems.

INTRODUCTION

The involvement of plasmids in the catabolism of naphthalene by *Pseudomonas* strains has been well documented (Dunn & Gunsalus, 1973; Boronin *et al.*, 1977, 1980). In *Pseudomonas* strain NCIB 9816, the genes coding for the breakdown of naphthalene are carried on an 87 kbp IncP9 plasmid, pWW60 (Cane & Williams, 1982). The ability to degrade naphthalene has been transferred by conjugation into a plasmid-free *Pseudomonas putida* strain, PaW340, to give the transconjugant PaW701. The plasmid in PaW701, pWW60-1, is responsible for the conversion of naphthalene as far as catechol, which is then metabolized by the enzymes of the chromosomally encoded *ortho* pathway. This has the consequence that growth on 2-methylnaphthalene is not supported since the *ortho* pathway has an absolute specificity for catechol and cannot utilize its methylated derivatives (Feist & Hegeman, 1969). However, spontaneous mutants of PaW701, selected through their ability to grow on 2-methylnaphthalene, use a plasmid-coded *meta* pathway for the metabolism of catechol. These mutants have undergone a small deletion (1·2–1·6 kbp) in the plasmid between the genes encoding salicylate hydroxylase (SH) and catechol 2,3-oxygenase (C23O) facilitating the expression of previously silent *meta* pathway genes; pWW60-22, the plasmid used in the present study, is the deleted plasmid in one such mutant.

Restriction endonuclease mapping and transposon mutagenesis of pWW60-1 have shown the early enzymes of the naphthalene catabolic pathway (naphthalene to salicylate) and the *meta* pathway genes (salicylate to pyruvate) to be clustered in two distinct and spatially separated operons (Cane & Williams, 1986). A similar situation exists in the TOL plasmid pWW53-4, which encodes a set of inducible enzymes required for the oxidative degradation of toluene via

Abbreviations: SH, salicylate hydroxylase; C23O, catechol 2,3-oxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; 4OT, 4-oxalocrotonate tautomerase; 4OD, 4-oxalocrotonate decarboxylase; OPH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase.

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meta-cleavage of catechol (Keil *et al.*, 1985*b*). The genes responsible for this oxidation comprise an upper pathway operon (toluene to benzoate) and a separate *meta*-cleavage pathway operon (benzoate to pyruvate), controlled respectively by the products of the regulatory genes *xylR* and *xylS* (Keil *et al.*, 1987).

The present study aimed to elucidate further the genetic organization of the naphthalene *meta* pathway operon on pWW60-22 and to compare it to the analogous pathway on TOL plasmid pWW53-4 in an attempt to understand the evolutionary relationships between the two systems.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* and *Pseudomonas putida* strains and the plasmids used during this study are detailed in Table 1.

Media and culture conditions. P. putida strains were grown on solid and liquid minimal medium (Worsey & Williams, 1975) with added streptomycin (150 $\mu g\ ml^{-1}$) to ensure maintenance of recombinant plasmids. The media and methods used for the cultivation of *E. coli* have been described previously (Keil *et al.*, 1985*a*).

Enzyme assays. Cell-free extracts were prepared according to Cane & Williams (1982) but the period of sonication was reduced to two 30 s bursts. The enzyme assay procedures for catechol 2,3-oxygenase (C23O), 2-hydroxymuconic semialdehyde hydrolase (HMSH), 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) and 4-hydroxy-2-oxovalerate aldolase (HOA) were taken from Sala-Trepat & Evans (1971). The modified assay of Harayama *et al.* (1984) was employed for 4-oxalocrotonate decarboxylase (4OD). For 4-oxalocrotonate tautomerase (4OT), reaction mixtures were as for Sala-Trepat & Evans (1971) but the procedure was modified such that the A_{295} was followed until it had fallen from 2·0 to 0·8. A tangent was then drawn to the curve at an absorbance of 1·0 and the slope of the tangent taken as the rate of tautomerization. Non-enzymic tautomerization was measured in an identical manner and subtracted from the rate obtained in the presence of cell-free extract. 2-Oxopent-4-enoate hydratase (OPH) was assayed according to Collinsworth *et al.* (1973). Substrates for enzyme assays were as described previously (Keil *et al.*, 1987). Protein concentrations were determined by the biuret procedure.

Plasmid extraction and DNA manipulation. Plasmid pWW60-22 was extracted by the method of Wheatcroft & Williams (1981). *E. coli* and *P. putida* strains were screened for the presence of small recombinant plasmids according to Holmes & Quigley (1981) and vector and cloned DNA prepared in quantity by CsCl density-gradient centrifugation of cleared lysates (Guerry *et al.*, 1973). Restriction endonuclease digestion and ligation with T4 ligase were done in accordance with the manufacturer's instructions. *E. coli* strains were transformed by standard procedures (Cohen *et al.*, 1972) and transformants selected on media containing antibiotics appropriate to the vector. Colonies carrying recombinant plasmids expressing C23O activity were detected by the catechol spray test (Franklin *et al.*, 1981).

Mobilization of recombinant plasmids. All pKT230-derived recombinant plasmids were mobilized into plasmid-free *P. putida* strain PaW130 using the unstable RP4 derivative pNJ5000 (Grinter, 1983) as described by Keil *et al.* (1985*b*).

