The lower pathway operon for benzoate catabolism in biphenyl-utilizing *Pseudomonas* sp. strain IC and the nucleotide sequence of the *bphE* gene for catechol 2,3-dioxygenase

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Pseudomonas sp. strain IC is able to grow on biphenyl, 3-methylbiphenyl and 4-methylbiphenyl. These are converted to benzoate and the corresponding methylbenzoates. The lower pathway genes for the catabolism of the benzoates were cloned on a 22 kb *Hind*III fragment. Hybridization with gene-specific probes from the *meta* pathways of other catabolic plasmids showed that the gene order was identical to that of the operons carrying the same function from TOL plasmids. The nucleotide sequence of a 1241 bp region carrying the whole of the *bphE* gene (for a catechol 2,3-dioxygenase) and the 5' end of the downstream *bphG* gene (for 2-hydroxy semialdehyde dehydrogenase) was determined. Both genes showed a high degree of homology with genes encoding isofunctional proteins from other *Pseudomonas* strains. The upper pathway genes for the conversion of biphenyl to benzoate have also been cloned but no linkage with the lower pathway operon has been detected. *Pseudomonas* strain IC contains a large plasmid pWW110 (> 200 kb) and there are indications that this plasmid carries the *bph* genes.

Keywords: *Pseudomonas* sp. strain IC, *bph* genes, benzoate catabolism, biphenyl catabolism, catechol 2,3-dioxygenase

INTRODUCTION

The pathway for the catabolism of biphenyl shares many features with the catabolic pathways of other aromatic compounds (see Higson, 1992). Much work has been done to delineate the early genes, *bphABCD*, the products of which convert biphenyl to benzoate (Furukawa *et al.*, 1989; Hayase *et al.*, 1990; Taira *et al.*, 1992; Erickson & Mondello, 1992). These conversions are analogous to, but enzymically distinct from, the well studied upper pathway operon (*xylCMABN*) (Harayama *et al.*, 1989) on TOL plasmids which encodes the conversion of toluene to benzoate. The *xyl* and *bph* pathways converge at benzoate which is assimilated *via* catechol and subsequent *meta* cleavage in both toluene and biphenyl degraders (Catelani

et al., 1971; Fig. 1). Whereas the lower pathway operon on TOL plasmids (*xylXYZLTEGFJQKIH*) (Harayama & Rekik, 1990), which encodes the further conversion of benzoate to pyruvate and acetyl-CoA, has been the subject of intensive investigation (Assinder & Williams, 1990) and has been completely sequenced (Nakai *et al.*, 1983; Horn *et al.*, 1991; Harayama *et al.*, 1991a, b; Neidle *et al.*, 1992; Harayama & Rekik, 1983), very little biochemistry or molecular biology has been performed on the analogous sequence of reactions of biphenyl catabolism.

Much of this pathway, from catechol to pyruvate and acetyl-CoA (the *meta* cleavage pathway), is shared by other aromatic catabolic pathways, in particular those for naphthalene and phenol degradation. The *dmp* genes on the phenol-catabolic plasmid pVI150 have been completely sequenced and, from the gene upstream of the catechol 2,3-dioxygenase (C23O) gene downstream (*dmpQBCDEFGHI*), they are in the same order as the *xyl* genes and are very similar to them (Bartilson & Shingler, 1989; Nordlund & Shingler, 1990; Shingler *et al.*, 1992). A similar situation appears to be the case with the same

Abbreviations: C23O, catechol 2,3-dioxygenase; HMSH, 2-hydroxy-muconic semialdehyde hydrolase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase.

The GenBank accession number for the sequence reported in this paper is U01825.

Fig. 1. Pathway for biphenyl catabolism.

sequence of genes in naphthalene catabolism where (a) DNA hybridization has been used to show colinearity between the *nah* and *xyl* genes (Assinder & Williams,

Table 1. Enzyme and gene designations for the meta pathway operons for toluene (*xyl*), biphenyl (*bph*), phenol (*dmp*) and naphthalene (*nah*) catabolism

