Biodegradation **5**: 195–217, 1994.

The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*

Peter A. Williams & Jon R. Sayers
School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK

Received 12 August 1993; accepted 3 January 1994

Key words: Aromatic catabolism, by bacteria (*Pseudomonas*), evolution, of catabolic pathways, hydrocarbons, catabolism of aromatic, *Pseudomonas*, evolution of catabolism in, oxygenases, evolution of

Abstract

The organisation and nucleotide sequences coding for the catabolism of benzene, toluene (and xylenes), naphthalene and biphenyl *via* catechol and the extradiol (*meta*) cleavage pathway in *Pseudomonas* are reviewed and the various factors which may have played a part in their evolution are considered. The data suggests that the complete pathways have evolved in a modular way probably from at least three elements. The common *meta* pathway operons, downstream from the ferredoxin-like protein adjacent to the gene for catechol 2,3-dioxygenase, are highly homologous and clearly share a common ancestry. This common module may have become fused to a gene or genes the product(s) of which could convert a stable chemical (benzoate, salicylate, toluene, benzene, phenol) to catechol, thus forming the lower pathway operons found in modern strains. The upper pathway operons might then have been acquired as a third module at a later stage thus increasing the catabolic versatility of the host strains.

Introduction

In recent years there has been a quantum leap in the information published about the catabolism of natural and xenobiotic aromatic compounds by bacteria. At present the most detailed investigations have been carried out on a relatively few metabolic pathways, which include those for the catabolism of the aromatic hydrocarbons, toluene and the xylenes, naphthalene and biphenyl, in a relatively limited range of bacteria mostly of the genus *Pseudomonas*. Molecular biological analysis of these systems has progressed sufficiently to enable their interrelationships to be assessed and, as a consequence, it is possible to speculate about the events which might have occurred during their evolution. Some of this ground has been covered elsewhere (Harayama & Timmis 1992; Harayama et al. 1992).

General principles of pathways for aerobic aromatic catabolism

The pathways in bacteria for the aerobic dissimilation of aromatic substrates can all be considered as consisting of three parts (Fig. 1; Dagley 1986; Harayama & Timmis 1992). In the first stage the substrate undergoes changes in its substituent groups, particularly through the introduction of hydroxyl groups by mono- or di-oxygenases, to produce a dihydroxyaromatic metabolite. Dihydroxybenzenes, usually catechols (1,2-dihydroxybenzenes), are metabolites in the aerobic catabolism of virtually all aromatic substrates.

The catechols are substrates for the second stage of the catabolism, the opening of the ring. This occurs by the action of dioxygenases which break one of the carbon-carbon bonds of the ring by the addition of molecular oxygen, producing an unsaturated aliphatic acid. There are two families of ring-cleavage enzymes, the intradiol (or *ortho*) dioxygenases which are Fe^{3+} enzymes and produce *cis,cis*-muconic acid (or a derivative) and the extradiol (or

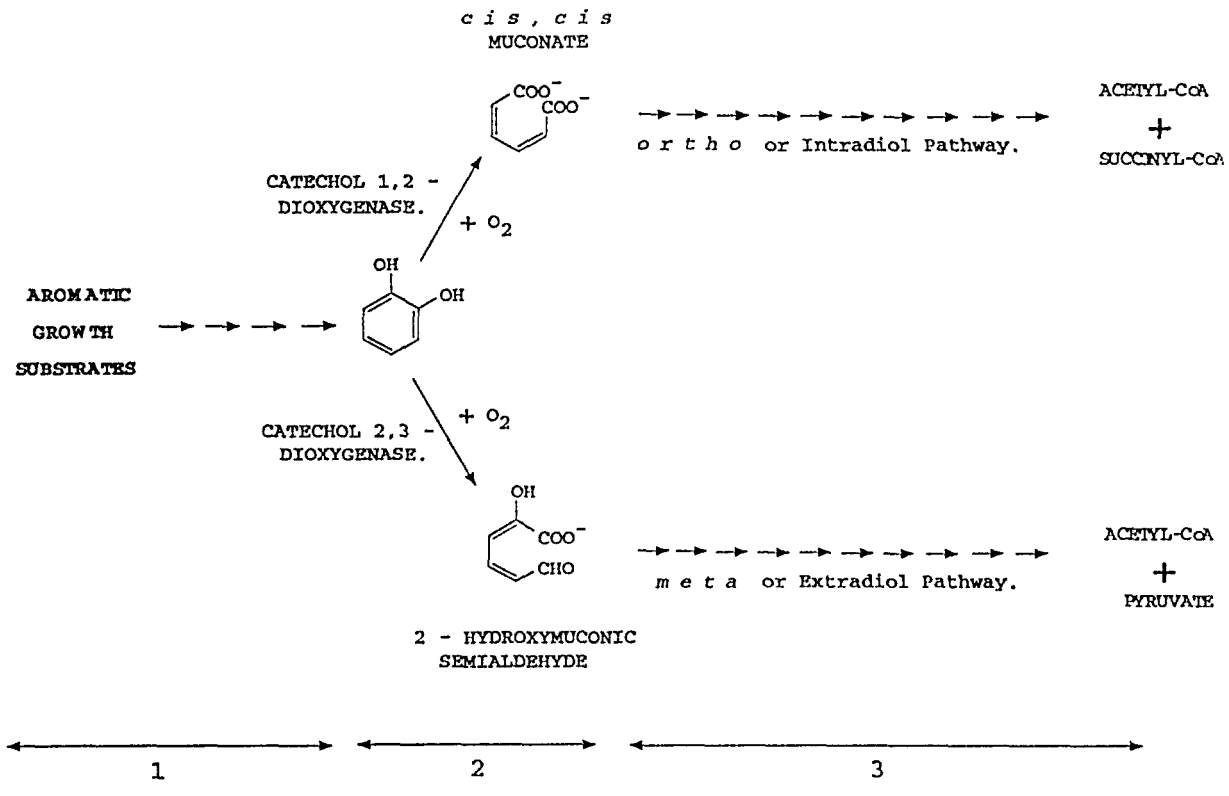


Fig. 1. General principle of aerobic aromatic catabolism in bacteria. The three stages are as described in the text: (1) The conversion of the growth substrate to catechol (or substituted catechol), (2) ring cleavage and (3) metabolism of the ring-cleavage product to central metabolites by either the *ortho* or *meta* pathways.

meta) dioxygenases which are Fe^{2+} enzymes and which produce 2-hydroxymuconic semialdehyde (or a derivative). The archetypal intradiol dioxygenase is catechol 1,2-dioxygenase (or pyrocatechase) (EC 1.13.11.1) and the archetype extradiol dioxygenase is catechol 2,3-dioxygenase (or metapyrocatechase) (EC 1.13.11.2).

After ring cleavage, the third stage of the catabolism consists of the conversion of the ring cleavage product to small aliphatic compounds which can directly enter central metabolism. Ring cleavage plus the subsequent metabolic steps for intradiol cleavage are often referred to as the β-ketoadipate (or *ortho*) pathway and for extradiol cleavage as the *meta* pathway. The biochemistry of these two reaction sequences appears to be conserved in all genera of eubacteria in which they are found. In broad outline therefore aerobic aromatic catabolism consists of a variety of pathways which converge on a limited number of common intermediates (dihydroxybenzenes) which are then further assimilated by a small number of common pathways.

Aromatic hydrocarbon catabolism in *Pseudomonas*

The commonest routes for catabolism of toluene, naphthalene and biphenyl in *Pseudomonas* converge on catechol which is further metabolised by extradiol ring cleavage and the *meta* pathway. Although four routes for toluene catabolism have been described, only two have been the subject of any degree of genetic analysis. In the *tod* pathway, the toluene molecule undergoes successive dihydroxylation and dehydrogenation to produce 3-methylcatechol (Fig. 2a): benzene can also be utilised by this pathway and is converted directly to catechol (Gibson et al. 1968, 1990). In the *xyl* pathway, toluene is metabolised to catechol by way of sequential oxidations of the methyl group, through benzyl alcohol and benzaldehyde to

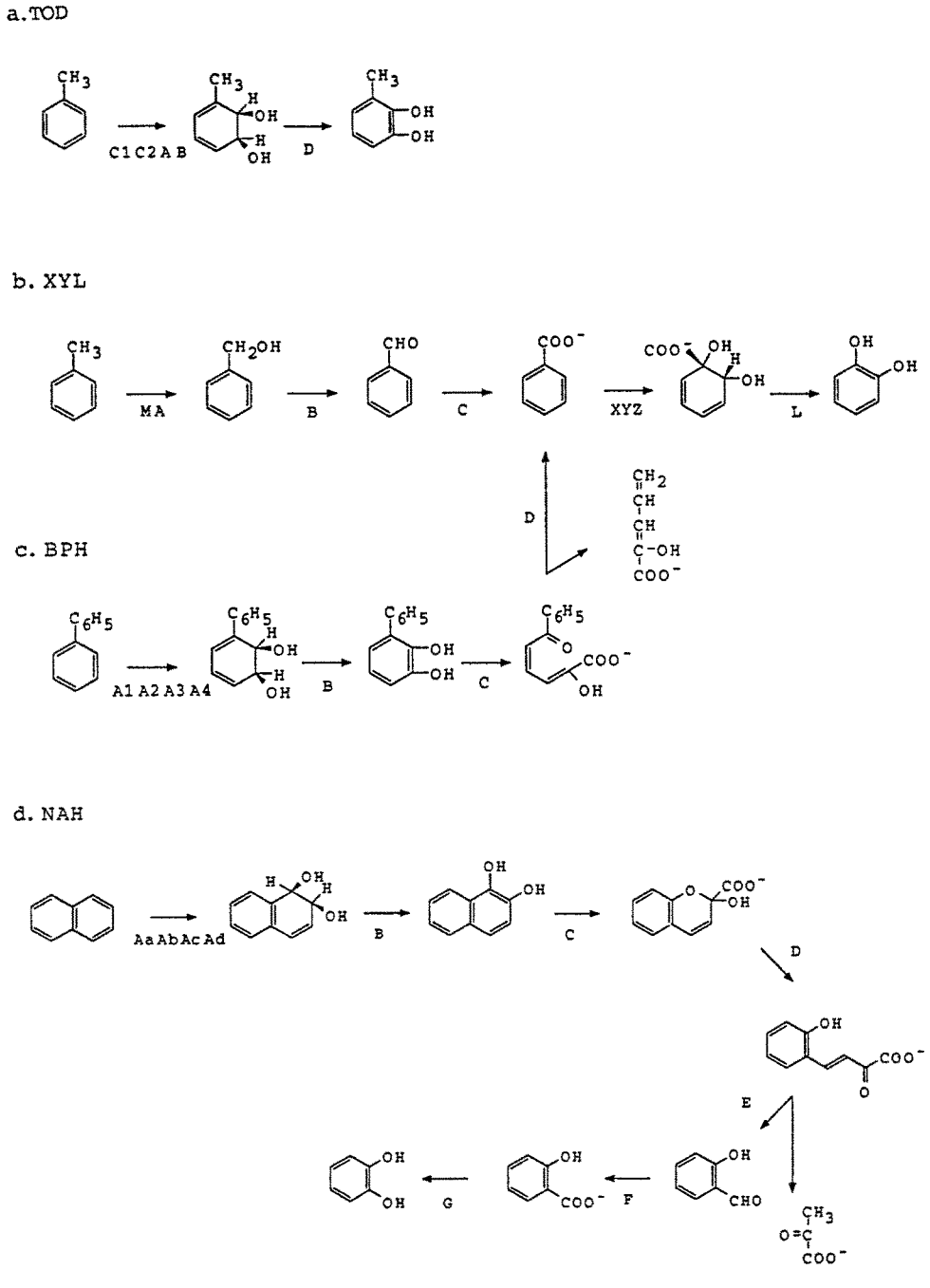


Fig. 2. The pathways for conversion of the aromatic hydrocarbons to catechol(s) (a) TOD pathway for toluene and benzene (b) XYL pathway for toluene and various alkyl substituted toluenes (c) BPH pathway for biphenyl and (d) NAH pathway for naphthalene (Eaton & Chapman 1993). The gene(s) encoding the various enzymatic steps are below each conversion.

benzoate; this is then converted to catechol in two steps, a dihydroxylation followed by a dehydrogenation/decarboxylation (Fig. 2b). This pathway has a broad substrate range and is also able to dissimilate various alkyl derivatives of toluene, *m*-xylene (1,3-dimethylbenzene), *p*-xylene (1,4-dimethylbenzene), 3-ethyltoluene and 1,2,4-trimethylbenzene (Worsey & Williams 1975; Kunz & Chapman 1981).