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Notes and references
E. coli		
ED8654	–	Murray *et al.* (1977)
JM103	–	Messing *et al.* (1981)
P. putida		
PaW11	–	Cane & Williams (1982)
PaW130	–	Keil *et al.* (1985*b*)
PaW701	pWW60-1	Nah^+ 2MeNah$^-$ Sal^+ 4MeSal$^-$ Cane & Williams (1982)
PaW719	pWW60-22	Nah^+ 2MeNah$^+$ Sal^+ 4MeSal$^+$ Cane & Williams (1982)
	pWW53-4	Keil *et al.* (1985*b*)
	pWW53-3506	Keil *et al.* (1987)
	pWW53-3508	Keil *et al.* (1987)
	pWW53-3510	Keil *et al.* (1985*b*)
	pKT230	Km^R Sm^R: Bagdasarian *et al.* (1981)
	pUC18	Yanisch-Perron *et al.* (1985)
	pBR322	Bolivar *et al.* (1977)
	pNJ5000	Grinter (1983)

DNA–DNA hybridization. Restriction fragments were separated by agarose gel electrophoresis and transferred by Southern blotting (Southern, 1975) to Biodyne filter membranes (Pall Ultrafine Filtration Corp.). Cloned fragments to be used as radio-labelled probes were separated from vector DNA by agarose gel electrophoresis and extracted according to Girvitz *et al.* (1980).

The procedures for nick-translation and hybridization have been described previously (Keil & Williams, 1985).

RESULTS

Molecular cloning of BamHI fragment BC″ from pWW60-22

Plasmid pWW60-22 is derived from pWW60-1 by spontaneous deletion of 1·6 kbp of DNA between the C23O and SH genes located on *Bam*HI fragment BC (Cane & Williams, 1982). The resultant novel *Bam*HI fragment in pWW60-22 is designated BC″. Purified pWW60-22 DNA was ligated into the broad-host-range vector pKT230 after restriction with *Bam*HI. *E. coli* transformants were sprayed with 100 mM-catechol to detect those capable of converting catechol to the yellow compound 2-hydroxymuconic semialdehyde. One such transformant was selected which contained a recombinant plasmid (designated pWW60-3026) carrying the 17·5 kbp *Bam*HI fragment BC″. Mobilization of pWW60-3026 into plasmid-free *P. putida* strain PaW130 conferred on it the ability to grow on salicylate, 4-methylsalicylate and 5-methylsalicylate as sole

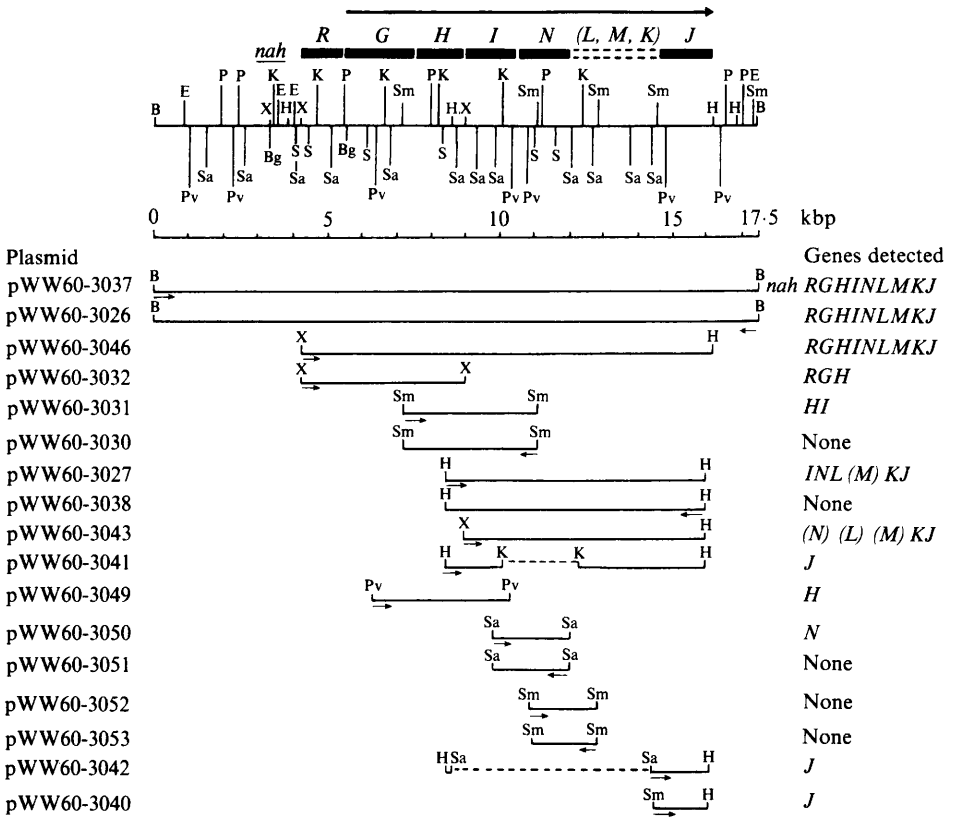

Fig. 1. Restriction map of *Bam*HI fragment BC″ from naphthalene plasmid pWW60-22 and its derivative subclones. The small arrows indicate the direction of transcription from the vector promoters. The abbreviations for the restriction enzymes are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Pv, *Pvu*II; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I. The locations assigned to the genes of the *meta* pathway operon are shown above the map, and the genes detected on each of the subclones are listed. Where genes are in parentheses their presence has not been proven experimentally for the particular subclone, but deduced by comparison with other recombinant plasmids.

