Implications of the *xyI*Q gene of TOL plasmid pWW102 for the evolution of aromatic catabolic pathways

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***Pseudomonas putida* strain O₂C₂ is able to grow on toluene, *m*-xylene and *p*-xylene through benzoate and the corresponding methylbenzoates (toluates). The catabolic genes are encoded on a large TOL plasmid, pWW102, of >220 kb. The complete catabolic genes were cloned on four large overlapping restriction fragments covering a total of 28 kb of the plasmid, which was carefully mapped by restriction enzyme analysis. The presence of the *xyI* genes on the cloned DNA was confirmed by assay of representative enzymes of both operons. Virtually all the genes were located on the cloned DNA by hybridization of Southern blots with gene-specific probes from related pathways of other catabolic plasmids. Within the limitations of available restriction sites, the analysis showed that the genes are in two blocks. The major block carries the *meta* pathway operon *xyI*XYZLTEGFJQKIH with the two regulatory genes *xyI*SR immediately downstream. The upper pathway operon *xyI*UWCMAB(N) is about 2–3 kb downstream of the regulatory genes and transcribed in the same direction as the *meta* pathway operon. Within each operon the gene order appears to be identical to that found in other TOL plasmids, but the relative location of the operons most closely resembles that found on plasmid pWW53, although there is no evidence of any *xyI* duplications on pWW102. The nucleotide sequence of the *xyI*Q gene for the acetaldehyde dehydrogenase (acylating; ADA), together with the 3'-end of the upstream *xyI*J (for 2-oxopent-4-enoate hydratase) and the 5'-end of the downstream *xyI*K (for 4-hydroxy-2-oxovalerate aldolase), was determined. The *xyI*Q gene was ligated into expression vector pTrc99a and high levels of XylQ protein were detected by enzyme assay and by SDS-PAGE. All three genes *xyI*JQK showed a high degree of homology with genes encoding isofunctional proteins from other *Pseudomonas meta* pathways, the highest being with the naphthalene catabolic genes *nah*LOM from the plasmid of *Pseudomonas* sp. NCIB 9816. The implications of the sequence homologies to the evolution of these pathways are discussed.**

Keywords: TOL plasmid, *Pseudomonas putida*, toluene/xylene catabolism, acetaldehyde dehydrogenase (acylating), *xyI*Q

INTRODUCTION

The pathway (Fig. 1) for catabolism of toluene and some substituted toluenes, such as *m*- and *p*-xylenes, in *Pseudomonas* appears to be almost always plasmid-

determined (Williams & Worsey, 1976) and shares many features with the catabolic pathways of other aromatic compounds (Assinder & Williams, 1990; Harayama & Rekik, 1993). The genes encoding the catabolic pathway on the archetype of these TOL plasmids, pWW0, have

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Abbreviation: ADA, acetaldehyde dehydrogenase (acylating).

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Fig. 1. *xyl* catabolic pathway encoded by TOL plasmids. The enzymes catalysing each of the transformations are denoted by their gene-associated nomenclature. *xyl* genes not encoding known metabolic reactions are *xylU*, *W*, *N*, *T*, *R*, *S*. The function of XylT is proposed as being involved in reactivating oxidized catechol 2,3-dioxygenase (XylE) (Polissi & Harayama, 1993). XylR and XylS are positive regulatory proteins. The functions of XylU, XylW and XylN are not known.

been studied in great detail (see Assinder & Williams, 1990) and a number of other TOL plasmids have been investigated to a lesser extent (Pickup & Williams, 1982; Keil & Williams, 1985; Keil *et al.*, 1985; Osborne *et al.*, 1988). They all share a common organization into two operons: an 'upper pathway' operon, *xylUWCMABN* (Harayama *et al.*, 1989; Williams *et al.*, 1997), encoding the sequential oxidation of the methyl substituent to a carboxylic acid (e.g. toluene to benzoate), and a 'meta pathway' operon, *xylXYZLTEGFQKIJH* (Harayama & Rekik, 1990), encoding the conversion of the carboxylic acids to central metabolites. Whereas the gene order within each of the operons appears to be con-

served, the relative orientations of the two operons and the distances between them differ from plasmid to plasmid, and most carry duplications of structural and/or regulatory genes (Chatfield & Williams, 1986; Osborne *et al.*, 1988; O'Donnell & Williams, 1991).