Biphenyl and naphthalene, with two aromatic rings, present an additional biochemical problem. This is solved by initial reactions analogous to the first two steps of the *tod* pathway producing 1,2-dihydroxybiphenyl and 1,2-dihydroxynaphthalene respectively (Figs 2c, d). The catechol ring of each of these undergoes cleavage by an extradiol dioxygenase to produce a single ring aromatic product with an unsaturated side chain, which is subsequently removed producing benzoate and salicylate respectively (Catelani et al. 1971; Eaton & Chapman 1992). In naphthalene catabolism, salicylate is converted to catechol directly by the action of a single monooxygenase (salicylate hydroxylase) whereas in biphenyl catabolism the benzoate is converted to catechol by the same sequence of reactions described above for the *xyl* pathway.

The four catabolic pathways share an identical sequence of reactions from catechol downwards (Table 1), but each has a unique series of reactions for the conversion of the aromatic hydrocarbon to catechol. Naphthalene and biphenyl catabolism each contain two ring cleavage steps with identical chemistry which might therefore be expected to have related enzymes. One other pathway which shares this pattern and which has been particularly well characterised genetically is the *dmp* pathway for phenols described in another review in this volume (Shingler 1993); the initial reaction is the monohydroxylation of phenol to catechol which is then assimilated by the *meta* pathway (Shingler et al. 1992).

An additional feature common to many of these pathways, certainly in *Pseudomonas* hosts, is their genetic location upon large plasmids.

Genetic organisation of pathway genes

The xyl genes of TOL plasmids

The *xyl* genes for toluene/xylene catabolism appear to be almost always encoded by large plasmids collectively called the TOL plasmids: their structures and properties have been reviewed (Assinder & Williams 1990). The archetype TOL plasmid pWW0 from *P. putida* mt-2 is a 117 kbp conjugative plasmid which has been the subject of extensive analysis while two other plasmids, pWW53 and pDK1, have been investigated to a lesser extent.

The organisation of the catabolic genes on pWW0 demonstrates a common pattern found with the other pathways being considered. There are two operons, the first of which encodes the conversion of the growth substrate to a compound which is the last chemically stable metabolite of the pathway. A second operon then converts this compound through a sequence of chemically unstable metabolites to link with central metabolism. The first of the two *xyl* operons is the 'upper pathway' operon, *xylCMABN*, and encodes the enzymes for the conversion of toluene (and its alkyl derivatives) to benzoate (and the alkylbenzoates) (Harayama et al. 1989). The 'lower pathway' operon (also called the *meta* pathway operon), *xylXYZLTEGFJQKIH*, codes for the further metabolism of the benzoates to central metabolites through catechol and the *meta* pathway (Harayama & Rekik 1990). Two positive regulator proteins, the products of *xylR* and *xylS*, are responsible for the induction of both operons (Ramos et al. 1987; Inouye et al. 1987; Holtel et al. 1990; Abril et al. 1991).

TOL plasmids pWW53 and pDK1 share the same operon structure with pWW0. However the relative locations and orientations of their operons differ from pWW0 and both carry duplications of *xyl* genes. pWW53 has a single *xylCMABN* operon but has two *xylXYZLTEGFJQKIH* operons which are homologous but not identical (Osborne et al. 1988). It carries a single *xylR* gene but has three regions homologous to *xylS*. Two of these, *xylS1* and *xylS3*, encode functional regulators (Assinder et al. 1993) but *xylS2* is only the residue of a third homologue which has been disrupted by an insertion event (Assinder et al. 1992). Like pWW0, pDK1 has only single copies of the two operons of catabolic genes but has two regions homologous to *xylS*, one of which is functional and the other is identical to the interrupted *xylS2* region of pWW53 (Assinder et al. 1993). The significance of these will be discussed below.

The tod genes of P. putida F1

The genes for toluene catabolism in *P. putida* F1 appear to be in a single chromosomal operon. The first enzyme of the pathway is the multicomponent toluene dioxygenase which converts toluene to *cis* toluene dihydrodiol. Its genes (*todC1C2BA*) are located in between two

Table 1. Protein and gene designations for the *meta* pathway genes for catechol catabolism in pathways for toluene/xylene (XYL), biphenyl (BPH), phenol (DMP), naphthalene (NAH) and toluene (TOD) catabolism.

Ferredoxin-like protein (FLP)
Catechol 2,3-oxygenase (C23O)
2-Hydroxymuconic semialdehyde dehydrogenase (HMSD)
2-Hydroxymuconic semialdehyde hydrolase (HMSH)
2-oxopent-4-enoate hydratase (OPH)
Acetaldehyde dehydrogenase (ADA)
4-Hydroxy-2-oxovalerate aldolase (HOA)
4-Oxalocrotonate decarboxylase (4OD)
4-Oxalocrotonate isomerase (4OI)

	Genes				
Protein	TOL	BPH	DMP	NAH	TOD
FLP	*xylT*	*bphT*	*dmpQ*	*nahT*	NI
C23O	*xylE*	*bphE*	*dmpB*	*nahH*	*todE*
HMSD	*xylG*	*bphG*	*dmpC*	*nahI*	*todG*
HMSH	*xylF*	*bphF*	*dmpD*	*nahN*	*todF*
OEH	*xylJ*	*bphJ*	*dmpE*	*nahL*	NI
ADA	*xylQ*	*bphQ*	*dmpF*	*nahO*	NI
HOA	*xylK*	*bphK*	*dmpG*	*nahM*	NI
4OD	*xylI*	*bphI*	*dmpH*	*nahK*	NI
4OI	*xylH*	*bphH*	*dmpI*	*nahJ*	NI

NI, not investigated.

genes for the subsequent transformations in the order *todFC1C2BADEJ* (Zylstra & Gibson 1989). However the locations of the other *meta* pathway genes have not been reported nor is the regulation known. Five contiguous genes (*bnzABCDE*) encoding the conversion of benzene to catechol from a *P. putida* strain have been sequenced (Irie et al. 1987). These correspond to *todC1C2BAD*, are homologous to them and in the same order and also appear to be chromosomal. The location of the other genes for subsequent catabolism of the catechol has not been reported.

The nah genes of naphthalene catabolic plasmids

Like the TOL plasmids, the archetype naphthalene catabolic plasmid NAH7 from *P. putida* PpG7 has its genes organised into two operons (Yen & Gunsalus 1982; Schell & Wender 1986). The first, *nahAaAbAcAdBCDEF*, codes for the enzymes which convert naphthalene to salicylate (Yen & Gunsalus 1982; Simon et al. 1993) and the second, *nahGTHINLJKM*, for the conversion of salicylate to central metabolites via the *meta* pathway (Yen & Gunsalus 1982; Harayama et al. 1987). The regulation of the two operons appears to be less complex than that of the *xyl* operons: a single protein NahR acts as a positive regulator at both promoters and salicylate is the only effector (Yen

& Gunsalus 1985; Schell & Wender 1986). The same two-operon structure is found on a second plasmid pWW60-1 from *P. putida* NCIB9816, although their relative positions and orientations differ from NAH7 (Cane & Williams 1986).

The bph genes for biphenyl catabolism

Most research into biphenyl catabolism has concentrated upon the early genes which encode the conversions of biphenyl into benzoate. The reason for this is that the nonspecificity of many of the enzymes results in many biphenyl degraders being able to carry out partial catabolism of some polychlorinated biphenyls congeners (PCBs) and thus present a possible route for bioremediation of PCB contamination. The range of biphenyl-degrading bacteria is much wider than for toluene and naphthalene and includes *Pseudomonas, Acinetobacter, Achromobacter, Moraxella* and *Alcaligenes* as well as Gram positives (Furukawa et al. 1989). The location of the genes also differs, being found in both plasmid and chromosomes in different strains.

The conversion of biphenyl to benzoate requires four enzymes which are encoded by a single operon which has been compared in a number of strains. In most the order of genes is conserved as *bphA1A2A3A4BCD*. However in some strains the genes are interrupted by various insertions. Whereas in *P. putida* KF715 the gene order is as described (Hayase et al. 1990), in both *P. pseudoalcaligenes* KF707 and *Pseudomonas* sp. LB400 there is a short open reading frame between *bphA2* and *bphA3* and a long insertion of about 3.3 kbp between *bphC* and *bphD* (Taira et al. 1992; Erickson & Mondello 1992; Hofer et al. 1993). DNA:DNA hybridisations with probes from the *bphABCD* operon from strain KF707 showed that homologous DNA was present in 6/8 biphenyl-utilising Pseudomonas strains but in only 2/6 strains from genera other than *Pseudomonas* (Furukawa et al. 1989). This shows that there are at least two non-hybridising classes of *bph* genes in the bacterial population and Furukawa et al. (1992) have suggested there are as many as four different classes. However within the broad genus *Pseudomonas* there is a high degree of conservation of homologous genes.

Much less work has been done on the lower pathway for the further catabolism of the benzoate formed from biphenyl. However a complete operon encoding benzoate catabolism through the *meta* pathway (*bphXYZLTEGFJQKIH*) has been cloned from a *Pseudomonas* catabolic plasmid pWW110 (Carrington et al. 1994) and hybridisation using gene-specific probes have shown a complete linearity with the lower pathway operon of TOL plasmids. Nothing appears to have been reported on the regulation of either operon.

The evolution of the catabolic pathways

Two different classes of evidence can be used to piece together the past history of the pathways. The first is to use the nucleotide and amino acid sequences of the individual genes. The second is to consider the physical organisation of the genes, as they have been outlined above, and the factors which can influence it.

Sequence homologies of individual genes

One of the advances which amino acid and nucleotide sequence determination has made possible is the derivation of relationships between structural genes and their products which are not apparent from their specificity or occasionally even from their mechanism of action. A large databank of DNA and inferred protein sequences is available and comparisons can be performed automatically using several different computational algorithms. In the case of two proteins with related functions but with very limited homology it can be debatable whether the observed similarity is due to divergence from a common ancestor or is the result of selection pressures towards an identical function acting upon unrelated ancestors and thus imposing constraints on the protein structure which result in sequence similarities (i.e. convergent evolution). Harayama & Timmis (1992) suggest that common ancestries for two proteins can be assumed if the following criteria are met:

- They catalyse a similar reaction.
- They have similar subunit molecular weights.
- Their amino acid sequences can be aligned without the introduction of multiple gaps.
- Amino acid identity is > 30%.

Hydroxylating oxygenases

In the pathways under consideration the initial steps of all the operons involve hydroxylation reactions. Dioxygenases acting on biphenyl, naphthalene, toluene (Tod), and toluates (benzoate) produce *cis*-dihydrodiols as products whereas monooxygenases introduce a single hydroxyl group into salicylate and xylene (Fig. 2).

Table 2. Enzymes/genes involved in mono- and di-hydroxylation reactions.