Table 2. *Recombinant plasmids carrying cloned nah genes*

Plasmid*	Notes
pWW60-3037	*Bam*HI fragment BC″ of pWW60-22 in pKT230
pWW60-3026	BC″ in reverse orientation in pKT230
pWW60-3046	*Xho*I fragment XC″ of pWW60-3032 cloned into pWW60-3043
pWW60-3044	*Hin*dIII fragment HS of pWW60-1 in pBR322
pWW60-3032	*Xho*I fragment XC″ of pWW60-3037 in pKT230
pWW60-3031	3·7 kbp *Sma*I fragment of pWW60-3037 in pKT230
pWW60-3030	3·7 kbp *Sma*I fragment in reverse orientation in pKT230
pWW60-3027	*Hin*dIII fragment HD of pWW60-3037 in pKT230
pWW60-3038	HD in reverse orientation in pKT230
pWW60-3043	7·2 kbp *Xho*I–*Hin*dIII fragment of pWW60-3027 obtained by *Xho*I digestion and religation
pWW60-3041	Internal 2·4 kbp *Kpn*I fragment of pWW60-3027 deleted from pWW60-3027 by *Kpn*I digestion and religation
pWW60-3049	4·0 kbp *Pvu*II fragment of pWW60-3037 obtained by *Pvu*II digestion and ligation into *Sma*I site of pKT230
pWW60-3050	2·1 kbp *Sal*I fragment of pWW60-3027 obtained by *Sal*I digestion and ligation into pUC18
pWW60-3051	2·1 kbp *Sal*I fragment in reverse orientation in pUC18
pWW60-3052	1·8 kbp *Sma*I fragment of pWW60-3027 in pKT230
pWW60-3053	1·8 kbp *Sma*I fragment in reverse orientation in pKT230
pWW60-3042	1·8 kbp *Sal*I–*Hin*dIII fragment of pWW60-3027 obtained by *Sal*I digestion and religation
pWW60-3040	1·7 kbp *Sma*I–*Hin*dIII fragment of pWW60-3027 obtained by *Sma*I digestion and religation
pWW60-3036	*Hin*dIII fragment HU of pWW60-3037 in pKT230

* Recombinant plasmids were maintained in *E. coli* ED8654 or *P. putida* PaW130 with the exception of pWW60-3050 and pWW60-3051, which were isolated in *E. coli* JM103.

carbon sources. The recombinant plasmid also conferred growth on salicylate and its methylated derivatives to a plasmid-free mutant PaW11 which has a non-functional *ortho* pathway (Cane & Williams, 1982).

A detailed physical map of BC″ for 11 restriction enzymes was derived and a number of subclones constructed (Fig. 1, Table 2).

Sequential hybridization experiments using TOL plasmid meta pathway genes

DNA homology was demonstrated previously (Cane & Williams, 1986) between the C23O gene (*nahH*) on plasmid pWW60-22 and the equivalent gene (*xylE*) on the archetypal TOL plasmid pWW0. To extend this analysis, a sequential series of DNA probes was derived from the *Hin*dIII fragment HA of pWW53-4 which encodes a functional *meta* pathway operon (Keil *et al.*, 1985*b*). Table 3 lists the NAH *meta* pathway genes and their analogues on the TOL operon. The majority of the pWW53-4 probes hybridized strongly to BC″, indicating a considerable degree of DNA homology (Fig. 2). The pattern of hybridization observed was consistent with the genes being located in the same linear array within the two operons. The *meta* pathway operon on BC″ was seen to extend from the C23O gene commencing around co-ordinate 7·4 to co-ordinate 16·2 at the end of *Hin*dIII fragment HD. No hybridization was observed when plasmid pWW60-3036, carrying the adjacent 0·9 kbp *Hin*dIII fragment (HU) as its insert, was used as a probe against HA of pWW53-4 (probe 7; Fig. 2). Probes derived from the pWW53-4 regulatory gene *xylR* (probe 8, Fig. 2) and from the regulatory gene *xylS* of TOL plasmid pWW0 (Spooner *et al.*, 1986; not shown on Fig. 2) also failed to show any homology to BC″

The TOL genes *xylD* and *xylL* encode enzymes responsible for the breakdown of benzoate to catechol. A large DNA probe from pWW53-4 HA carrying *xylD* and *xylL* (not shown on Fig. 2) failed to hybridize to BC″. However, it did hybridize weakly to BC of pWW60-1 and the region of homology was shown to be limited to the *Hin*dIII fragment HS which is deleted when pWW60-22 is formed from pWW60-1 (Cane & Williams, 1982). When fragment HS of pWW60-1 was subcloned (pWW60-3044) and used as a probe against multiple digests of pWW53-4 HA, hybridization was localized to a 0·8 kbp region at the upstream end of the fragment (probe 1, Fig. 2).