Enzymes				
	Toluate 1,2-dioxygenase			
	Toluate <i>cis</i> -diol dehydrogenase			
	Ferredoxin-like ORF			
	Catechol 2,3-dioxygenase			
	2-Hydroxymuconic semialdehyde dehydrogenase			
	2-Hydroxymuconic semialdehyde hydrolase			
	2-Oxopent-4-enoate hydratase			
	Acetaldehyde dehydrogenase			
	4-Hydroxy-2-oxovalerate aldolase			
	4-Oxalocrotonate decarboxylase			
	4-Oxalocrotonate isomerase			
Genes	<i>xyl</i>	<i>bph</i>	<i>dmp</i>	<i>nah</i>
TO	<i>xylXYZ</i>	<i>bphXYZ</i>		
TDD	<i>xylL</i>	<i>bphL</i>		
FLO	<i>xylT</i>	<i>bphT</i>	<i>dmpQ</i>	<i>nahT</i>
C23O	<i>xylE</i>	<i>bphE</i>	<i>dmpB</i>	<i>nahH</i>
HMSD	<i>xylG</i>	<i>bphG</i>	<i>dmpC</i>	<i>nahI</i>
HMSH	<i>xylF</i>	<i>bphF</i>	<i>dmpD</i>	<i>nahN</i>
OEH	<i>xylJ</i>	<i>bphJ</i>	<i>dmpE</i>	<i>nahL</i>
ADA	<i>xylQ</i>	<i>bphQ</i>	<i>dmpF</i>	<i>nahO</i>
HOA	<i>xylK</i>	<i>bphK</i>	<i>dmpG</i>	<i>nahM</i>
4OD	<i>xylI</i>	<i>bphI</i>	<i>dmpH</i>	<i>nahK</i>
4OI	<i>xylH</i>	<i>bphH</i>	<i>dmpI</i>	<i>nahJ</i>

1988) and (b) the only gene to be sequenced, *nahH* for the C23O, is homologous to the equivalent *xyl* and *dmp* genes (Ghosal *et al.*, 1987).

The results presented in this paper indicate that both the biochemical route and the gene organization for benzoate catabolism in biphenyl-degrading strain IC are identical to those previously reported for TOL plasmids. We therefore propose that the gene nomenclature for each of the *bph* genes in this catabolic sequence be the same as the isofunctional gene in the *xyl* pathway (Table 1).

METHODS

Organisms. *Pseudomonas* sp. strain IC was isolated from agricultural soil by selective enrichment with biphenyl as sole carbon source. It produces a green-yellow diffusible pigment on medium B (King *et al.*, 1954) typical of fluorescent *Pseudomonas* strains such as *P. putida* and *P. fluorescens*. All the bacteria and plasmids used during this study are detailed in Table 2.

Media and culture conditions. *Pseudomonas* sp. strain IC was grown in liquid minimal medium (Sala-Trepat *et al.*, 1972) with biphenyl added as a solid and on minimal agar plates with crystals of biphenyl in the inverted lid. All strains were routinely maintained on nutrient agar or on sensitivity test agar (Amersham) with added antibiotics as appropriate.

DNA extraction and manipulation. Whole-cell DNA was

Table 2. Bacteria and plasmids

Strain or plasmid	Relevant details	Source
Bacterial strains		
<i>Pseudomonas</i> IC	Wild-type Bph ⁺ strain	This study
<i>P. putida</i> PaW95	Ben ⁻	Keil <i>et al.</i> (1985)
<i>P. putida</i> PaW130	Rif ^r	Keil <i>et al.</i> (1985)
<i>P. putida</i> PaW340	Trp ⁻ Str ^r	Jeenes & Williams (1982)
<i>E. coli</i> ED8654		Murray <i>et al.</i> (1977)
<i>E. coli</i> NM522		Vieira & Messing (1982)
Vector plasmids		
pKT230		Bagdasarian <i>et al.</i> (1981)
pUC18		Vieira & Messing (1982)
pNJ5000		Grinter (1983)
Recombinant plasmids		
pWW0-3004	<i>xyIE</i> from TOL plasmid pWW0	Chatfield & Williams (1986)
pWW53-3825	<i>xyiL</i> from pWW53	Keil <i>et al.</i> (1987)
pWW53-3514	<i>xyiXYZ</i> from pWW53	Keil <i>et al.</i> (1987)
pWW53-3511	<i>xyiE</i> from pWW53	Williams <i>et al.</i> (1990)
pVI242	3.6 kb <i>SstI</i> - <i>NotI</i> in pMMB66HE, <i>dmpBCD</i>	Bartilson <i>et al.</i> (1990)
pVI308	1.02 kb <i>HpaI</i> - <i>SacI</i> in pMMB66HE, <i>dmpD</i>	Shingler <i>et al.</i> (1992)
pVI309	1.02 kb <i>DdeI</i> - <i>EcoRI</i> in pMMB66HE, <i>dmpE</i>	
pVI311	2.6 kb <i>NruI</i> - <i>NotI</i> in pMMB66HE, <i>dmpF</i>	
pVI312	1.14 kb <i>SauI</i> - <i>BstEII</i> in pMMB66HE, <i>dmpG</i>	
pVI313	1.08 kb <i>NotI</i> - <i>XhoI</i> in pMMB66HE, <i>dmpH</i>	
pVI314	0.82 kb <i>ScaI</i> - <i>PvuII</i> in pMMB66HE, <i>dmpI</i>	Donated by V. Shingler
Con 147	0.87 kb <i>PstI</i> - <i>NdeI</i> , <i>dmpQ</i>	
pWWX1021	<i>xyiX</i> in M13mp19	Donated by S. Harayama
pWW110	Wild-type plasmid from strain IC: confers Bph ⁺	This study
pWW110-1000	7.2 kb <i>XhoI</i> fragment of pWW110 in pKT230, <i>bphABC</i>	
pWW110-1001	2.2 kb <i>PstI</i> fragment of pWW110-1000 in pUC18, <i>bphC</i>	
pWW110-2025	19 kb <i>HindIII</i> fragment of pWW110 in pKT230, <i>bphABCD</i>	
pWW110-3000	19 kb <i>BamHI</i> fragment of pWW110 in pKT230, <i>bphE-H</i>	
pWW110-3010	9 kb <i>KpnI</i> subclone from pWW110-3000 in pUC18, <i>bphE-H</i>	
pWW110-4000	2.2 kb <i>XhoI</i> subclone in pKT230, <i>bphE</i>	
pWW110-4010	2.2 kb <i>XhoI</i> subclone in pUC18, <i>bphE</i>	
pWW110-4011	2.2 kb <i>XhoI</i> subclone in pUC19, <i>bphE</i> (+)	
pWW110-4012	2.2 kb <i>XhoI</i> subclone in pUC19, <i>bphE</i> (-)	
pWW110-5000	22 kb <i>HindIII</i> fragment of pWW110 in pKT230, <i>bphX-H</i>	
pWW110-5001	7.1 kb <i>SstI</i> fragment of pWW110 in pKT230, <i>bphX-F</i>	