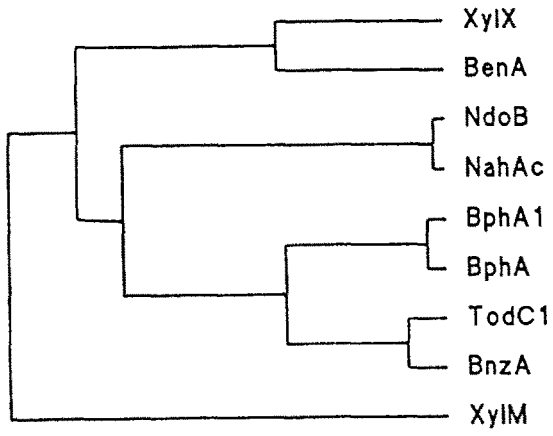
Pathway	Hydroxylase α subunit	Hydroxylase ß subunit	Electron transport protein	Reductase	
BPH:Biphenyl 1,2-dioxygenase					
	bphA1	*bphA2*	*bphA3*	*bphA4*	(1)
	bphA	*bphE*	*bphF*	*bphG*	(2)
TOD:Toluene (benzene) dioxygenase					
	todC1	*todC2*	*todB*	*todA*	(3)
	bnzA	*bnzB*	*bnzC*	*bnzD*	(4)
NAH:Naphthalene 1,2-dioxygenase					
	nahAc	*nahAd*	*nahAb*	*nahAa*	(5)
	ndoB	*ndoC*	*ndoA*	NI	(6)
NAH:Salicylate hydroxylase					
	----------*nahG*----------				(7)
TOL:Xylene monooxygenase					
	---------*xylM*----------		---------*xylA*---------		(8)
TOL:Toluate dioxygenase					
	xylX	*xylY*	---------*xylZ*---------		(9)
BEN:Benzoate 1,2-dioxygenase					
	benA	*benB*	---------*benC*---------		(10)
DMP:Phenol hydroxylase					
	-------*dmpLMNO*---------		---------*dmoP*---------		(11)

References: 1. Taira et al. (1992); 2. Erickson & Mondello (1992); 3. Zylstra & Gibson (1989); 4. Irie et al. (1987); 5. Simon et al. (1993); 6. Kurkela et al. (1988); 7. You et al. (1991); 8. Suzuki et al. (1991); 9. Harayama et al. (1991); 10. Neidle et al. (1991); 11. Nordlund et al. (1990), Powlowski & Shingler (1990). (NI) not investigated.

Salicylate hydroxylase (NahG) is the odd member of this group since it has only a single subunit: the rest of the hydroxylases are multicomponent (Table 2). Naphthalene, biphenyl and toluene dioxygenases have four components (see Mason & Cammack 1992). The direct oxygenation is catalysed by a protein made up of two subunits (α and β) each with an iron-sulphur centre. The other components comprise an electron transport chain of a small redox protein linked to NADH by a reductase. The toluate (benzoate) dioxygenase has three components: the oxygenase has two subunits, only one of which has an iron-sulphur centre, and the electron transport to NADH is performed by a single protein. Xylene monooxygenase requires only two components, an oxygenase with a single subunit and a single reductase.

A comparison of the amino acid sequences of groups of the various components are presented in Figs 3–6. Each figure presents a clustering relationship derived from the multiple alignment of all the sequences in the group produced by the GCG program PILEUP (Devereux et al. 1984). As an alternative a tablulation of the fractional divergences calculated from pairwise alignment generated by the programme CLUSTAL V (Higgins et al. 1992) is presented.

Comparison of the protein sequences and the gene organisations reveals a patchwork of relationships. The biphenyl and the toluene(tod)/benzene dioxygenases are clearly very closely related. The genes are in the



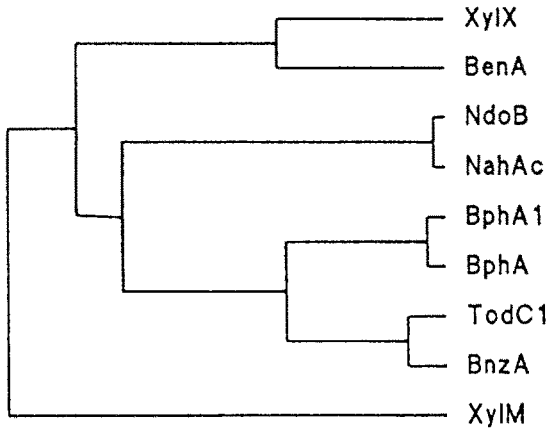
	BphA1	BphA	BnzA	TodC1	NdoB	NahAc	BenA	XylX	XylM
BphA1	0								
BphA	0.04	0							
BnzA	0.40	0.40	0						
TodC1	0.34	0.34	0.08	0					
NdoB	0.67	0.68	0.66	0.65	0				
NahAc	0.67	0.68	0.66	0.65	0.03	0			
BenA	0.77	0.76	0.77	0.75	0.76	0.76	0		
XylX	0.75	0.75	0.77	0.76	0.73	0.74	0.37	0	
XylM	0.87	0.87	0.87	0.86	0.87	0.86	0.87	0.89	0

Fig. 3. Homologies between α subunits of aromatic hydroxylases. The dendrogram was obtained using the GCG program PILEUP (Devereux et al. 1984) and shows the clustering relationships between the proteins. The protein distances in the Table have been calculated from pairwise comparisons in the multiple alignment produced by CLUSTALV (Higgins et al. 1992). The value for each pairwise distance is the (percentage divergence of each pair)/100. The proteins are designated according to their gene designations from Table 1.

same order (ISP large subunit, ISP small subunit, electron transport protein, reductase) and each gene/protein is homologous to the corresponding gene/protein in the other system (Figs 3–6).

The naphthalene dioxygenase components are more distantly related. Firstly the genes are in a different order (reductase, electron transport protein, ISP large subunit, ISP small subunit) to the Bph/Tod genes and the amino acid homology of both of their ISP subunits is lower (Figs 3, 4). The small electron transport proteins of the Bph/Tod and Nah systems are related (Fig. 5) and appear to be novel proteins unlike ferredoxins but with iron-sulphur centres. However the reductases differ significantly and the NahAa protein is more closely related to the reductases of 2-, 3- and 5-component systems such as those of xylene monooxygenase (XylA), toluate dioxygenase (XylZ) and phenol hydroxylase (DmpP) than it is to the Bph/Tod reductases (Fig. 6).

All three components of the toluate dioxygenase (XylXYZ) show strong homology with the isofunctional chromosomal benzoate dioxygenase (BenABC) from *Acinetobacter calcoaceticus*. The two hydroxylase components (XylXY) show low and uneven homology with the corresponding Bph/Tod/Nah subunits which is greater at the N-terminal regions of the α subunits than at the C-terminal regions or with the β subunits (Neidle et al. 1991). The reductases (XylZ/BenC) appear to be hybrids: their N-termini resemble chloroplast-type ferredoxins whereas their C-termini share homology with ferredoxin reductases. They do not resemble the reductases of

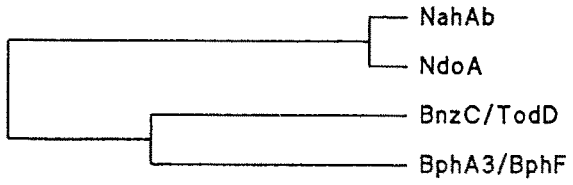


	BphA1	BphA	BnzA	TodC1	NdoB	NahAc	BenA	XylX	XylM
BphA1	0								
BphA	0.04	0							
BnzA	0.40	0.40	0						
TodC1	0.34	0.34	0.08	0					
NdoB	0.67	0.68	0.66	0.65	0				
NahAc	0.67	0.68	0.66	0.65	0.03	0			
BenA	0.77	0.76	0.77	0.75	0.76	0.76	0		
XylX	0.75	0.75	0.77	0.76	0.73	0.74	0.37	0	
XylM	0.87	0.87	0.87	0.86	0.87	0.86	0.87	0.89	0

Fig. 4. Homologies between β subunits of hydroxylases. The tree and Table are derived as in Fig. 3. The proteins are designated according to their gene designations from Table 1.

the Bph/Tod/Nah dioxygenases but do share homology throughout their length with the xylene monooxygenase reductase (XylA) and with reductase components of other monooxygenases such as the multicomponent phenol hydroxylase (DmpP) and the toluene-4-monooxygenase (TmoF) (Yen & Karl 1992) and the methane monooxygenase (MmoC) (Stainthorpe et al. 1990).

The xylene monooxygenase enzyme (XylM), the only one with a single subunit, is the least similar to either of the subunits of the other aromatic multicomponent hydroxylases (Figs 3, 4). However it does show much greater homology to the AlkB protein encoded by the OCT plasmid of *P. putida* (Suzuki et al. 1991). AlkB is involved in a very similar catalytic reaction to XylM, the monohydroxylation of a methyl group to a hydroxymethyl group, but in this case it is the methyl of a linear alkane. The patchwork nature of all these enzymes is further illustrated by the XylM/AlkB comparison: whereas the electron transfer between XylM and NADH is carried out by a single protein (XylA) the sequence of which, like that of XylX, resembles a hybrid between a ferredoxin-like protein and a ferredoxin reductase, the electron transfer between AlkB



	BnzC	BphA2	NahAb	NdoC
BnzC/TodD	0			
BphA2/BphF	0.41	0		
NahAb	0.66	0.63	0	
NdoA	0.62	0.62	0.06	0

Fig. 5. Homologies between small electron transport proteins of the 4-component hydroxylases. The Table are derived as in Fig. 3. The proteins are designated according to their gene designations from Table 1.

and NADH involves two components, a rubredoxin and a rubredoxin reductase neither of which are similar to XylA.

Salicylate hydroxylase (NahG) has no similarities with any of the other hydroxylases under consideration. It is a flavoprotein and the only other protein in the databanks with which it shows homology is *p*-hydroxybenzoate hydroxylase from *P. putida* which catalyses a similar reaction (You et al. 1991).

Ring cleavage dioxygenases

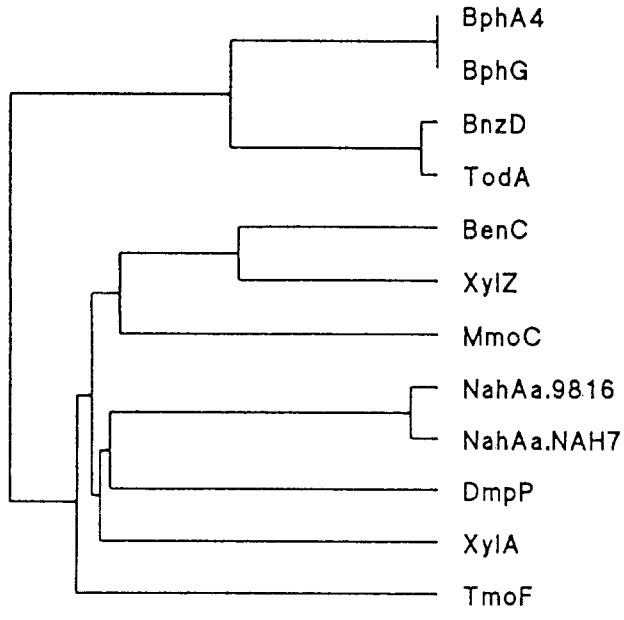
The extradiol dioxygenases have overlapping substrate specificities and are able to catalyse the *meta* cleavage of catechol, alkylcatechols and polycyclic catechols to varying degrees. Such enzymes have been described in a wide variety of Gram negative genera and also in Gram positives. The sequencing of a large number of these genes from a wide variety of sources should provide interesting evidence about the evolution and spread of nonessential catabolic genes.