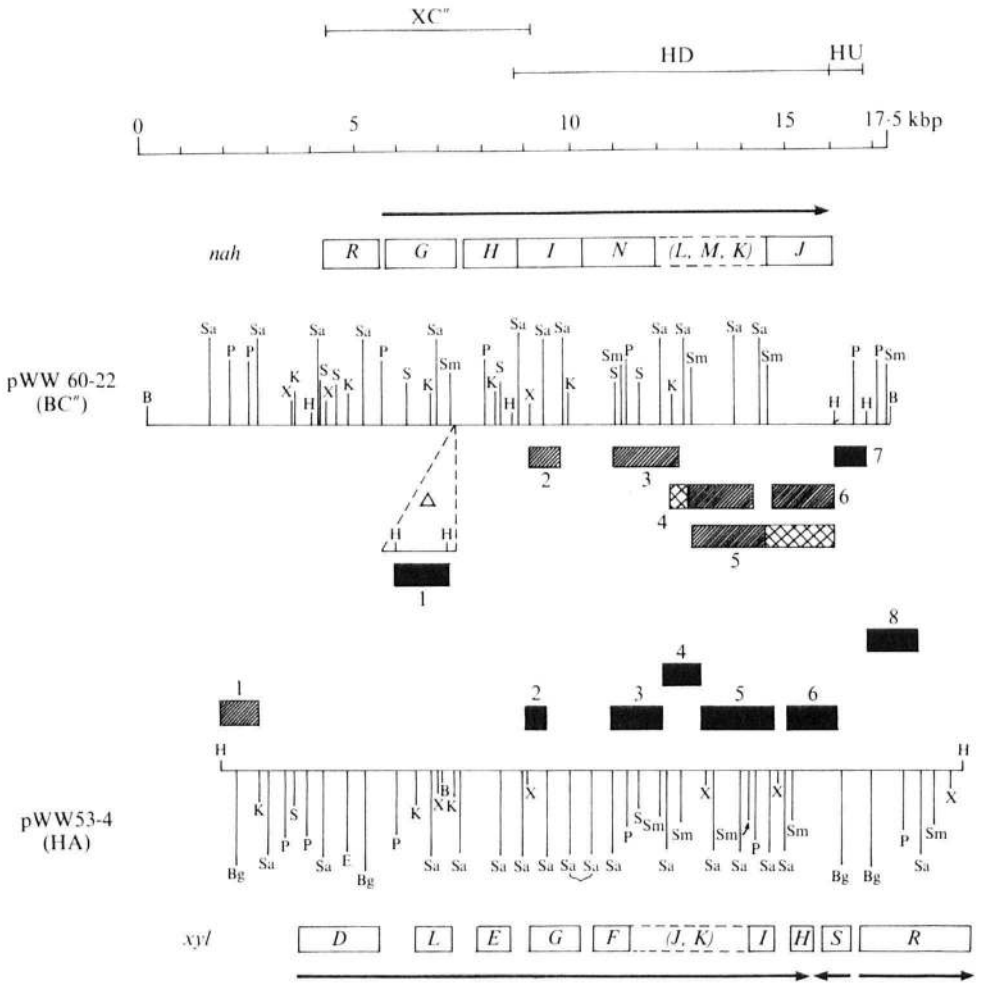

Fig. 2. Colinearity of hybridization between *meta* pathway genes of pWW53-4 and pWW60-22. The restriction map and positions of the pWW53-4 genes are taken from Keil *et al.* (1987): two alternative locations for a single *Sal*I site which have not been distinguished are bracketed. The solid black boxes represent probe DNA and the regions of DNA to which they hybridized strongly are hatched. Regions of weak hybridization found for some of the probes are cross-hatched. DNA probes are as follows: 1, 1·0 kbp *Hin*dIII fragment HS from pWW60-1 (Cane & Williams, 1982); 2, 0·5 kbp *Xho*I–*Sal*I fragment from pWW53-3510 (Keil *et al.*, 1985*b*); 3, 1·1 kbp *Sal*I fragment from pWW53-3510; 4, 0·8 kbp *Sal*I–*Xho*I fragment from pWW53-3510; 5, 1·6 kbp *Xho*I fragment from pWW53-3506 (Keil *et al.*, 1987); 6, 1·3 kbp *Sma*I–*Bgl*II fragment from pWW53-3508 (Keil *et al.*, 1987); 7, 0·7 kbp *Hin*dIII fragment HU from pWW60-3036 (this study); 8, 1·6 kbp *Bgl*II–*Sma*I fragment from pWW53-3508. All probes were prepared by restriction enzyme digestion of the appropriate plasmid followed by electroelution of the desired DNA band from agarose gels.

Localization of naphthalene meta pathway genes

The enzyme activities in *E. coli* and *P. putida* strains carrying recombinant plasmids are detailed in Table 4. Cells carrying pWW60-3037, which contains BC″, expressed all of the enzymes of the *meta* pathway operon, albeit at very low levels for HMSH (*nahN*). Similar specific activities were demonstrated in both *E. coli* and *P. putida* carrying plasmid pWW60-3026, which constitutes BC″ cloned in the opposite orientation relative to the Km^R promoter of pKT230 compared with pWW60-3037. The specific activities of C23O, HMSD (*nahI*) and OPH (*nahL*) were similar regardless of whether the host strain was *E. coli* or *P. putida*. For 4OT (*nahJ*) and OPH (*nahL*), the activities were two- to three-fold higher in *P. putida* hosts. HMSH levels

Table 3. *Gene designations for the meta pathway enzymes of NAH and TOL plasmids*

Enzyme	TOL	NAH
Catechol 2,3-oxygenase (C23O)	*xylE*	*nahH*
2-Hydroxymuconic semialdehyde hydrolase (HMSH)	*xylF*	*nahN*
2-Hydroxymuconic semialdehyde dehydrogenase (HMSD)	*xylG*	*nahI*
4-Oxalocrotonate tautomerase (4OT)	*xylH*	*nahJ*
4-Oxalocrotonate decarboxylase (4OD)	*xylI*	*nahK*
2-Oxopent-4-enoate hydratase (OPH)	*xylJ*	*nahL*
4-Hydroxy-2-oxovalerate aldolase (HOA)	*xylK*	*nahM*

Table 4. *Specific activities of cloned meta pathway enzymes in E. coli and P. putida*