isolated by the method of Ausubel *et al.* (1989). Plasmid pWW110 was extracted by the method of Wheatcroft & Williams (1981). *Escherichia coli* and *P. putida* strains were screened for the presence of recombinant plasmids according to Holmes & Quigley (1981). The vector and cloned DNA were prepared from CsCl density-gradient centrifugation of cleared lysates (Guerry *et al.*, 1973). Restriction endonuclease digestions and ligations with T4 ligase were performed in accordance with the manufacturer's instructions. *E. coli* was 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 spraying with 100 mM catechol: C23O⁺ colonies turn yellow because of the formation of 2-hydroxymuconic semialdehyde.

Mobilization of plasmids. pKT230-derived recombinant plasmids were mobilized into plasmid-free *P. putida* strains PaW95

and PaW130 using the RP4 derivative pNJ5000 (Grinter, 1983) which fails to replicate in the absence of tetracycline selection.

DNA-DNA hybridization. Restriction fragments were separated by agarose gel electrophoresis and transferred by Southern blotting (Southern, 1975) to Zeta-probe membrane (Bio-Rad). Bacterial colonies were blotted onto nitrocellulose (Maniatis *et al.*, 1982). Cloned fragments to be used as DNA probes were separated from vector DNA by agarose gel electrophoresis and extracted according to the method of Girvitz *et al.* (1980). Probes were non-radioactively labelled using dioxygenin according to the manufacturer's instructions (Boehringer Mannheim) or radiolabelled by nick-translation using [³²P]dGTP according to Rigby *et al.* (1977).

Preparation of hybridization probes. Gene-specific probes were prepared from the plasmids in Table 2 by restriction digestion followed by band extraction of the probe as a linear

fragment. *xylL*, a 1 kb *KpnI* fragment of pWW53-3825; *xylX*, 0.3 kb *SalI*-*HindIII* fragment of pWWX1021; *xylXYZ*, a 2 kb *PstI* fragment of pWW53-3514; *xylE*, a 0.5 kb *SalI* fragment of pWW53-3511; *dmpB*, a 0.4 kb *SalI* fragment of pVI242; *dmpC*, a 1.2 kb *SalI*-*PvuII* fragment of pVI308; *dmpD*, a 0.7 kb *PvuII*-*SalI* fragment of pVI308; *dmpE*, a 0.8 kb *SacI*-*SalI* fragment of pVI309; *dmpF*, a 1.1 kb *SalI* fragment of pVI311; *dmpG*, a 0.8 kb *SalI*-*NotI* fragment of pVI312; *dmpH*, a 0.6 kb *BstEII*-*KpnI* fragment of pVI313; *dmpI*, a 0.5 kb *XhoI*-*KpnI* fragment of pVI314; *dmpQ*, 0.3 kb *SmaI*-*HindIII* fragment of Con147.

Nucleotide sequencing. Sequencing was performed on both DNA strands using the dideoxy chain-termination procedure (Sanger *et al.*, 1977) with M13 forward and reverse primers. Nested deletions of plasmids pWW110-4011 and pWW110-4012 were generated using S1 nuclease and exonuclease III (Henikoff, 1984) from commercial kits.

Induction studies. *E. coli* ED8654 and *P. putida* PaW95 containing the recombinant plasmid pWW110-5000 were grown under aeration in 2 l pots containing M9 minimal salts medium (Maniatis *et al.*, 1982) and 0.1 % casamino acids, or minimal salts medium with 10 mM sodium acetate as the carbon source. After 8 h, the inducers [solid biphenyl or solutions of the sodium salts of *m*-toluate or benzoate (to 5 mM)] were added, along with a further 13 mM sodium acetate if the inducer did not act as a carbon source. The cells were harvested after a further 16 h. After centrifugation, cell pellets were washed, recentrifuged and the pellet was stored at -20 °C until required.