At present most of the sequenced genes come from *Pseudomonas* strains and, in particular, from the pathways being considered here. Several papers have analysed the relationships between the published sequences (Harayama & Rekik 1989; Hofer et al. 1993). The homologies within the *Pseudomonas* enzymes (Fig. 7) correspond closely to their function as either cleaving single ring catechols or those with two rings. However within each of these two main clusters a number of subgroups can be seen:

- Within the catechol 2,3-dioxygenases those which are from complete *meta* pathway operons are very closely related but there are three which are more distant (CmpE, TdnC and C23OII). Certainly in the latter case the gene does not appear to be adjacent to any other *meta* pathway enzymes and its function is not known.
- Within the polycyclic catechol dioxygenases, the BphC proteins are closely related and surprisingly, the TodE protein, the natural substrate of which is catechol or 3-methyl catechol, is also homologous: this augments the strong homology noted above between all four hydroxylase components of the Bph and Tod pathways and suggests a common ancestry of the *bph* and *tod* genes.
- A subcluster which is less closely related to the BphC/TodE enzymes includes the NahC protein from plasmid NAH7 and a putative BphC from *P. paucimobilis* Q1. The overlapping specificities of extradiol dioxygenases are a potential source for misinterpreting their function particularly since many strains, not only those with polycyclic growth substrates, contain two (or more) different genes for extradiol ring cleavage dioxygenases (Keil et al. 1985; Chatfield & Williams 1986; Asturias & Timmis 1993). Unless a cloned gene is mapped as part of an operon of known function there is a danger of wrongly assigning its function. The gene from *P. paucimobilis* Q1 may be an example of this (Hofer et al. 1993): Kuhm et al. (1991) have shown that strain Q1 grows on naphthalene as well as biphenyl so it is possible that its BphC may be functional in the catabolism of naphthalene (or of both naphthalene and biphenyl) and hence its similarity with NahC might be explained.

The gene for C23OII from plasmid pWW15 falls in between the *meta* pathway catechol 2,3-dioxygenase genes and the enzymes cleaving catechols with two aromatic rings. There is no evidence that it is part of a *meta* pathway operon and the sequence gives no more clues as to its biological role than did the biochemical studies (Keil et al. 1985).

Two enzymes from another genus are included in this analysis, metapyrocatechases 1 and 2 from *Alcaligenes eutrophus*. These are very distantly related to the



	BphA4	BnzD	TodA	BenC	XylZ	MmoC	NahAa[1]	NahAa[2]	DmpP	XylA	TmoF
BphA4	0										
BnzD	0.50	0									
TodA	0.48	0.02	0								
BenC	0.88	0.90	0.90	0							
XylZ	0.86	0.85	0.85	0.47	0						
MmoC	0.87	0.88	0.88	0.68	0.67	0					
NahAa[1]	0.89	0.89	0.90	0.70	0.70	0.73	0				
NahAa[2]	0.89	0.89	0.90	0.70	0.71	0.73	0.07	0			
DmpP	0.87	0.88	0.89	0.70	0.68	0.72	0.71	0.71	0		
XylA	0.86	0.88	0.89	0.68	0.69	0.71	0.71	0.71	0.68	0	
TmoF	0.91	0.90	0.90	0.72	0.73	0.73	0.73	0.74	0.70	0.70	0

[1] From NCIB 9816

[2] From PpG7

Fig. 6. Homologies between reductase components of hydroxylases. The tree and Table are derived as in Fig. 3. The proteins are designated according to their gene designations from Table 1.

Pseudomonas enzymes, although they share a small number of conserved residues with all of them.

Other proteins

Sequence analysis of other enzymes in the hydrocarbon catabolic pathways have been shown to be homologous to other proteins mostly not involved in aromatic catabolism. These are listed in Table 3.

Gene organisation

Transposition

Much of our fundamental understanding of transposition results from study of transposons such as Tn*1*, Tn*3* and Tn*5* carrying antibiotic resistance genes and originally found on many R plasmids. By analogy with these, it might be expected that some of the catabolic plasmids also have associated transposition functions. Certain catabolic pathways such as those for naph-

Table 3.

Catabolic protein	Homologous proteins
XylB (Benzyl alcohol dehydrogenase)	Zinc-containing long chain alcohol dehydrogenases (Shaw *et al.* 1993)
XylC (Benzaldehyde dehydrogenase) XylG,DmpD(2HMS dehydrogenases)	Aldehyde dehydrogenases (Nordlund & Shingler, 1990; Horn *et al.* 1991)
XylL,BphB,TodD,BnzD (*cis*-diol dehydrogenases)	Short chain alcohol dehydrogenases (Neidle *et al.* 1992)
XylT,NahT,DmpQ (Regeneration of catechol 2,3-dioxygenase?)	Chloroplast-type ferredoxins (Harayama *et al.* 1991b; Polissi & Harayama 1993)
XylF,DmpC/TodF (2HMS hydrolases)	Atropinesterase (serine proteases?) (Horn *et al.* 1991)
NahG (salicylate hydroxylase)	*p*-hydroxybenzoate hydroxylase (You *et al.* 1991)
XylS (lower operon regulator)	AraC family of transcriptional regulators (Gallegos *et al.* 1993)
XylR (upper operon regulator) DmpR (phenol operon regulator)	NtrC/NifA family of enhancer-binding transcriptional activators (Inouye *et al.* 1988; Shingler *et al.* 1993)
NahR (upper/lower operon regulator)	LysR family of regulatory proteins (Schell, 1990).

thalene and toluene (by the TOL pathway) seem to be almost universally encoded on plasmids in *Pseudomonas*, much as is antibiotic resistance in clinical isolates of enteric bacteria. Early work with the TOL plasmid pWW0 demonstrated that it could recombine with various R plasmids to form cointegrate plasmids which carried all the genes for toluene/xylene catabolism (White & Dunn 1977; Nakazawa et al. 1978). Subsequently it was shown that the same genes could move into the chromosome and be rescued from

the chromosome on another R plasmid (Jeenes & Williams 1982; Sinclair et al. 1986). However the experiments of Tsuda & Iino (1987, 1988, 1989) showed that the reported recombinations were due to the presence of two overlapping transposons on pWW0, both of which carry the complete complement of *xyl* genes: Tn*4651* is a 56 kb transposon and Tn*4653* is 70 kb and encompasses Tn*4651*.

A second TOL plasmid pWW53 also forms cointegrates with the R plasmid RP4 and has been shown to carry a 39 kb transposon Tn*4656* (Tsuda 1993). pWW53 differs from pWW0 because it carries two complete and functional *meta* pathway operons but only one upper pathway operon (Osborne et al. 1988). Tn*4656* however carries only one of the *meta* pathway operons plus the adjacent upper pathway operon and one copy of each regulatory gene. It therefore appears to be functionally identical to the two pWW0 transposons although the relative positions of the operons on the transposon differ.

Tsuda (1993) has also shown that the naphthalene plasmid NAH7 carries a defective transposon. All the functions for transposition apart from a transposase gene are encoded on the plasmid: when one is provided *in trans* Tn*4655*, a 37.5 kb fragment of NAH7 carrying the complete *nah* genes, transposes. This transposon bears some relationship to the TOL transposons since the transposase from Tn*4651* will supply the missing function.

Recently it has been reported that the genes for conversion of biphenyl to benzoate (*bphABCD*) from the chromosome of an *Alcaligenes eutrophus* strain are on a 59 kb transposon Tn*4371* (Springael et al. 1993). In our laboratory we have shown that cointegrate plasmids can be formed between a *P. putida* biphenyl catabolic plasmid pWW100 and RP4 which appear to possess a single contiguous piece of the catabolic plasmid and carry the genes for the complete catabolism of biphenyl (Lloyd-Jones et al. 1994), suggesting the presence of a transposon.

Catabolic transposons have also been described for chlorobenzoate (Nakatsu et al. 1991), halogenated alkanoates (Thomas et al. 1992), trichlorophenoxyacetate (Haughland et al. 1990) and trichlorobenzene (van der Meer et al. 1991) and they may well prove to be fairly widespread in strains capable of biodegradations.

Recombination and rearrangements

There are a number of examples from within the hydrocarbon catabolic genes of where recombinational events appear to have taken place.

As documented in previous sections, the *tod* genes show a remarkable similarity to the *bph* genes. The complete sequence of the *todC1C2BADE* gene cluster encoding the conversion of toluene to the ring fission product of 3-methylcatechol bears a striking homology to the *bphA1A2A3A4BC* cluster for the conversion of biphenyl to its first ring fission product (see Figs 3–7; Zylstra & Gibson 1989). The *todC1C2BADE* genes are between *todF* and *todJ* which are isofunctional to *xylF* and *xylJ* in the toluene/xylene *meta* pathway operon (Men et al. 1991; Horn et al. 1991). However TodF is considerably more homologous (~ 60%) to the proteins from *meta* pathway operons (XylF, DmpD) than to BphD (~ 30%) from the *bph* upper operon (Men et al. 1991). It is therefore likely that the *tod* gene cluster is the result of insertion of six *bph* genes (or of a common ancestor of both) into a *meta* pathway operon. A second example of a novel gene order which might have arisen by the insertion of one cassette of genes adjacent to another has been found in biphenyl degrader *Pseudomonas* sp. KKS102 by Kikuchi and colleagues (personal communication). In this strain the early genes for biphenyl catabolism are situated downstream of *meta* pathway genes equivalent and homologous to *xylJKQ*. However the situation is more complex than could be explained by a single recombination event because the early genes are in the order *bph(ORF4)A1A2A3BCD(ORF1)A4*. This differs from the order found in *P. alcaligenes* strain KF707 in three respects:

- *bphA4* is downstream of *bphBCD*,
- ORF4 is homologous to the ORF found in KF707 between *bphA2* and *bphA3* but is in a different position, and
- a novel ORF (ORF1) is present within the cluster.

In order to explain the difference in the order of the genes in this strain, one has to invoke one or more rearrangements or insertions in addition to the primary insertion.

The sequence of reactions by which catechol is converted to central metabolites is identical in the catabolism of toluene/xylene, naphthalene and biphenyl (and many other aromatics). As far as has been determined the gene order also appears to have been conserved in *Pseudomonas* and its plasmids (Table 1). The evidence for this is:

- The complete nucleotide sequences for the catabolism of catechol have been determined on the TOL plasmid pWW0 (Nakai et al. 1983; Horn et al. 1991; Harayama & Rekik 1993), on the phenol plasmid pVI150 (Shingler et al. 1992), on the TOL plasmid pDK1 (R.J. Benjamin, personal communication).
- The complete gene order for pWW0 and the sizes of the gene products has been confirmed by subcloning the DNA and determining the sizes of the gene products by SDS-PAGE electrophoresis (Harayama & Rekik 1990).
- The restriction sites on both of the *meta* pathway operons on TOL plasmid pWW53 have been shown to be very similar to those on pWW0. Most of the genes have been subcloned and the location of the majority determined by biochemical assay of the gene products (Osborne et al. 1988).
- Hybridisations with subcloned fragments have been carried out on *meta* pathway genes from two naphthalene plasmids, NAH7 (Harayama et al. 1987) and pWW60-22 (Assinder & Williams 1988) and a biphenyl plasmid pWW110 (Carrington et al. 1994).