			Specific activity [milliunits (mg protein)$^{-1}$]†						
Plasmid	Host*	Inducer	C230	HMSD	HMSH	4OT	4OD	OPH	HOA
pWW60-1	*P. putida*	None	3	3	<0·1	640	14	400	1·3
		Salicylate	4	2	<0·1	500	7	350	3·8
pWW60-22	*P. putida*	None	14	3	0·5	6000	33	980	0·9
		Salicylate	330	140	1·2	260000	730	12000	4·7
pWW60-3037	*E. coli*	None	74	22	0·5	34000	130	990	ND
	P. putida	None	61	24	0·5	71000	300	2400	3·0
		Salicylate	570	390	4·8	880000	2300	23000	66·0
pWW60-3026	*E. coli*	None	71	12	<0·1	33000	140	1100	ND
	P. putida	None	71	31	1·9	100000	420	3000	5·6
		Salicylate	670	550	14	720000	2900	30000	57·0
pWW60-3046	*E. coli*	None	110	19	1·2	36000	180	860	ND
	P. putida	None	61	32	0·5	62000	580	3200	1·1
		Salicylate	610	490	5·6	950000	5500	26000	16·0
pWW60-3032	*E. coli*	None	47	<0·1	<0·1	ND	ND	ND	ND
	P. putida	None	20	<0·1	ND	ND	ND	ND	ND
		Anthranilate	271	<0·1	ND	ND	ND	ND	ND
pWW60-3031	*E. coli*	None	330	59	<0·1	ND	ND	ND	ND
	P. putida	None	20	13	<0·1	ND	ND	ND	ND
		Anthranilate	12	20	<0·1	ND	ND	ND	ND
pWW60-3030	*E. coli*	None	1	<0·1	<0·1	ND	ND	ND	ND
pWW60-3027	*E. coli*	None	<0·1	9	0·5	16000	75	710	ND
	P. putida	None	<0·1	8	1·0	13000	250	620	1·4
		Salicylate	<0·1	8	1·1	8000	90	630	0·8
pWW60-3038	*E. coli*	None	<0·1	<0·1	<0·1	<100	<5	<50	ND
pWW60-3043	*E. coli*	None	<0·1	<0·1	<0·1	6000	34	ND	ND
pWW60-3041	*E. coli*	None	<0·1	<0·1	ND	27000	<5	<50	ND
	P. putida	None	ND	ND	ND	6000	<5	ND	ND
pWW60-3049	*E. coli*	None	140	<0·1	ND	ND	ND	ND	ND
pWW60-3050	*E. coli*	None	<0·1	<0·1	5·1	ND	ND	ND	ND
pWW60-3051	*E. coli*	None	<0·1	<0·1	<0·1	ND	ND	ND	ND
pWW60-3052	*E. coli*	None	ND	ND	ND	<100	<5	<50	ND
pWW60-3053	*E. coli*	None	ND	ND	ND	<100	<5	<50	ND
pWW60-3042	*E. coli*	None	ND	ND	ND	120000	11	ND	ND
	P. putida	None	ND	ND	ND	4900	<5	ND	ND
pWW60-3040	*E. coli*	None	ND	ND	ND	160000	6	ND	ND
	P. putida	None	ND	ND	ND	2600	<5	ND	ND
None	*E. coli*	None	<0·1	<0·1	<0·1	<100	<5	<50	ND
None	*P. putida*	None	<0·1	<0·1	<0·1	<100	<5	<50	1·0

ND, Not determined.

* *E. coli* ED8654 or JM103 (for pWW60-3050 and pWW60-3051 only) and *P. putida* PaW130.

† All values represent the mean of three replicates.

were too low in both hosts for such comparisons to be valid, whereas HOA (*nahM*) cannot be measured in *E. coli* because of its high NADH oxidase activity.

The genes for SH (*nahG*) and C23O (*nahH*) were located by Cane & Williams (1986) as shown on Fig. 1. Evidence for the location of the remaining genes was obtained by assaying appropriate subclones of BC″.

The product of *nahI*, HMSD, was detected in strains carrying pWW60-3027 but not pWW60-3043, implying that the *Xho*I site at co-ordinate 9·0 is within the gene. However, *nahI* must extend only a small distance upstream since the *Xho*I fragment XC″ showed no hybridization to the upstream end of the isofunctional and homologous gene *xylG* from pWW53-4 (probe 2; Fig. 2). Strains carrying pWW60-3041 and pWW60-3049 failed to express *nahI* but high HMSD activity was observed on the *Sma*I subclone in pWW60-3031. This fragment did not express *nahI* in the opposite orientation relative to the vector promoter (pWW60-3030). This implies that the gene terminates between the *Pvu*II site at 10·4 and the *Sma*I site at 11·1.

The product of *nahN*, HMSH, was detected only at very low levels in strains carrying plasmids pWW60-3027 and pWW60-3046. However, when the *Sal*I fragment of BC″ between co-ordinates 9·9 and 12·0 was subcloned into the high-copy-number vector pUC18 (pWW60-3050), higher HMSH levels were recorded, showing *nahN* to be located within this region of BC″. With respect to *nahJ*, 4OT activity was detected in strains carrying pWW60-3027 and four of its subclones, pWW60-3040, pWW60-3041, pWW60-3042 and pWW60-3043. This localizes the gene to between the *Sma*I site at co-ordinate 14·5 and the *Hin*dIII site at 16·2.