Enzyme assays. Cell-free extracts were prepared according to Cane & Williams (1982). Assays for C23O (BphE) and for 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) were carried out as described by Sala-Trepat & Evans (1971). 2-Hydroxymuconic semialdehyde hydrolase (HMSH) and dehydrogenase (HMSD) were assayed according to Duggleby & Williams (1986) and Salat-Trepat & Evans (1971) using the ring-cleavage products of 3-methylcatechol and catechol as respective substrates. The absorbance for catechol was measured at 375 nm ($\epsilon_m = 36 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$); for 3-methylcatechol at 388 nm ($\epsilon_m = 15 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$); 4-methylcatechol at 382 nm ($\epsilon_m = 31.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$); and 2,3-dihydroxybiphenyl at 434 nm ($\epsilon_m = 22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Protein was determined by the Biuret method.

RESULTS

Growth substrate range of *Pseudomonas* strain IC

This bacterium was isolated by selective enrichment on biphenyl and was able to grow on the following as sole carbon sources: biphenyl, 3-methylbiphenyl, 4-methylbiphenyl, benzoate, *m*-toluate (3-methylbenzoate), *p*-toluate (4-methylbenzoate). It was unable to grow on naphthalene, toluene, *m*- or *p*-xylenes, phenol or any of the three monochlorobiphenyls. This indicated that strain IC expressed a biphenyl pathway with a broad specificity, able to tolerate methyl substituents on the biphenyl nucleus or on the ring of its main metabolite, benzoate.

Catabolic pathway for biphenyl catabolism

When wild-type IC was grown on biphenyl, benzoate or *m*-toluate there was significant induction of the first three enzymes of the *meta* pathway (Table 3), confirming that

this pathway is used for their catabolism as would be expected from the pathway of Catelani *et al.* (1971). However, the high induced activities of C23O measured in this way (with catechol as substrate) does not reflect the expression of only BphE: because of the overlapping specificities of BphE and BphC (see Table 4) some of the activity against catechol will be due to BphC. This is apparent from Table 4 where the substrate specificity of ring cleavage dioxygenase activity in biphenyl-grown IC (column 4) was clearly intermediate between that of the two separate enzymes (columns 2 and 3).

Table 3. Expression of *meta* pathway enzymes in *Pseudomonas* IC (IC), *E. coli* ED8654(pWW110-5000) (E) and *P. putida* PaW95(pWW110-5000) (P)

Substrate	Host	Specific activity*		
		C23O	HMSH	HMSD
Acetate	IC	4	3	2
<i>m</i> -Toluate	IC	3900	240	180
Benzoate	IC	12900	540	400
Biphenyl	IC	450	50	40
Acetate	E	< 1	< 1	< 1
<i>m</i> -Toluate	E	< 1	< 1	< 1
Benzoate	E	< 1	< 1	< 1
Biphenyl	E	< 1	< 1	< 1
Acetate	P	170	13	10
<i>m</i> -Toluate	P	600	67	36
Benzoate	P	480	64	40
Biphenyl	P	80	9	6

* Expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. The data are means of at least two determinations.

Table 4. Relative specific activities of ring cleavage dioxygenases from strain IC compared to XylE from pWW0

Substrate	Relative specific activity*			
	XylE†	BphC‡	BphE§	BphC/BphE
Catechol	100	100	100	100
3-Methylcatechol	59	400	15	88
4-Methylcatechol	72	0	33	29
2,3-Dihydroxybiphenyl	4	690	0.6	97

* Measured relative to catechol (= 100). Each value is the mean of four independent experiments.

† In *E. coli* (pWW0-3004).

‡ In *E. coli* (pWW110-1001).

§ In *E. coli* (pWW110-4000).

|| In biphenyl-grown *Pseudomonas* strain IC.

Fig. 2. Maps of the *meta* pathway genes of the biphenyl plasmid pWW110. (a) Summary of hybridizations of Southern blots with gene-specific probes from the phenol plasmid pVI150, and TOL plasmids pWW53 and pWW0. The map of the relevant part of pWW110 is shown between the *Pst*I site (coordinate 0) and the *Hind*III site (coordinate 18.0); this region is one end of the large 22 kb insert of pWW110-5000 and overlaps the end of the *Bam*HI insert of pWW110-3000. Other cloned fragments which overlap this region are the inserts of pWW110-3010 (coordinates 6.2–15.2), pWW110-4000 (5.9–8.1, not shown) and pWW110-5001 (3.1–10.2, not shown). The boxes below the map denote the regions which hybridized strongly to the probes from the three comparison plasmids (see Tables 1 and 2) and the letters correspond to the *xyl* or *dmp* genes used as probes. The open boxes correspond to the *dmp* probes, the vertically hatched boxes to the pWW53 *xyl* probes and the cross-hatched box to the pWW0 *xyl* probe. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Sc, *Sc*I; N, *Not*I. The *Sc*I site at coordinate 6.7 and the *Not*I site at coordinate 7.7 are not unique to the fragment but are unique to the *Xho*I fragment (pWW110-4000) and accurately delineate the *bphE* gene. The *Pst*I and *Sal*I sites at the left hand end are also not unique and have been mapped only on subcloned fragments. (b) Positions of the *bph* genes deduced from hybridization results.