This points to a high degree of genetic homology between these operons (as also evidenced by the sequences for the catechol 2,3-dioxygenase genes in Fig. 7) and an identical gene order. This identity only starts at the gene upstream of the catechol 2,3-dioxygenase gene which is *xylT* on the TOL plasmids; upstream of the initiation codon for this gene the homology between the *xyl, nah* and *dmp* sequences is lost (Fig. 8). The function of the XylT protein has not yet been definitively demonstrated but Polissi & Harayama (1993) have strong evidence that it is involved in reactivating catechol 2,3-dioxygenase molecules which have been inactivated by oxidation of the essential Fe^{2+} atoms. If so then it would be expected to be an essential part of the *meta* pathway genes and therefore conserved. It has been found upstream of the gene for catechol 2,3-dioxygenase on all the completed sequences (*xyl* genes of pWW0 and pDK1, *dmp* genes of pVI150, *nah* genes of NAH7) and we have found homologous DNA in the *bph meta* operon using a specific probe from the *dmp* pathway (unpublished results).

The only piece of evidence which suggests that a completely conserved gene sequence is not found in all the *Pseudomonas meta* pathways is the single report that the genes for 4-oxalocrotonate tautomerase (isomerase) and 4-oxalocrotonate dehydrogenase are in a different order on the NAH7 plasmid to that found on the other *meta* pathway operons (Yen & Gunsalus 1982).

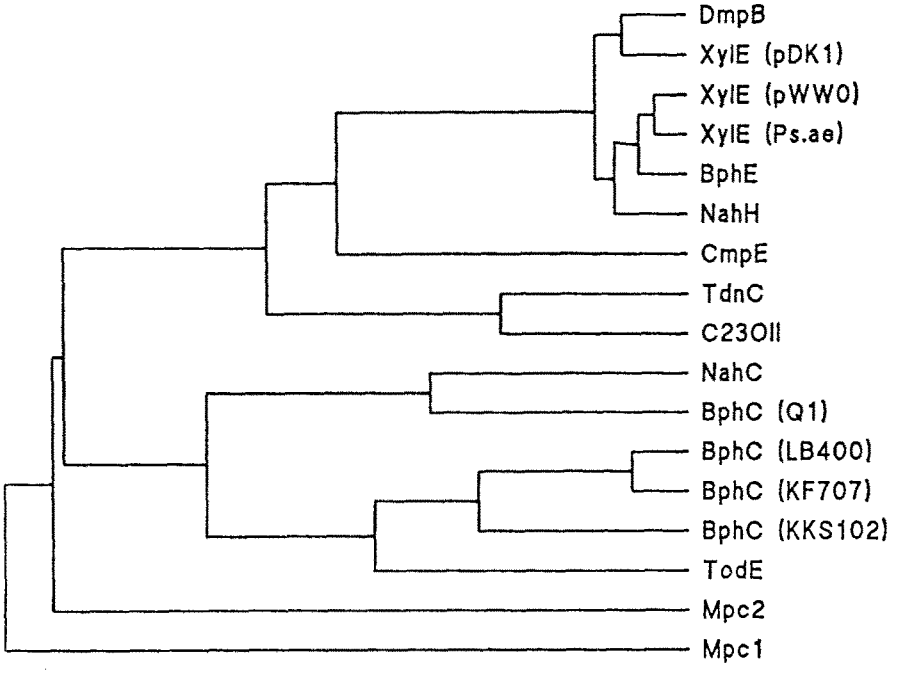
However the orientations and relative locations of the pathway operons (upper and lower pathway) differ from plasmid to plasmid. On naphthalene plasmids NAH7 and pWW60-1 the two operons are in different orientations relative to each other (Yen & Gunsalus 1982; Cane & Williams 1986). A similar situation exists with the three TOL plasmids which have been extensively mapped, pWW0, pWW53 and pDK1 (see Assinder & Williams 1990). The upper and lower pathway operons could have been acquired by the plasmids at different times by different recombinational events or, if they have been acquired by a similar event such as a transposition, then subsequent rearrangements of the complete operons have taken place.

Recombination can also occur between genes to produce fusions or to produce hybrid genes. As implied earlier, some of the reductase proteins (XylZ, XylA) for the hydroxylases may well have resulted from the fusion of a ferredoxin gene and a flavin reductase gene to produce bifunctional protein which performs the same function as two separate proteins for the other hydroxylases.

Recombination between homologous genes can give rise to hybrids. From Fig. 7 it is apparent that the XylE from plasmid pDK1 is as distant from the two other XylE proteins as it is from NahH and BphE. A detailed comparison of the sequence (Benjamin et al. 1991) shows that in its C-terminal region $XylE_{pDK1}$ is closer to $XylE_{pWW0}$ whereas at its N-terminus it is closer to NahH. This raises the possibility that recombination between two coexisting homologous *nah* and *xyl* operons may have taken place within the gene for their catechol 2,3-dioxygenases to give rise to the pDK1 operon.

Comparison of the complete sequences of the *meta* pathway operons on TOL plasmid pWW0 with the equivalent genes on the phenol plasmid pVI150 and the partial sequence available for the *nah* genes on plasmid NAH7 also suggests that recombination may have taken place in their past (Harayama & Rekik 1993). The sequences are consistent with the contemporary *xylTEGFJQ* cluster being derived from a recombination product of the ancestral *dmpQBCDEF* and *nahTHINJZ* sequences.

The formation of just such a hybrid *meta* pathway operon has been seen to occur in the laboratory. On TOL plasmid pWW53 spontaneous recombination occurs between the two homologous copies of its two



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.	0																
2.	0.12	0															
3.	0.17	0.17	0														
4.	0.16	0.19	0.06	0													
5.	0.13	0.18	0.10	0.07	0												
6.	0.13	0.17	0.15	0.12	0.12	0											
7.	0.48	0.48	0.51	0.50	0.49	0.50	0										
8.	0.59	0.58	0.58	0.58	0.59	0.58	0.62	0									
9.	0.55	0.56	0.57	0.57	0.56	0.57	0.60	0.32	0								
10.	0.83	0.84	0.83	0.84	0.82	0.85	0.83	0.83	0.84	0							
11.	0.83	0.83	0.82	0.82	0.83	0.85	0.81	0.82	0.84	0.39	0						
12.	0.81	0.81	0.80	0.79	0.81	0.82	0.80	0.82	0.82	0.65	0.63	0					
13.	0.82	0.82	0.81	0.80	0.81	0.82	0.81	0.83	0.83	0.66	0.64	0.09	0				
14.	0.81	0.81	0.81	0.81	0.82	0.82	0.81	0.82	0.82	0.63	0.61	0.34	0.34	0			
15.	0.80	0.81	0.80	0.79	0.80	0.81	0.80	0.83	0.83	0.65	0.65	0.45	0.45	0.51	0		
16.	0.79	0.81	0.82	0.81	0.81	0.80	0.83	0.79	0.82	0.81	0.82	0.79	0.80	0.83	0.81	0	
17.	0.90	0.87	0.88	0.88	0.88	0.88	0.88	0.86	0.86	0.88	0.87	0.87	0.88	0.90	0.89	0.90	0

1. DmpB:plasmid pVI150 (Shingler et al. 1992)
2. XylE:plasmid pDK1 (Benjamin et al. 1991)
3. XylE:plasmid pWW0. (Nakai et al. 1983)
4. XylE:P.aeruginosa strain JI104 (unpublished sequence,EMBL no.60704)
5. BphE:plasmid pWW110 (Carrington et al. 1993)
6. NahH:plasmid NAH7 (Ghosal et al. 1988)
7. TdnC:plasmid pTDN1 (Saint et al.,1990, Genbank no.L10655)
8. CmpE:plasmid pSKY4 (unpublished sequence, Genbank no.L10655)
9. C23OII:TOL plasmid pWW15 (unpublished sequence, Genbank no.U01826) (Keil et al. 1985)
10. NahC:plasmid NAH7 (Harayama & Rekik, 1989)
11. BphC:P.paucimobilis Q1 (Taira et al. 1988)
12. BphC:Pseudomonas sp. LB400 (Hofer et al. 1993)
13. BphC:P.pseudoalcaligenes KF707 (Furukawa et al. 1987)
14. BphC:Pseudomonas sp. KKS102 (Kimbara et al. 1989)
15. TodE:P.putida F1 (Zylstra & Gibson, 1989)
16. Mpc2:Alcaligenes eutrophus (Kabisch & Fortnagel, 1990a)
17. Mpc1:Alcaligenes eutrophus (Kabisch & Fortnagel, 1990b)

Fig. 7. Homologies between extradiol dioxygenases. The tree and Table are derived as in Fig. 3.

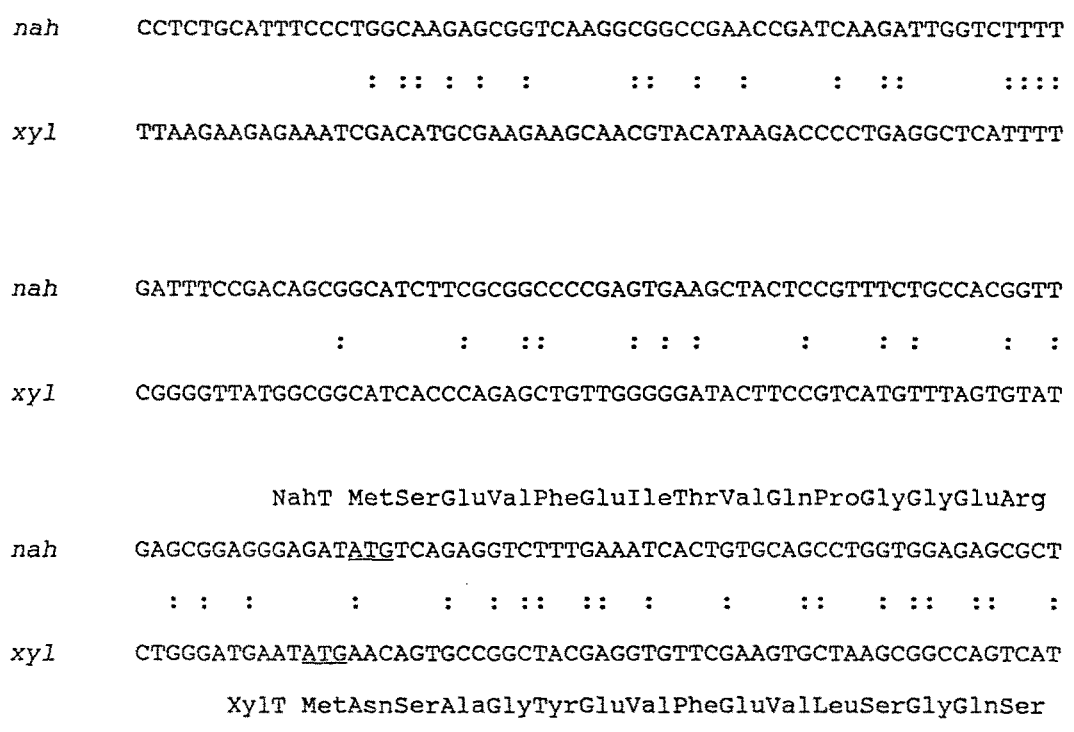


Fig. 8. Region upstream of the postulated start of the DNA sequence common to all *Pseudomonas meta* pathway at around the start codon for the gene for the ferredoxin like open reading frame (NahT, XylT), itself upstream of the gene for catechol 2,3-dioxygenase. The sequence for the *dmp* genes is not included as the termination codon (TGA) for the upstream gene (*dmpP*) is only 9 bases before the ATG start codon for the homologous gene to *xylT* (*dmpQ*).

directly repeated *xylXYZLTEGFQKIH* operons which removes the DNA between (including the *xylCMABN* upper pathway operon) and produces a fully functional operon which is a hybrid between the two original copies (Osborne et al. 1988). The hybrid operons on two plasmids which have undergone recombination have been carefully mapped and the recombinational crossover between the two parental copies has been shown to occur in a different place on each.

pDK1 is host to a *xyl* gene which is clearly a hybrid between two other genes. Comparison of the nucleotide sequence of the gene $xylS1_{pDK1}$, encoding the regulator of the *meta* pathway operon, with other *xylS* genes shows that its 3' end is highly homologous to the archetypal *xylS* gene from pWW0 and to one of the functional homologues from plasmid pWW53 ($xylS1_{pWW53}$). However the 300 nucleotides at the 5' end are 100% identical to the second homologue from pWW53 ($xylS3_{pWW53}$) and diverge significantly from the $xylS_{pWW0}$ and $xylS1_{pWW53}$ genes (Assinder et al. 1993). When the overall gene organisations on pDK1 and pWW53 are compared, it is possible to rationalise that pDK1 arose from a pWW53-like plasmid by recombination between the two *xylS* homologues on the latter. This would not only account for the hybrid nature of the $xylS1_{pDK1}$ gene but would also explain the difference in the relative arrangement of their *xyl* operons (Fig. 9).