It was not possible to localize the remaining three enzyme activities to specific small subclones. The gene products of *nahK* (4OD) and *nahL* (OPH) were detected in strains carrying pWW60-3027 but were surprisingly both absent from pWW60-3041. Fine gene localization was not attempted for *nahM* since the enzyme levels were only measurable under conditions of salicylate induction. The only credible position for the genes is between *nahN* and *nahJ*, although their relative order cannot be ascertained from the evidence to date.

Regulation of the meta pathway genes

Growth in the presence of salicylate of *P. putida* carrying fragment BC″ in either orientation (pWW60-3037 or pWW60-3026) resulted in an elevation of specific activities for all enzymes of approximately 10-fold. For pWW60-22 higher levels of induction were observed, ranging from 20-fold for C23O and 4OD to 40-fold for 4OT and HMSD. Cells carrying the parent plasmid pWW60-1 exhibited low basal levels of expression for all enzymes and these were not affected by the presence of salicylate.

Cells carrying pWW60-3046, which lacks the upstream 4·2 kbp of BC″, exhibited similar levels of induction to those carrying pWW60-3037. This implies that any regulatory element(s) lies downstream of the *Xho*I site at co-ordinate 4·2. *P. putida* strains lacking the full *meta* pathway operon (e.g. pWW60-3031) grew poorly in the presence of salicylate, possibly due to the accumulation of toxic intermediates. With such strains, 5 mM-anthranilate was used as the inducing molecule since this has been shown to be a gratuitous inducer of the *meta* pathway (Shamsuzzaman & Barnsley, 1974). Cells carrying pWW60-3032 demonstrated a 10-fold increase in C23O activity in the presence of anthranilate, whereas with pWW60-3031 the level was unaffected by the presence of inducer. This suggests that a regulatory locus (designated *nahR*) lies between the *Xho*I site at co-ordinate 4·2 and the upstream end of the SH gene.

DISCUSSION

The 17·5 kbp *Bam*HI fragment BC″ from the naphthalene plasmid pWW60-22 was cloned in *E. coli* in the broad-host-range vector pKT230. This fragment was shown to contain all the genetic information required for the metabolism of salicylate via the *meta* cleavage of catechol. Comparison of the restriction map of BC″ of pWW60-22 with that of TOL plasmid pWW53-4 *Hin*dIII fragment HA reveals some degree of similarity in the location of restriction sites, notably the *Xho*I, *Pst*I, *Sst*I and *Sma*I sites at co-ordinates 9·0, 11·2, 11·6 and 14·6 of BC″

respectively (Fig. 2). Both operons also display a relative abundance of sites for endonuclease *Sal*I, suggesting a general similarity in base-pair composition and/or codon usage. Comparison of the restriction map of BC″ with that of the *meta* pathway operon of naphthalene plasmid NAH7 reveals a greater degree of restriction site conservation (Harayama *et al.*, 1987*b*), possibly indicative of a more recent evolutionary link.

DNA hybridization has been used previously to demonstrate homology between the *meta* cleavage pathway genes of NAH and TOL plasmids (Bayley *et al.*, 1979; Farrell & Chakrabarty, 1979; Lehrbach *et al.*, 1983; Cane & Williams, 1986). The present study aimed to elucidate the exact nature and extent of these structural similarities by employing a sequential series of specific DNA hybridization probes. A strong degree of DNA homology was observed, commencing at the C23O gene and extending along the full length of the NAH and TOL structural genes. The pattern of hybridization using DNA probes designed to hybridize only to small regions of specific genes was highly indicative of the same gene order on the two operons.

The demonstration of homology between the 1·6 kbp of DNA deleted in pWW60-1 to yield pWW60-22, and the region of pWW53-4 immediately preceding *xylD* at the upstream end of the *meta* pathway operon, is further evidence for a relationship between the two operons and suggests an attractive hypothesis for the evolutionary history of the *meta* cleavage pathway. It is possible that the naphthalene catabolic pathway was formed by the chance combination on a single replicon of three pre-evolved metabolic 'modules', one controlling the breakdown of naphthalene to salicylate, a second encoding SH under the regulatory control of *nahR* and a third element responsible for the *meta* pathway enzymes. This latter unit is presumed to share a common origin with the TOL *meta* pathway operon and may originally have included functional genes for *xylD* and *xylL* and an independent operator–promoter region. Selective pressure for the retention of *xylDL* would be minimal due to the existence of isofunctional chromosomal genes, thus allowing the accumulation of non-deleterious point mutations and deletions within this region. The eventual deletion of the vestigial DNA between the SH and C23O genes in pWW60-1 would then form a single operon under the regulatory control of *nahR*. This is the case on NAH7, where the order of the *meta* pathway genes is identical to pWW60-22 with the exception of *nahJ* and *nahK*, whose relative order is reversed. An evolutionary relationship has also been proposed between NAH7 and the archetypal TOL plasmid pWW0 on the basis of similarities in the nucleotide and amino acid sequences of their C23O genes (Harayama *et al.*, 1987*b*).

Assaying strains carrying BC″ and its subclones for enzyme activities allowed the location of the majority of the genes to small regions of DNA which corresponded to the positions indicated by hybridization with gene-specific probes. Although their activities could be assayed on BC″, it was not possible to locate the genes for *nahK* or *L* to specific small subclones. It is conceivable that the restriction sites available for cloning in pKT230 lie within the genes, but this seems unlikely to account for the lack of *nahK* activity in pWW60-3041. The analogous gene on TOL plasmid pWW0 (*xylI*) has been shown to lie immediately upstream of *xylH* encoding 4OT. A subclone of the pWW53-4 *meta* pathway operon derived from this region hybridized to the equivalent position on BC″ (probe 5; Fig. 2) but we have been unable to confirm experimentally the previous report (Keil *et al.*, 1987) that this subclone expresses 4OD activity. Hence, its hybridization does not necessarily imply the presence of *nahK* in this position although it is consistent with a co-linearity of gene order. It is possible that the arrangement of *nahK* and *nahL* may be more complicated than has previously been envisaged, perhaps involving more than one gene coding for each enzyme. It is clear that further work is needed in this respect, both on the NAH operon and on the TOL *meta* pathway where the full complexities of the system may not yet have been recognized.