Cloning of *bph* genes from plasmid preparations: the lower (*meta*) pathway operon

Strain IC contains a large plasmid pWW110 (> 200 kb) which was isolated with some difficulty by the method of Wheatcroft & Williams (1975). The lower pathway genes for the catabolism of benzoate formed from biphenyl were cloned from plasmid preparations into the broad host range vector pKT230 on two large overlapping fragments, a 19 kb *Bam*HI fragment (pWW110-3000) and a 22 kb *Hind*III fragment (pWW110-5000) (Table 2): these were completely mapped using seven restriction enzymes and partially mapped with four others (Fig. 2a).

Whereas an *E. coli* host carrying plasmid pWW110-3000 turned yellow when sprayed with catechol (due to the formation of 2-hydroxymuconic semialdehyde by the action of C23O), when carrying pWW110-5000 no yellow colour was produced. Transformants carrying pWW110-5000 were first identified by colony hybridization using a *xy*/E probe from TOL plasmid pWW53 (pWW53-3511, Table 2). To investigate whether pWW110-5000 carried the complete pathway for benzoate dissimilation, it was

mobilized into two host strains, both derivatives of *P. putida* mt-2. In strain PaW340, which contains a functional β -ketoadipate pathway, the presence of pWW110-5000 supported growth, not only on benzoate but also on *m*-toluate. Since only the *meta* pathway and not the β -ketoadipate pathway is able to metabolize alkyl-substituted benzoates, this demonstrated that PaW340(pWW110-5000) expressed the *meta* pathway. It is possible, however, that the benzoate and *m*-toluate are converted to catechols in this strain by the chromosomal enzymes (*ben*ABCD) which convert benzoate to catechol before entry into the β -ketoadipate pathway. To confirm that pWW110-5000 carried at least some of this genetic information we also mobilized it into PaW95, a strain defective in the early steps of benzoate utilization: the resultant strain, PaW95(pWW110-5000), also grew on benzoate and *m*-toluate.

The overlapping *Bam*HI fragment pWW110-3000 did not appear to carry all the genes necessary for benzoate utilization since its mobilization into PaW95 did not permit growth on benzoate or *m*-toluate. This was confirmed by hybridization experiments described below which showed that only the genes from *bphE* downstream were present (Fig. 2a).

Expression of cloned *meta* pathway genes

Confirmation of the presence of the *meta* pathway genes on pWW110-5000 was obtained by assaying the three representative enzymes (Table 3). In benzoate- and *m*-toluate-grown cells of PaW95(pWW110-5000) all three enzymes were induced. This suggested that either there was a regulator protein on pWW110-5000 which, in the presence of benzoate and *m*-toluate, induced the *meta* pathway enzymes, or that some regulatory protein present in the genome of PaW95 was acting in this capacity. We tried to induce the same activities in *E. coli* ED8654(pWW110-5000) under a variety of conditions and were unable to detect significant expression of C23O, HMSH or HMSD, whether grown in the presence or absence of benzoate, *m*-toluate or biphenyl (Table 3). This agrees with the observation that no yellow colour was obtained on spraying *E. coli* hosts carrying this plasmid with catechol. If a regulator protein is encoded on pWW110-5000 then it appears to be unable to function in an *E. coli* host.

Gene order of the *bph* lower pathway operon

The gene order on the lower pathway fragments was determined by a series of hybridization experiments with plasmids pWW110-3010 (carrying a 9 kb *Kpn*I fragment subcloned from pWW110-3000) and pWW110-5000. Gene-specific probes were obtained by the excision of fragments from cloned *meta* pathway genes of the phenol-catabolic plasmid pVI150 carrying parts or complete genes from *dmpB* (for C23O) downstream. Gene-specific probes for the benzoate-utilization pathway were obtained from the TOL plasmids pWW53 (*xy*/XYZ, *xy*/L) and pWW0 (*xy*/X).

Fig. 3. Typical hybridization results upon which Fig. 2 is based. (a) Restriction digest of recombinant plasmids carrying *meta* pathway genes from pWW110. (b) Autoradiogram of Southern blot of same digest probed with pWW53-3825 carrying *xylL* from pWW53. Lanes 1, 1 kb DNA ladder (Gibco); 2, *XhoI* digest of pWW110-5000; 3, *SaI* digest of pWW110-5000; 4, *SstI* digest of pWW110-5000; 5, *KpnI* digest of pWW110-5000; 6, *BamHI* digest of pWW110-5000; 7, *PstI* digest of pWW110-5000; 8, *XhoI* digest of pWW110-5001; 9, *SaI* digest of pWW110-5001; 10, *PstI* digest of pWW110-5001; 11, *XhoI* digest of pWW110-3000; 12, *HindIII* digest of bacteriophage λ DNA.