Both pWW53 and pDK1 carry a functionless homologue of *xylS*. Their nucleotide sequences reveal that after about 70% of an open reading frame for a XylS-like protein the sequence is disrupted by an ORF which is highly homologous to a family of transposon resolvases (Assinder et al. 1992). The implication is that a functional *xylS* gene was inactivated by the insertion of a transposon.

Evolution of pathways: a synthesis

From the data presented above it is possible to make some generalisations. The first is that the homologies within the proteins with a particular function in any pathway are greater than between pathways. Thus for

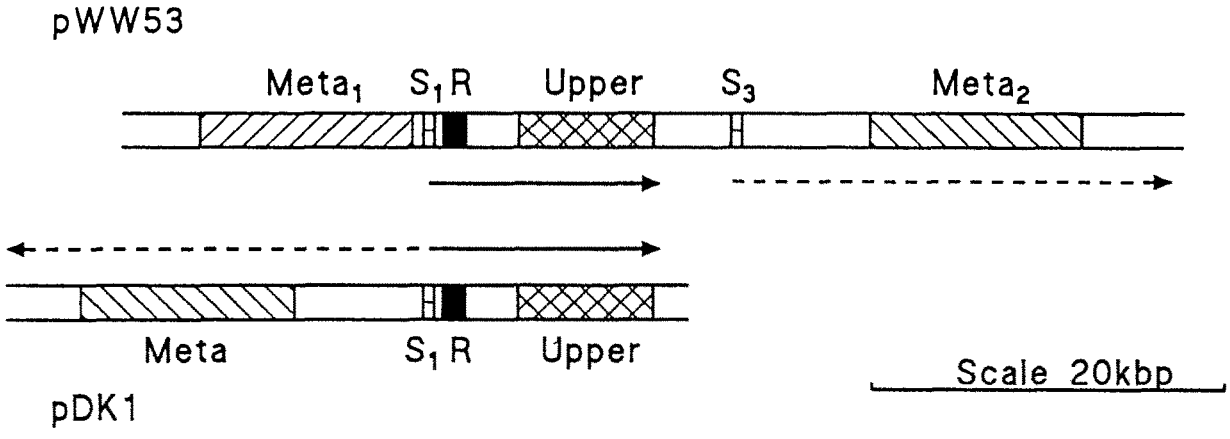


Fig. 9. Postulated recombination between *xylS* homologues leading to formation of pDK1 from a pWW53-like plasmid. The plasmids share two large areas denoted by the two arrows: the areas beside the two bold arrows are homologous and carry the two upper pathway operons (*xylCMABN*) and the two *xylR* genes and the areas beside the dashed arrows each carry a *meta* pathway operon (*xylXYZLTEGFJQKIH*). pWW53 has two *meta* pathway operons, denoted 1 and 2. *Meta2* shares all its restrictions with the *meta* pathway operon on pDK1 whereas *meta1* is much less homologous. The *xylS1* gene on pDK1 is a hybrid between the *xylS1* and the *xylS3* genes on pWW53 and lies at the junction of the two areas of homology which pDK1 shares with pWW53. It is proposed that an internal recombination between the two homologues of *xylS* on a plasmid similar to pWW53 produced the hybrid *xylS2* genes of pDK1 and, at the same time, moved the *meta* operon 2 into the position to the left of the *xylS1R* genes.

example the BphC proteins are closer to each other than they are to the functionally similar NahC proteins or to the various catechol 2,3-dioxygenases (Fig. 7). In this particular example this could be the result of a divergent evolution in which the NahC proteins shared a common ancestor more recently than they shared a common ancestor with the BphC proteins. However one must be careful in bringing time into the argument since the selective pressure for catalysis of different substrates is likely to be an important element in determining the rates at which mutations are fixed and therefore in determining the similarities in the gene products. Even with this proviso it is difficult to avoid the conclusion that early genes of the naphthalene upper operon have diverged considerably from the essentially isofunctional genes for biphenyl. The gene order for the two hydrocarbon dioxygenases is different, although each order appears to be conserved, and the comparison of the reductase proteins (Fig. 6) shows that the naphthalene protein NahAa is very different from the BphA4 proteins and is closer to the reductases from other hydroxylating enzymes including monooxygenases. The picture which the hydroxylase components present suggests a scenario where they each evolved independently by the recruitment of genes from a mixed pool: this has resulted in the patchwork nature of the homologies referred to earlier.

The second major generalisation is the great similarity in the genes/proteins of the *meta* pathway sequences for all the pathways considered. This indicates that it evolved from a common ancestral sequence. Harayama & Rekik (1993) have used a model which compares the degree of identity of two genes in the third base of synonomous codons to estimate that the divergence from this common ancestor occurred about 50×10^6 years ago. However when the *meta* pathway sequences are aligned the close homology downstream of the *xylT* homologues is lost upstream. In all these lower pathway operons the upstream gene(s) differs (Table 4): for TOL plasmids and biphenyl pathways the upstream genes encode the conversion of benzoates to catechols (*xylXYZL* and *bphXYZL*), for NAH plasmids it is *nahG*, for the conversion of salicylate to catechol, and for the phenol pathway of pVI150 there are the phenol hydroxylase components *dmpLMNOP*. Although the complete TOL and Bph *meta* pathway operons appear homologous over their complete length and are likely to share a close common ancestry (Carrington et al. 1994) the upstream genes (and proteins) of the other systems show no homology. This must mean that these operons evolved by the fusion into a single regulon of a common ancestral sequence with a gene or genes from elsewhere. Thus in the case of the TOL (and presumably the Bph) lower pathway operons, it is clear that

the *xylXYZL* cluster is related to the chromosomal *benABCD* cluster. Since (a) benzoate utilization is very widely spread amongst saprophytic bacterial (and fungal) genera and (b) simple aromatics which could give rise to benzoate as a metabolite have presumably been around in the biosphere for a longer period of time than has toluene, it is likely that these genes originally evolved for benzoate degradation and were later acquired as part of the pathway for toluene/xylene or biphenyl catabolism.

It is interesting that catechol and all the later intermediates of the *meta* pathway up to central metabolism are relatively unstable chemicals. A metabolic sequence for aerobic dissimilation of catechols would be likely to be of little environmental advantage to an organism unless catechol was being produced within the same cell or unless the organism was close to a source producing exogeneous catechols. However the selective advantage of acquiring an enzyme which itself generated catechol from a *compound which could exist stably in the environment* (i.e. benzoate, salicylate, phenol etc.) is obvious. This might well have been the selective pressure which caused such fusions and the evolution of the *meta* pathway operons as we now see them. These operons could have been and could still be self-sufficient (e.g. the *dmp* operon). However, if they coexisted with a second gene cluster, the products of which converted another stable compound found in the environment into the substrate for the operon (i.e. toluene or biphenyl to benzoate, naphthalene to salicylate), then the selective advantage would be even greater because the combination of the two would give any host cell a much augmented catabolic versatility.

The picture therefore suggests that the catabolic structural genes for the modern pathways are the result of accretion of at least three modules:

- the catechol *meta* pathway genes headed by
- another gene (or set of genes) to form the lower pathway operon and
- an upper pathway operon acquired subsequently.

Having formed such a unit, presumably there would be a selective advantage to the host strain if it were regulated rather than constitutive. The difference in mode of regulation and of the regulatory elements between the *nah* and *xyl* systems suggests that regulation was imposed after the assembly of the structural genes by recruitment of elements of other regulatory systems already present in the host genome (see Table 3).

This scenario fails to answer the fundamental question of how the modules themselves evolved. The evolution of the catechol *meta* pathway genes as a regulon seems a particularly unlikely event given the probable low availability of all its chemically unstable intermediates as potential growth substrates in the environment. Thus retrograde evolution (Horowitz 1945) would seem to be excluded as a possibility. Neither is there any evidence for tandem duplication of the pathway genes (Horowitz 1963) having played an important role. Only two proteins in the *meta* pathway are possible contenders for having a common ancestor (DmpE and DmpH; Shingler et al. 1992) but the low degree of similarity between them is not convincing. Even in the case of the biphenyl and naphthalene pathways with two extradiol ring cleavage dioxygenases, the low homology between the genes (Fig. 7) suggests a common but remote ancestry which has probably not occurred by an internal duplication followed by a mutational divergence. As the sequence databanks increase in size, more ancestral relationships will be revealed. The genes/proteins of these hydrocarbon catabolic pathways indicate that many belong to quite diverse superfamilies (Table 3). At present all that can be said with certainty is that the modules themselves must have evolved by the recruitment of genes already part of the host genome which for purposes of coordinate expression became linked into a single regulon.

Given that modular construction of these pathways is an approximate description of their evolution, detailed examination of the nucleotide sequences indicates that a variety of other phenomena played a part in moulding the genes and operons into the forms in which they presently exist. Point mutations are the most basic evolutionary changes which take place. However other mechanisms of base pair exchange have been proposed. Ornston and his colleagues have carried out detailed analyses of the nucleotide sequences for the chromosomally-encoded genes for benzoate catabolism in both *P. putida* and *Acinetobacter calcoaceticus* (Neidle et al. 1988; Ornston et al. 1990) and have suggested that a mechanism of DNA slippage and strand exchange of short oligonucleotides appears to play an important role in the evolution of divergent DNA sequences. Similar analysis of the TOL plasmid *xylXYZ* and the homologous *A. calcoaceticus benABC* shows the sequences to be entirely consistent with such a mechanism (Harayama et al. 1991). Using a different analysis of the later genes of the *xyl* lower pathway operon Harayama & Rekik (1993) also conclude that multi-substituting mutations play an important part in driving protein evolution.

Table 4. Enzymes/genes forming catechol in *meta* pathway operons.

	Gene	Enzyme
XYL	*xylXYZ*	Toluate 1,2-dioxygenase
	xylL	Toluate *cis*-diol dehydrogenase
NAH	*nahG*	Salicylate hydroxylase
BPH	*bphXYZ*	Toluate (benzoate) 1,2-dioxygenase
	bphL	Toluate (benzoate) *cis*-diol dehydrogenase
TOD	*todC1C2BA*	Toluene dioxygenase
	todD	*cis*-Toluene dihydrodiol dehydrogenase
DMP	*dmpKLMNOP*	Phenol hydroxylase

The existence of catabolic genes on transposons and plasmids provides a mobility which must accelerate their evolutionary potential. Different host strains with a variety of genetic and biochemical environments together with possible locations on either plasmids or chromosomes must increase the selective pressures which are acting upon the genes by comparison with genes which are obligately chromosomal genes. If nothing else it at least increases the possibilities for recombination events. The duplications of *xyl* genes on TOL plasmid pWW53 described above could have resulted from an internal gene duplication or could have arisen by the acquisition of a second operon during the transient coexistence of two different TOL plasmids in the host cell. The presence of two copies of homologous genes within a cell can also lead to new alleles or hybrid operons by homologous recombination: several such events appear to have operated in the past history of the genes described above.