The specific activities of HMSH in strains carrying pWW60-22 and its subclones were low, although a functional *nahN* gene was localized by subcloning an appropriate fragment into a high-copy-number vector (pWW60-3050). This is consistent with the observation that the hydrolytic branch of the pathway is of no metabolic significance in strain NCIB 9816 (Catterall *et al.*, 1971). It has been shown for TOL plasmid pWW0 that only 3-methylcatechol is dissimilated via the hydrolytic branch and that catechol and 4-methylcatechol are catabolized

almost exclusively by the 4-oxalocrotonate pathway (Murray *et al.*, 1972; Wigmore *et al.*, 1974; Harayama *et al.*, 1987*a*). The low activity of HMSH in pWW60-22 may thus have evolved as a consequence of a limited availability of 1-methylnaphthalene as a naturally-occurring substrate compared to naphthalene and 2-methylnaphthalene.

The existence of a regulatory gene *nahR* was clearly demonstrated by the induction of enzyme activities in the presence of salicylate. The *nahR* locus was shown to lie immediately upstream of the *nahG* gene in an identical position to the analogous gene on NAH7 (Yen & Gunsalus, 1985). The levels of induction of cloned genes were generally approximately two- to fourfold lower than observed for the native plasmid pWW60-22 in a *P. putida* host. However, it is likely that the nature of the cloning vehicle and, particularly, its copy number may have a major effect on the level of expression and this result does not necessarily imply that additional regulatory elements exist elsewhere in the plasmid. There certainly do not appear to be regulatory genes homologous to *xylR* and *xylS* of pWW53-4. Specific DNA probes for these genes failed to hybridize and the downstream end of BC″ does not exhibit the two highly conserved restriction sites for *Bgl*II which are characteristic of the *xylSR* regulatory region (Keil *et al.*, 1987). This suggests that the regulatory control of certain metabolic pathways may evolve subsequent to the development of their catalytic functions.

The concept that a structurally heterogeneous group of catabolic plasmids may share a common evolutionary origin is becoming increasingly substantiated by fact. The present study provides strong evidence for an evolutionary relationship between TOL plasmid pWW53-4 and the naphthalene plasmid pWW60-22. Hybridization evidence has been complemented with enzyme assay data to show that the two *meta* pathway operons are related at the DNA level and identical in their structural gene organization. This paper thus adds to an accumulating body of data which should ultimately yield important information regarding the evolution of peripheral catabolic pathways.

We would like to thank Mrs L. Shaw for technical assistance. This work was funded by SERC grant no. GR/D17557.

REFERENCES

BAGDASARIAN, M., LURZ, R., RÜCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose cloning vectors. II. Broad host range, high copy number RSF1010-derived vectors and a host:vector system for gene cloning. *Gene* **16**, 237–247.

BAGDASARIAN, M. M., AMANN, E., LURZ, R., RÜCKERT, B. & BAGDASARIAN, M. (1983). Activity of the hybrid *trp–lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad host range, regulatable expression vectors. *Gene* **26**, 273–282.

BAYLEY, S. A., MORRIS, D. W. & BRODA, P. (1979). The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group. *Nature, London* **280**, 338–339.

BOLIVAR, F., RODRIGUEZ, R. L., GREEN, P. J., BETLACH, H. C., HEYNECKER, H. L., BOYER, H. W., CROSA, J. J. & FALKOW, S. (1977). Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.

BORONIN, A. M., KOCHETOV, V. V., STAROVOITOV, I. I. & SKRYABIN, G. K. (1977). Plasmids pBS2 and pBS3, controlling the oxidation of naphthalene in bacteria of the genus *Pseudomonas*. *Doklady Academii nauk SSSR* **237**, 1205–1208.

BORONIN, A. M., KOCHETOV, V. V. & SKRYABIN, G. K. (1980). Incompatibility groups of naphthalene degradative plasmids in *Pseudomonas*. *FEMS Microbiology Letters* **7**, 249–252.

CANE, P. A. & WILLIAMS, P. A. (1982). The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. *Journal of General Microbiology* **128**, 2281–2290.

CANE, P. A. & WILLIAMS, P. A. (1986). A restriction map of naphthalene catabolic plasmid pWW60-1 and the location of some of its catabolic genes. *Journal of General Microbiology* **132**, 2919–2929.

CATTERALL, F. A., SALA-TREPAT, J. M. & WILLIAMS, P. A. (1971). The coexistence of two pathways for the metabolism of 2-hydroxymuconic semialdehyde in a naphthalene-grown pseudomonad. *Biochemical and Biophysical Research Communications* **43**, 463–469.

COHEN, S. N., CHANG, A. C. Y. & HSU, C. L. (1972). Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2110–2114.

COLLINSWORTH, W. L., CHAPMAN, P. J. & DAGLEY, S. (1973). Stereospecific enzymes in the degradation of aromatic compounds by *Pseudomonas putida*. *Journal of Bacteriology* **113**, 922–931.