A typical blot is shown in Fig. 3 and the final conclusion of the hybridizations is summarized in Fig. 2(b). Apart from uncertainty as to the order of *bphYZ*, all the other genes are in an identical order to that demonstrated for the lower pathway operon on TOL plasmids (Harayama & Rekik, 1990, 1993). The insert of pWW110-3010 carried only the sequence *bphEGFJQKIH* (from the C23O gene down) and not the genes converting benzoate to catechol (*bphXYZL*).

Nucleotide sequence of the *bphE* gene

The gene for C23O was subcloned on a 2.2 kb *XhoI* fragment in plasmids pWW110-4000 and pWW110-4010, and the nucleotide sequence of 1241 bp was determined (Fig. 4). This encoded an open reading frame of 290 amino acids which is highly homologous to several C23O proteins from other *Pseudomonas* strains (Fig. 5). Those which clustered with BphE are part of *meta* pathway operons which have been sequenced or mapped and which also have the same gene order: this is not certain for the closely related XylE from *P. aeruginosa* as there is no information about its adjacent genes, although it is from a TOL plasmid and it is likely that the genes are organized as in pWW0. There are three proteins, all plasmid-encoded, in the comparison shown in Fig. 5 which are less closely related.

Downstream of BphE is a second open reading frame (BphG) of which only the first 84 codons were sequenced. However this is very similar to the published sequences of

HMSD (XylG, Horn *et al.*, 1991; DmpC, Nordlund & Shingler, 1990). The N-terminal sequence of BphG shows 71/84 identities with XylG from TOL plasmid pWW0 and 11 of the 13 differences are clustered in a short region between residues 9 and 27.

Substrate specificity of BphE

The substrate specificity of BphE is shown in Table 4. It shows strong specificity for catechol over the methylcatechols and very low activity against 2,3-dihydroxybiphenyl. Preliminary results indicated that the enzyme in cell-free extracts was particularly susceptible to inhibition by excess substrate and to inactivation by high levels of substrates, particularly the methylcatechols (data not presented).

The biphenyl upper pathway

The complete upper pathway was cloned using pKT230 on a 19 kb *HindIII* fragment. When the resulting recombinant plasmid pWW110-2025 was introduced into a plasmid-free *P. putida* strain (PaW130) it supported growth on biphenyl, presumably using *bphABCD* to convert the biphenyl to benzoate and the chromosomal β -ketoadipate pathway to further metabolize the benzoate. The *bphC* gene encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase was subcloned on a 2.2 kb *PstI* fragment in plasmid pWW110-1001 (Table 2). Assay of extracts containing BphC showed a distinctly different substrate specificity to BphE, with a much greater activity against