However it does not seem likely that *intergeneric* gene transfer is a major force in disseminating these genes. If genes from genera other than the saprophytic members of *Pseudomonas* of RNA group I (Palleroni 1992) are compared, then they are found to be distantly related. Thus the divergence of the *xylXYZ* and the *A. calcoaceticus benABC* genes has been estimated as 2×10^8 years which is the same order of magnitude as the divergence between *Pseudomonas* and *Acinetobacter* estimated to be 7×10^8 years from 16S rRNA (Harayama & Rekik 1993). This is also reinforced by for example

- the very low homology between the two catechol 2,3-dioxygenase genes from *Alcaligenes eutrophus* and the various *Pseudomonas* enzymes in Fig. 7, and
- the differences in the *meta* pathway genes between the strains described in this review and in *P. picketti* (Kukor & Olsen 1991): *P. pickettii* is in RNA group II and taxonomically far removed from the group I *Pseudomonas* (see Palleroni 1992).

A scenario of promiscuous catabolic genes frequently transferring between diverse Gram negative genera and participating in recombination and transposition is therefore unrealistic. Probably these genes have been imprisoned within a very limited taxonomic range of hosts for a long period of time.

Adaptation to novel xenobiotics: evolution in the short time scale

The pathways discussed in this review have undoubtedly been in existence for a considerable period of evolutionary time. Numerous examples in the literature show that strains capable of degrading toluene, xylenes, naphthalene and biphenyl can easily be obtained from any healthy soil or water samples either directly or by selective enrichment with little or no lag phase before the appearance of competent degraders. They appear

to be ubiquitous and must use the catabolic capability frequently otherwise its loss might be selectively advantageous. However industry is constantly producing chemicals for deliberate or inadvertent release into the environment, sometimes in very large quantities. Evolution is unable to predict the diversity of novel chemical structures which industrial man is presenting to the microbial population as potential growth sources. Remarkably however a large proportion of these compounds prove to be biodegradable. This must mean that natural evolution (i.e. before the advent of synthetic organic chemistry) has resulted in

- a sufficiently diverse set of protein catalysts within the entire microbial population to enable it to adapt relatively easily when a xenobiotic is first introduced into the environment and
- sufficiently flexible genetic systems to enable those adaptations to occur.

The adaptive mechanisms which occur in the highly heterogeneous soil population between the first introduction of a novel xenobiotic and the advent of a strain or a consortium of strains capable of its catabolism are extremely difficult to monitor experimentally. What occurs during this adaptation must be no different in kind from the events which have been described above, a mixture of mutation, conjugation, transposition, recombination, changes in regulation and enzyme recruitment but over a short time scale and involving changes of a relatively minor nature: given sufficient cells in the population with a sufficient wealth of catabolic diversity and genetic flexibility, it may not require much modification to find one route for degrading any particular xenobiotic. Many laboratory studies have used relatively simple systems to demonstrate various aspects of such adaptations (see Clarke & Slater 1986; van der Meer et al. 1992). Studies on strains with 'newly-evolved' catabolic phenotypes are rarer. One that is worthy of note is the analysis of *Pseudomonas* sp. strain P51 isolated from Rhine river sediment after a long selective enrichment on dichlorobenzenes (van der Meer et al. 1987; 1991a, b, c, d). The results suggest that the pathway for chlorobenzene catabolism arose by the combination within a single cell of two preexisting gene clusters, each encoding a part of the pathway. One of these was plasmid-coded and the plasmid subsequently acquired the second on a transposon: this primary event may then have been followed by minor mutational changes affecting both the structural and regulatory genes.

The last three decades have transformed our understanding of the remarkable flexibility of organisms in being able to respond to selective pressures by the discoveries of the molecular mechanisms by which this occurs. Not all DNA is slowly turning over by the acquisition of neutral mutations in the same way that the DNA encoding, for example, essential 'housekeeping' proteins such as the cytochromes C. The bacterial catabolism of xenobiotics is certainly one area where the rapidity of adaptive response is remarkably short by normal evolutionary standards. Studies of preexisting catabolic pathways and of newly evolved ones will undoubtedly be an important element in our understanding of evolutionary processes at the protein and DNA level.

Acknowledgements

The authors would like to thank the many colleagues who have provided reprints, preprints, unpublished results, faxes and discussions during the course of writing this review. Dr. Vicky Shingler has been preeminent in this respect but also Drs. J. Archer, S.J. Assinder, R.J. Benjamin, D.T. Gibson, Y. Kikuchi, J.L. Ramos, K.N. Timmis and M. Tsuda. This review benefitted from the use of the SEQNET facility provided be SERC Daresbury laboratory.

References

Abril M-A, Buck M & Ramos JL (1991) Activation of the *Pseudomonas* TOL plasmid upper pathway operon. Identification of binding sites for the positive regulator XylR and for integration host factor protein. Journal of Biological Chemistry 266: 15832–15838

Assinder SJ & Williams PA (1988) Comparison of the *meta* pathway operons on NAH plasmid pWW60-22 and TOL plasmid pWW53-4 and its evolutionary significance. Journal of General Microbiology 134: 2769–2778

Assinder SJ & Williams PA (1990) The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Advances in Microbial Physiology 31: 1–69

Assinder SJ, Marco P de, Sayers JR, Shaw LE, Winson MK & Williams PA (1992) Identical resolvases are encoded by *Pseudomonas* TOL plasmids pWW53 and pDK1. Nucleic Acids Research 20: 5476

Assinder SJ, Marco P de, Osborne DJ, Poh CL, Shaw LE, Winson MK & Williams PA (1993) A comparison of the multiple alleles of *xylS* carried by TOL plasmids pWW53 and pDK1 and its implications for their evolutionary relationship. Journal of General Microbiology 139: 557–568

Asturias JA & Timmis KN (1993) Three different 2,3-dihydroxybiphenyl dioxygenase genes in the Gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus globerulus* P6. Journal of Bacteriology 175: 4631–4648

Benjamin RC, Voss JA & Kunz DA (1991) Nucleotide sequence of *xylE* from the TOL pDK1 plasmid and structural comparison with isofunctional catechol 2,3-dioxygenase genes from TOL pWW0 and NAH7. Journal of Bacteriology 173: 2724–2728

Cane PA & Williams PA (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

Carrington B, Lowe A & Williams PA (1994) The lower pathway operon for benzoate catabolism in biphenyl-utilising *Pseudomonas* sp. strain IC. Microbiology 140: 499–508

Catelani D, Sorlini C & Treccani V (1971) The metabolism of biphenyl by *Pseudomonas putida*. Experientia 27: 1173–1174

Chatfield LK & Williams PA (1986) Naturally occurring TOL plasmids isolated from soil carry either two homologous or two non-homologous catechol 2,3-oxygenase genes. Journal of Bacteriology 168: 878–885

Clarke PH & Slater JH (1986) Evolution of enzyme structure and function in *Pseudomonas*. In: Gunsalus IC, Sokatch JR & Ornston LN (Eds) The Bacteria, Vol 10 (pp 71–144). Academic press, New York

Dagley S (1986) Biochemistry of aromatic hydrocarbon degradation in Pseudomonads. In: Gunsalus IC, Sokatch JR & Ornston LN (Eds) The Bacteria, Vol 10 (pp 527–556). Academic press, New York

Devereux J, Haeberli P & Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Research 12: 387–395

Eaton RW & Chapman PJ (1992) Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. Journal of Bacteriology 174: 7542–7554

Erickson BD & Mondello FJ (1992) Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. Journal of Bacteriology 174: 2903–2912

Furukawa K, Arimura N & Miyazaki T (1987) Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*. Journal of Bacteriology 169: 427–429

Furukawa K, Hayase N, Taira K & Tomizuka N (1989) Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: some bacteria possess a highly conserved *bph* operon. Journal of Bacteriology 171: 5467–5472

Furukawa K, Hayashida S & Taira K (1992) Biochemical and genetic basis for the degradation of polychlorinated biphenyls in soil bacteria. In: Galli E, Silver S & Witholt B (Eds) *Pseudomonas*. Molecular Biology and Biotechnology (pp 259–267). American Society for Microbiology, Washington

Gallegos M-T, Michan C & Ramos JL (1993) The XylS/AraC family of regulators. Nucleic Acid Research 21: 807–810

Ghosal D, You I-S & Gunsalus IC (1987) Nucleotide sequence and expression of gene *nahH* of plasmid NAH7 and homology with gene *xylE* of TOL pWW0. Gene 55: 19-28

Gibson DT, Koch JR & Kallio RE (1968) Oxidative degradation of aromatic hydrocarbon from microorganisms. I. Enzymatic formation of catechol from benzene. Biochemistry 7: 2653–2662

Gibson DT, Zylstra GJ & Chauhan S (1990) Biotransformations catalysed by toluene dioxygenase from *Pseudomonas putida* F1. In: Silver S, Cakrabarty AM, Iglewski B & Kaplan S (Eds) *Pseudomonas*: Biotransformations, Pathogenesis and Evolving Biotechnology (pp 121–132). American Society for Microbiology, Washington

Harayama S & Rekik M (1989) Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. Journal of Biological Chemistry 264: 15328–15333

Harayama S & Rekik M (1990) The *meta* cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Molecular and General Genetics 221: 113–120

Harayama S & Rekik M (1993) Comparison of the nucleotide sequences of the *meta*-cleavage pathway genes of the TOL plasmid pWW0 from *Pseudomonas putida* with other *meta*-cleavage genes suggests that both single and multiple nucleotide substitutions contribute to enzyme evolution. Molecular & General Genetics 239: 81–89

Harayama S & Timmis KN (1992) Aerobic biodegradation of aromatic hydrocarbons by bacteria. In: Sigel H & Sigel A (Eds) Metal Ions in Biological Systems, Vol 28. Degradation of environmental pollutants by microorganisms and their metalloenzymes (pp 99–156). Marcel Dekker Inc, New York

Harayama S, Rekik M, Wasserfallen A & Bairoch A (1987) Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7. Molecular and General Genetics 210: 241–247

Harayama S, Rekik M, Wubbolts M, Rose K, Leppik RA & Timmis KN (1989) Characterization of five genes from the upper pathway operon of TOL plasmid pWW0 from *Pseudomonas putida* and identification of the gene products. Journal of Bacteriology 171: 5048–5055

Harayama S, Rekik M, Bairoch A, Neidle EL & Ornston LN (1991a) Potential DNA slippage structures acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benABC* and *Pseudomonas putida* TOL pWW0 plasmid *xylXYZ* genes encoding benzoate dioxygenases. Journal of Bacteriology 173: 7540–7548

Harayama S, Polissi A & Rekik M (1991b) Divergent evolution of chloroplast-type ferredoxins. FEBS Letters 285: 85–88

Harayama S, Kok M & Neidle (1992) Functional and evolutionary relationships among diverse oxygenases. Annual Reviews of Microbiology 46: 565–601

Haughland RA, Sangodkar UMX & Chakrabarty AM (1990) Repeated sequences including RS1100 from *Pseudomonas cepacia* AC1100 function as IS elements. Molecular and General Genetics 220: 222–228

Higgins DG, Bleasby AJ & Fuchs R (1992) CLUSTAL V – improved software for multiple sequence alignment. Computer Applications in the Biosciences 8: 189–191

Hayase N, Taira K & Furukawa K (1990) *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis and expression in soil bacteria. Journal of Bacteriology 172: 1160–1164

Hofer B, Eltis LD, Dowling DN & Timmis KN (1993) Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl-polychlorinated biphenyl degradation. Gene 130: 47–55