DUNN, N. W. & GUNSALUS, I. C. (1973). Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. *Journal of Bacteriology* **114**, 974–979.

FARRELL, R. & CHAKRABARTY, A. M. (1979). Degrada-

tive plasmids: molecular nature and mode of evolution. In *Plasmids of Medical, Environmental and Commercial Importance*, pp. 97–109. Edited by K. N. Timmis & A. Puhler. Amsterdam: Elsevier/North Holland Biomedical.

FEIST, C. F. & HEGEMAN, G. D. (1969). Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. *Journal of Bacteriology* **100**, 869–877.

FRANKLIN, F. C. H., BAGDASARIAN, M., BAGDASARIAN, M. M. & TIMMIS, K. N. (1981). Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 7458–7462.

GIRVITZ, S. C., BACCHETTI, S., RAINBOW, A. J. & GRAHAM, F. W. (1980). A rapid and efficient procedure for the purification of DNA from agarose gels. *Analytical Biochemistry* **106**, 492–496.

GRINTER, N. J. (1983). A broad-host range cloning vector transposable to various replicons. *Gene* **21**, 133–143.

GUERRY, P., LEBLANC, D. J. & FALKOW, S. (1973). General method for the isolation of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **116**, 1064–1066.

HARAYAMA, S., LEHRBACH, P. R. & TIMMIS, K. N. (1984). Transposon mutagenesis of *meta*-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. *Journal of Bacteriology* **160**, 251–255.

HARAYAMA, S., MERMOD, N., REKIK, M., LEHRBACH, P. R. & TIMMIS, K. N. (1987*a*). Roles of the divergent branches of the *meta*-cleavage pathway in the degradation of benzoate and substituted benzoates. *Journal of Bacteriology* **169**, 558–564.

HARAYAMA, S., REKIK, M., WASSERFALLEN, A. & BAIROCH, A. (1987*b*). Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7 plasmids. *Molecular and General Genetics* **210**, 241–247.

HOLMES, D. S. & QUIGLEY, N. (1981). A rapid boiling method for preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193–197.

KEIL, H. & WILLIAMS, P. A. (1985). A new class of TOL plasmid deletion mutants in *Pseudomonas putida* MT15 and their reversion by tandem gene amplification. *Journal of General Microbiology* **131**, 1023–1033.

KEIL, H., LEBENS, M. R. & WILLIAMS, P. A. (1985*a*). TOL plasmid pWW15 contains two non-homologous catechol 2,3-oxygenase genes independently regulated. *Journal of Bacteriology* **163**, 248–255.

KEIL, H., KEIL, S., PICKUP, R. W. & WILLIAMS, P. A. (1985*b*). The complete *meta* pathway operon of the toluene/xylene catabolic pathway cloned from TOL plasmid pWW53. *Journal of Bacteriology* **164**, 887–895.

KEIL, H., KEIL, S. & WILLIAMS, P. A. (1987). Molecular analysis of regulatory and structural *xyl* genes of the TOL plasmid pWW53-4. *Journal of General Microbiology* **133**, 1149–1158.

LEHRBACH, P. R., MCGREGOR, I., WARD, J. M. & BRODA, P. (1983). Molecular relationships between *Pseudomonas* IncP-9 degradative plasmids TOL, NAH and SAL. *Plasmid* **10**, 164–174.

MESSING, J., CREA, R. & SEEBURG, P. H. (1981). A system for shotgun DNA sequencing. *Nucleic Acids Research* **9**, 309–321.

MURRAY, K., DUGGLEBY, C. J., SALA-TREPAT, J. M. & WILLIAMS, P. A. (1972). The metabolism of benzoate and methylbenzoates *via* the *meta*-cleavage pathway by *Pseudomonas arvilla* mt-2. *European Journal of Biochemistry* **28**, 301–310.

MURRAY, N. E., BRAMMAR, W. J. & MURRAY, K. (1977). Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Molecular and General Genetics* **150**, 53–61.

SALA-TREPAT, J. M. & EVANS, W. C. (1971). The *meta* cleavage of catechol by *Azotobacter* species: 4-oxalocrotonate pathway. *European Journal of Biochemistry* **20**, 400–413.

SCHELL, M. A. & WENDER, P. E. (1986). Identification of the *nahR* gene product and nucleotide sequences required for its activation of the *sal* operon. *Journal of Bacteriology* **166**, 9–14.

SHAMSUZZAMAN, K. M. & BARNSLEY, E. A. (1974). The regulation of naphthalene oxygenase in pseudomonads. *Journal of General Microbiology* **83**, 165–170.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–571.

SPOONER, R. A., LINDSAY, K. & FRANKLIN, F. C. H. (1986). Genetic, functional and sequence analysis of the *xylR* and *xylS* regulatory genes of the TOL plasmid pWW0. *Journal of General Microbiology* **132**, 1347–1358.

WHEATCROFT, R. & WILLIAMS, P. A. (1981). Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. *Journal of General Microbiology* **124**, 433–437.

WIGMORE, G. J., BAYLY, R. C. & DI BERARDINO, D. (1974). *Pseudomonas putida* mutants defective in the metabolism of the products of *meta* fission of catechol and its methyl analogs. *Journal of Bacteriology* **120**, 31–37.

WORSEY, M. J. & WILLIAMS, P. A. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. *Journal of Bacteriology* **124**, 7–13.

YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

YEN, M. & GUNSALUS, I. C. (1985). Regulation of naphthalene catabolic genes of plasmid NAH7. *Journal of Bacteriology* **162**, 1008–1013.