BphE M N K G V M R P G H
 CAAACCTGACAACAGAGAAAAGGTGACGTGATGAACAAAGGTGTAATGCGCCCCGCCAT 60
 V Q L R V L D M S K A L E H Y V E L L G
 GTGCAGCTGCGTGTACTGGACATGAGCAAGGCCTTGGAACTACTCGAGTGTCTGGGC 120
 L I E M D R D D Q G R V Y L K A W T E V
 CTAATCGAGATGGACCGTGACGATCAGGGCCGTGTCTATCTCAAGGCTTGGACCGAAGTG 180
 D K F S L V L R E A D E P G M D F M G F
 GACAAGTTTTCCCTGGTGTGCGTGAAGCGGATGAGCCGGGCATGGATTTCATGGGTTTC 240
 K V I D D E C L V R L T Q D L I D Y G C
 AAGGTGATCGATGATGATGCTGCTGCTGACCCAGGACCTGATCGACTACGGCTGC 300
 L I E T I P A G E L R G C G R R V R F Q
 CTGATCGAGACCATTCGCCCGGAGAACTCAGGGGCTGTGGCCGTCGCGTGCCTTCCAG 360
 A S S G H H F E L Y A D K E Y T G K W G
 GCATCCTCGGGCATCACTTCGAGTTGTATGCAGACAAGGAATATACTGGAAGTGGGGT 420
 V N E V N P E A W P R D L K G M A A V R
 GTGAATGAGGTCAATCCGAGGATGGCCGCGCATCTGAAAGGCATGGCGCTGTGCGT 480
 F D H C L L Y G D E L P A T Y D L F T K
 TTCGATCATTGCCTACTGTATGGCGACGAATTGCCGGCGACCTATGACCTGTTACCAAG 540
 V L G F Y L A E Q V L D E N G T R V A Q
 GTGCTCGGCTTCTATCTGGCCGAACAGGTGCTGGACGAAATGGCAACGCGCTCGCCAG 600
 F L S L S T K A H D V A F I H H P E K G
 TTCCTCAGTCTGTCGACCAAGGCCACGACGTGGCCTTCATTCACCATCCGAAAAAGGC 660
 R L H H V S F H L E T W E D V L R A A D
 CGCCTCCATCATGTGCTCTTTCACCTCGAAACCTGGGAAGACGTGCTTCGCGCCGCCGAC 720
 L I S M T D T S I D I G P T R H G L T H
 CTGATCTCCATGACCGACACCTCGATCGACATCGGCCCGACCCGTCATGGCCTGACCCAC 780
 G K T I Y F F D P S G N R S E V F C G G
 GGCAAGACCATCTACTTCTTCGACCCGTCGCGTAACCGCAGCGAGGTGTTCTGCGGCGGC 840
 N Y S Y P D H K P V T W L A K D L G K A
 AACTACAGCTATCCGATCACAAGCCGGTGACCTGGCTGGCTAAGGACCTGGGCAAGGCG 900
 I F Y H D R V L N E R F M T V L T *
 ATCTTCTATCACGACCGTGTGCTCAACGAACGATTTCATGACCGTGTGACCTGATGGTTC 960
 BphG M K E I H F I N G A F
 GGTTTCGACTTATTGCAGAGATTGCGCAGATGAAAGAGATCAAGCATTTCATTAACGGTGC 1020
 V D S A S G R T F E D I N P V N G Q V I
 CTTCTGCGATTTCGGCCAGCGGCCGACCTTCGAGGACATCAACCCGGTCAATGGCCAGGT 1080
 G R V H E A G R A E V D A A V R A A R A
 GATCGGCCCGGTGCACGAGGCCGCCGCCGCGAGGTGACGCGCGGTGAGGGCGGCACG 1140
 A A L K G P W G K M T V A E R A E I L H
 CGCTGCGCTGAAGGGACCATGGGGGAAGATGACGGTGGCCGAGCGCGCTGAGATTTCGA 1200
 R V A D G V T A R F D E F
 TCGCGTGGCCGATGGCGTACGGCGCGCTTCGATGAGTTTC 1241

Fig. 4. Nucleotide sequence of region including *bphE* gene and the 5' end of the downstream *bphG* gene. The *NotI* site (5' GCGGCCGC 3') downstream of *bphE* (see Fig. 2) is underlined.

2,3-dihydroxybiphenyl as compared with the catechols (Table 4).

We attempted to use DNA-DNA hybridization to find whether the upper pathway operon and the lower pathway operon are closely linked, as is found with both the *xyl* operons (Franklin *et al.*, 1983) and the *nab* operons (Yen & Gunsalus, 1982; Cane & Williams, 1986). So far this approach has given no indication of such a linkage although nearly 50 kb of plasmid pWW110 has been cloned and mapped.

DISCUSSION

Pseudomonas strain IC carries the genes for the catabolism of biphenyl and methyl-substituted biphenyls by the pathway proposed originally by Catelani *et al.* (1971). Biphenyl is converted to benzoate by the action of four

enzymes (BphABCD), the genetics of which have been extensively described in a number of bacteria (Furukawa *et al.*, 1989; Hayase *et al.*, 1990; Taira *et al.*, 1992; Erickson & Mondello, 1992), and the benzoate is then further metabolized by conversion to catechol and subsequent *meta* cleavage as found in the *xyl* pathway of TOL plasmids. The relaxed specificity of the enzymes allow the catabolism of 3-methylbiphenyl (presumably through *m*-toluate) and 4-methylbiphenyl (through *p*-toluate) just as the *xyl* pathway dissimilates toluene, *m*-xylene and *p*-xylene (Assinder & Williams, 1990).

There are indications that the *bph* genes are encoded by the large plasmid pWW110 found in strain IC. Both the catabolic operons cloned in this study were from sucrose gradient preparations of the plasmid. Southern hybridizations of restriction digests of plasmid preparations using the cloned fragments as probes appear to implicate

Fig. 5. Sequence relationships between C23Os from *Pseudomonas* strains. (a) The dendrogram was obtained using the UPGMA method in PHYLIP (Felsenstein, 1993) to analyse the data from pairwise comparisons of C23O sequences. The derived protein distances in the accompanying Table (b) were calculated from pairwise comparisons in the multiple alignment produced by CLUSTAL V (Higgins *et al.*, 1992). The distances were calculated as (percentage sequence divergence of each pair)/100. C23O sequences were obtained from the following sources: XylE, pWW0 (Nakai *et al.*, 1983; Swiss prot. P06622); XylE, *P. aeruginosa* J1104 (P.a.) (P27887); BphE, pWW110 (this paper; U01825); NahH, NAH7 (Ghosal *et al.*, 1987; P08127); DmpB, pVI150 (Shingler *et al.*, 1992; P17262); XylE, pDK1 (Benjamin *et al.*, 1991; M65205); CmpE, pSKY4 (L10655); TdnC, pTDN1 (Saint *et al.*, 1990; X59790); C23OII, pWW15 (Keil *et al.*, 1985; U01826).

plasmid restriction fragments (data not presented) and we have also shown that growth on benzoate can cause loss of the Bph⁺ phenotype (data not presented) in a way analogous to that found for the TOL plasmids (Assinder & Williams, 1990). However, the evidence is not yet sufficiently strong for a definitive implication of pWW110 as the carrier of the catabolic genes.