Holtel A, Abril M-A, Marques S, Timmis KN & Ramos J-L (1990) Promoter-upstram activator sequences are required for expression of the *xylS* gene and upper-pathway operon on the *Pseudomonas* TOL plasmid. Molecular Microbiology 4: 1551–1556

Horn JA, Harayama S & Timmis KN (1991) DNA sequence determination of the TOL plasmid *xylGFH* genes of *Pseudomonas putida*: implications for the evolution of aromatic catabolism. Molecular Microbiology 5: 2459–2474

Horowitz NH (1945) On the evolution of biochemical syntheses. Proceedings of the National Academy of Sciences, U.S.A. 31: 153–157

— (1963) In: Bryson V & Vogel HJ (Eds) Evolving Genes and Proteins (pp 629). Academic Press, New York

Inouye S, Nakazawa A & Nakazawa T (1987) Overproduction of the *xylS* gene and activation of the *xylDLEGF* operon on the TOL plasmid. Journal of Bacteriology 169: 3587–3592

Inouye S, Nakazawa A & Nakazawa T (1988) Nucleotide sequence of the regulatory gene *xylR* of the TOL plasmid from *Pseudomonas putida*. Gene 66: 301–306

Irie S, Doi S, Yorifuji T, Takagi M & Yano K (1987) Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. Journal of Bacteriology 169: 5174–5179

Jeenes DJ & Williams PA (1982) Excision and integration of degradative pathway genes from TOL plasmid pWW0. Journal of Bacteriology 150: 188–194

Kabitch M & Fortnagel P (1990a) Nucleotide sequence of the metapyrocatechase I (catechol 2,3-dioxygenase I) gene from *Alcaligenes eutrophus* JMP222. Nucleic Acids Research 18: 5543

— (1990b) Nucleotide sequence of the metapyrocatechase II (catechol 2,3-dioxygenase II) gene from *Alcaligenes eutrophus* JMP222. Nucleic Acids Research 18: 3405–3406

Keil H, Lebens MR & Williams PA (1985) TOL plasmid pWW15 contains two non-homologous, independently regulated catechol 2,3-oxygenase genes. Journal of Bacteriology 163: 248–255

Kimbara K, Hashimoto T, Fukuda M, Koana T, Takagi M, Oishi M & Yano K (1989) Cloning and sequencing of two tandem genes involved in the degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. Journal of bacteriology 171: 2740–2747

Kuhm AE, Stolz A & Knackmuss H-J (1991) Metabolism of naphthalene by biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. Biodegradation 2: 115–120

Kukor JJ & Olsen RH (1991) Genetic organisation and regulation of a *meta* cleavage pathways for catechols produced from catabolism of toluene, benzene, phenol and cresols by *Pseudomonas pickettii* PKO1. Journal of Bacteriology 173: 4587–4594

Kunz DA & Chapman PJ (1981) Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida* (*arvilla* mt-2: evidence for new functions of the TOL (pWW0) plasmid. Journal of Bacteriology 146: 179–191

Kurkela S, Lehväslaiho H, Palva ET & Teeri TH (1988) Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene 73: 355–362

Lloyd-Jones G, Jong C de, Ogden RC, Duetz WA & Williams PA (1994) Recombination of the *Bph* (biphenyl catabolic genes from plasmid pWW100 and their deletion during growth on benzoate. Applied and Environmental Microbiology 60: 691–696

Mason JR & Cammack R (1992) The electron-transport proteins of hydroxylating bacterial dioxygenases. Annual Reviews of Microbiology 46: 277–305

Men F-M, Zylstra GJ & Gibson DT (1991) Location and sequence of the *todF* gene encoding 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in *Pseudomonas putida* F1. Gene 104: 91–94

Nakatsu C, Ng J, Singh R, Straus N & Wyndham C (1991) Chlorobenzoate catabolic transposon Tn*5271* is a composite class I element with flanking class II insertion sequences. Proceedings of the National Academy of Sciences, U.S.A. 88: 8312–8316

Nakai C, Kagimiyama H, Nozaki M, Nakazawa T, Inouye S, Ebina Y & Nakazawa A (1983) Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *P. putida* mt-2. Journal of Biological Chemistry 258: 2923–2928

Nakazawa T, Hayashi E, Yokota T, Ebina Y & Nakazawa T (1978) Isolation of TOL and RP4 recombinants by integrative suppression. Journal of Bacteriology 134: 270–277

Neidle EL, Hartnett CS, Bonitz S & Ornston LN (1988) DNA aequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. Journal of Bacteriology 170: 4874–4880

Neidle EL, Hartnett C, Ornston LN, Bairoch A, Rekik M & Harayama S (1991) Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. Journal of Bacteriology 173: 5385–5395

— (1992) *Cis*-diol dehydrogenases encoded by the TOL pWW0 plasmid *xylL* and the *Acinetobacter catlcoaceticus* chromosomal *benD* gene are members of the short-chain alcohol dehydrogenase superfamily. European Journal of Biochemistry 204: 113–120

Nordlund I & Shingler V (1990) Nucleotide sequence of the *meta* cleavage pathway enzymes 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase. Biochimica Biophysical Acta 1049: 227–230

Nordlund I, Powlowski J & Shingler V (1990) Complete nucleotide sequence and polypeptide analysis of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. Journal of Bacteriology 172: 6826–6833

Ornston LN, Neidle EL & Houghton JE (1990) Gene rearrangements, a force for evolutionary change: DNA sequence arrangements, a source of genetic constancy. In: Drilca K & Riley M (Eds) The Bacterial Chromosome (pp 325–334). American Society for Microbiology, Washington

Osborne DJ, Pickup RW & Williams PA (1988) The presence of two homologous *meta* pathway operons on TOL plasmid pWW53. Journal of General Microbiology 134: 2965–2975

Palleroni NJ (1992) Present situation of the taxonomy of aerobic pseudomonads. In: Galli E, Silver S & Witholt B (Eds) *Pseudomonas*: Molecular Biology and Biotechnology (pp 105–115). American Society for Microbiology, Washington

Polissi A & Harayama S (1993) In vitro reactivation of catechol 2,3-dioxygenase mediated by a chloroplast-type ferredoxin: a bacterial strategy to expand the substrate specificity of aromatic degradative pathways. EMBO Journal 12: 3339–3347

Powlowski J & Shingler V (1990) In vitro analysis of polypeptide requirements of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. Journal of Bacteriology 172: 6834–6840

Ramos J-L, Mermod N & Timmis KN (1987) Regulatory circuit controlling transcription of TOL plasmid operon encoding *meta*-cleavage pathway for degradation of alkylbenzoates by *Pseudomonas*. Molecular Microbiology 1: 293–300

Saint CP, McClure NC & Venables WA (1990) Physical map of the aromatic amine and m-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique meta-cleavage pathway. Journal of General Microbiology 136: 615–625

Schell MA & Wender PE (1986) Identification of the *nahR* gene product and nucleotide sequences required for its activation of the *sal* operon. Journal of Bacteriology 166: 9–14

Shaw JP, Rekik M, Schwager F & Harayama S (1993) Kinetic studies on benzyl alcohol dehydrogenase encoded by TOL plasmid pWW0. A member of the zinc-containing long chain alcohol dehydrogenase family. Journal of Biological Chemistry 268: 10842–10850

Shingler V, Powlowski J & Marklund U (1992) Nucleotide sequence and functional analysis of the complete phenol/3,4-

dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. Journal of Bacteriology 174: 711–724

Shingler V, Bartilson M & Moore T (1993) Cloning a nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. Journal of Bacteriology 175: 1596–1604

Simon MJ, Oslund TD, Saunders R, Ensley BD, Suggs S, Harcourt A, Suen W, Cruden DL, Gibson DT & Zylstra GJ (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB9816-4. Gene 127: 31–37

Sinclair MI, Max PC, Lyon BR & Holloway BW (1986) Chromosomal location of TOL plasmid DNA in *Pseudomonas putida*. Journal of Bacteriology 168: 1302–1308

Springael D, Kreps S & Mergeay M (1993) Identification of a catabolic transposon Tn*4371* carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. Journal of Bacteriology 175: 1674–1681

Stainthorpe AC, Lees V, Salmond GPC, Dalton H & Murrell JC (1990) The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). Gene 91: 27–34

Suzuki M, Hayakawa T, Shaw JP, Rekik M & Harayama S (1991) Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylating system. Journal of Bacteriology 173: 1690–1695

Taira K, Hayase N, Arimura N, Yamashita S, Miyazaki T & Furukawa K (1988) Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry 27: 3990–3996

Taira K, Hirose J, Hayashida S & Furukawa K (1992) Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. Journal of Biological Chemistry 267: 4844–4853

Thomas AW, Slater JH & Weightman AJ (1992) The dehalogenase gene *dehI* from *Pseudomonas putida* PP3 is carried on an unusual mobile genetic element designated *DEH*. Journal of Bacteriology 174: 1932–1940

Tsuda M (1993) Catabolic transposons in Pseudomonads. In: Proceedings of the 2nd International Symposium on Genetic Engineering & Biotechnology

Tsuda M & Iino T (1987) Genetic analysis of a transposon carrying toluene degrading genes on TOL plasmid pWW0. Molecular and General Genetics 210: 270–276

— (1988) Identification and characterization of Tn*4653*, a transposon covering the toluene transposon Tn*4651* on TOL plasmid pWW0. Molecular and General Genetics 213: 72–77

— (1990) Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. Molecular and General Genetics 223: 33–39

Meer JR van der, Roelofsen W, Schraa G & Zehnder AJB (1987) Degradation of low concentrations of dichlorobenzenes by *Pseudomonas* sp. P51 in nonsterile soil columns. FEBS Microbial Ecology 45: 333–341

Meer JR van der, Neerven ARW van, Vries EJ de, Vos WM de & Zehnder AJB (1991a) Cloning and characterization of plasmid-coded genes for the degradation of 1,2-dichloro, 1,4-dichloro- and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. Journal of Bacteriology 173: 6–15

Meer JM van der, Eggen RIL, Zehnder AJB & Vos WM de (1991b) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster which encodes metabolism of chlorinated catechols: evidence for specialisation of catechol 1,2-dioxygenases for chlorinated substrates. Journal of Bacteriology 173: 2425–2434

Meer JM van der, Frijters ACJ, Leveau JHL, Eggen RIL, Zehnder AJB & Vos MW de (1991c) Characterization of the *Pseudomonas* sp. strain P51 gene *tcbR*, a LysR-type transcriptional activator of the *tcbCDEF* chlorocatechol oxidative operon and analysis of the regulatory region. Journal of Bacteriology 173: 3700–3708

Meer JM van der, Zehnder AJB & Vos WM de (1991d) Identification of a novel composite transposable element Tn*5280* carrying chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51. Journal of Bacteriology 173: 7077–7083

Meer JF van der, Vos WM de, Harayama S & Zehnder AJB (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiological Reviews 56: 677–694

White GP & Dunn NW (1977) Apparent fusion of the TOL plasmid with the R91 drug resistance plasmid in *Pseudomonas aeruginosa*. Australian Journal of Biological Science 30: 345–355

Yates JR & Mondello FJ (1989) Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. Journal of Bacteriology 171: 1733–1735

Worsey MJ & Williams PA (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

Yen M & Gunsalus IC (1982) Plasmid gene organisation: naphthalene/salicylate oxidation. Proceedings of the National Academy of Sciences of the USA 79: 874–878

Yen K-M & Gunsalus IC (1985) Regulation of naphthalene catabolic genes of plasmid NAH7. Journal of Bacteriology 162: 1008–1013

Yen K-M & Karl MR (1992) Identification of a new gene, *tmoF*, in the *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. Journal of Bacteriology 174: 7253–7261

Zylstra GJ & Gibson DT (1989) Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. Journal of Biological Chemistry 264: 14940–14946