The results do show clearly that the *meta* pathway genes of the four pathways (*bph*, *xyl*, *dmp*, *nab*), all found in fluorescent *Pseudomonas* of RNA group I, are homologous, co-linear and constitute a tightly clustered evolutionary unit. This is borne out by the strong homology of the nucleotide and protein sequences of their C23O (Fig. 5). Even the non-coding region between the TGA stop codon of the gene for C23O and the ATG start for the

HMSD on TOL plasmids pWW0 and pDK1, phenol plasmid pVI150 and the *bph* genes is highly conserved, being 34 bp in each case and having identical bases in 25/34 positions. The overall similarity of the *meta* pathway genes must point to a shared ancestry and adds further strength to the hypothesis that the complete catabolic pathways, of which the *meta* pathways form the terminal reactions, evolved in a modular fashion by the juxtaposition of pre-evolved 'catabolic' modules (Williams & Sayers, 1994). Two such assemblies would be necessary. A common *meta* pathway sequence (catechol downwards) could have become incorporated into the lower pathway operons by recombination with a gene (or genes) the product(s) of which convert a stable substrate (phenol, benzoate, salicylate) into catechol. Thus we find as the upstream genes in the three lower pathway operons, *dmpKLMNOP* for phenol hydroxylase (in phenol catabolism), *nabG* for salicylate hydroxylase (in naphthalene catabolism), and *xylXYZL* and *bphXYZL* for conversion of benzoate to catechol (in biphenyl and toluene catabolism): these upstream genes bear no relationship to each other and could have been acquired from chromosomal homologues [as the relationship between *benABC* and *xylXYZ* suggests (Neidle *et al.*, 1991)]. However the common downstream genes of the operons would be clearly related, as is observed. The upper pathway operons for the conversion of a further stable compound (toluene, naphthalene, biphenyl) would then subsequently be acquired as a separate operon to form the complete pathways and presumably at this stage some integrated regulation of expression would be acquired. It might be predicted according to this scenario that the two *meta* pathway operons headed by benzoate dioxygenase (*xyl* and *bph*) might be the closest relatives and the degree of homology between XylE and BphE proteins adds some degree of confirmation.

However, there are *meta* pathways which are not in this closely related family and where the gene order is different and the genes are less closely related. The order of the *thu meta* pathway sequence for toluene/benzene/phenol catabolism in *P. pickettii* PKO1 (Kukor & Olsen, 1991) is different but this reflects the taxonomic difference between the host, which is a member of the taxonomically unrelated RNA group II (Palleroni, 1992). The same is almost certainly true of the CmpE protein from plasmid pSKY4 which appears to be involved in naphthalene, salicylate and *m*-toluate catabolism. Mapping evidence suggests that *cmpE* is between the genes for HMSH and HMSD, a different order from the *xyl/dmp* family, but the host strain resembles *P. flava* and *Alcaligenes paradoxus* and therefore appears to be unrelated to either RNA groups I or II (K. Yrjälä, personal communication). These *meta* pathways may therefore represent either very distant divergences or possibly examples of convergent evolution.

In the case of the two less related C23O sequences from taxonomically similar *Pseudomonas* spp. (see Fig. 5) less can be said. TdnC is from the toluidine-catabolic plasmid pTDN1, originally isolated from a line of the *P. putida* mt-2 which is normally the carrier of the pWW0 TOL

plasmid (Saint *et al.*, 1990) and which carries a set of *meta* pathway genes but of undetermined order and with no homology with the *xyl meta*-pathway genes. The second is the C23OII from TOL plasmid pWW15 (Keil *et al.*, 1985) which does not appear to have an associated set of *meta*-pathway genes. In the case of these *Pseudomonas* genes/pathways it is impossible on the available data to state whether these alternative arrangements represent separate evolutionary assemblages of genes into *meta* pathways or whether, from the same genesis, subsequent selective pressures have caused a more rapid divergence affecting both the gene/protein sequences and the gene order.

During the course of this work, other papers on *bph meta* pathway genes have been published but only to the extent of presenting restriction maps of their fragments (Kim *et al.*, 1992; Chang *et al.*, 1992). It is not clear from the papers whether the ring cleavage dioxygenase genes they have cloned are *bphC* or *bphE* (Kim *et al.*, 1992) as 2,3-dihydroxybiphenyl was not used as a substrate in their enzyme assays. From our work we have been able to distinguish the upper and lower pathway genes because of the characteristic specificities of their gene products and will be extending their comparison at the biochemical and genetic levels.

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