Pseudomonas putida O₂C₂ is a bacterium isolated from soil from the Netherlands which is able to grow on toluene and methyl-substituted toluenes and which contains a large TOL plasmid, pWW102. This paper reports the cloning of the *xylQ* catabolic genes from pWW102. The nucleotide sequence of *xylQ*, for acetaldehyde dehydrogenase (acylating; ADA), is presented and the implications of comparison with genes for other ADA proteins to the evolution of aromatic pathways is considered.

METHODS

Maintenance of bacterial strains and plasmids. The strains and plasmids used in this work are listed in Table 1. Strains were routinely maintained on nutrient agar or on sensitivity test agar (Lab M) supplemented with appropriate antibiotics: kanamycin at $15 \mu\text{g ml}^{-1}$ and ampicillin at $100 \mu\text{g ml}^{-1}$.

Enzyme assays. Cell-free extracts, other than for ADA, were prepared according to Cane & Williams (1982). The assays for catechol 2,3-dioxygenase (XylE) and for 2-hydroxymuconic-semialdehyde dehydrogenase (XylG) were carried out as described by Sala-Trepat & Evans (1971) and for 2-hydroxymuconic-semialdehyde hydrolase (XylF) according to Duggleby & Williams (1986). For the ADA (XylQ) assay, *Escherichia coli* containing recombinant plasmids bearing *xylQ* was inoculated at 37 °C for 5 h in LB, when IPTG was added for induction of the *lacI*^q-regulated *tac* promoter of pTrc99a vector, and the cultures were incubated overnight at 30 °C in the presence of IPTG. Extracts were prepared and assayed for ADA activity by the method of Shingler *et al.* (1992).

Protein electrophoresis. Cell-free extracts containing ADA were prepared as described above. SDS-PAGE was carried out by standard procedures in a PROTEANII cell (Bio-Rad).

DNA extraction and manipulation. Shotgun cloning was performed using DNA of plasmid pWW102 which was prepared by the sucrose gradient method of Wheatcroft & Williams (1981). The vector chosen for cloning was pUC18. Restriction endonuclease digestions and DNA ligations with T4 ligase were performed by standard procedures (Sambrook *et al.*, 1989) and recombinant transformants were selected on media containing antibiotics appropriate to the vector. Plasmid DNA was prepared for rapid screening by the method of Holmes & Quigley (1981) and purified by CsCl centrifugation (Guerrey *et al.*, 1973) or using Qiagen kits. Colonies carrying recombinant plasmids expressing catechol 2,3-dioxygenase activity were detected as a result of their yellow colour after spraying with 10 mM catechol (Worsey *et al.*, 1978), whilst other recombinant plasmids were detected by loss of β -galactosidase activity or by colony hybridizations with probes constructed from homologous genes from other bacteria and pathways.

DNA-DNA hybridization. Restriction fragments were separated by agarose gel electrophoresis and transferred by Southern blotting (Southern, 1975) to Biotrans filter membranes.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant details	Source
Strains		
<i>P. putida</i> O ₂ C ₂	Wild-type Mtol ⁺ Mxy ⁺	This study
<i>E. coli</i> NM522		Vieira & Messing (1982)
<i>E. coli</i> JM109		Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pTrc99a	Expression vector	Pharmacia
pUC18	Cloning vector	Vieira & Messing (1982)
pWW102	Wild-type plasmid from strain O ₂ C ₂	This study
Recombinant plasmids		
pWW102-3	17 kb <i>Hind</i> III fragment of pWW102 in pUC18	This study
pWW102-6	11.6 kb <i>Bam</i> HI fragment of pWW102 in pUC18	This study
pWW102-7	8.6 kb <i>Hind</i> III fragment of pWW102 in pUC18	This study
pWW102-8	8.6 kb <i>Hind</i> III fragment of pWW102 in pUC18 (reverse orientation)	This study
pWW102-9	8.2 kb <i>Eco</i> RI fragment of pWW102 in pUC18	This study
pWW102-10	8.2 kb <i>Eco</i> RI fragment of pWW102 in pUC18 (reverse orientation)	This study
pWW102-61	4.0 kb <i>Bam</i> HI- <i>Sac</i> I fragment of pWW102-6 in pUC18 (coordinates 6.2-10.2 kb)	This study
pWW102-62	3.1 kb <i>Sac</i> I- <i>Sma</i> I fragment of pWW102-6 in pUC18 (coordinates 10.3-13.4 kb)	This study
pWW102-62E	3.1 kb <i>Sac</i> I- <i>Sma</i> I fragment of pWW102-6 in pTrc99a	This study
pWW102-622	2.2 kb <i>Kpn</i> I- <i>Sma</i> I fragment of pWW102-62 in pUC18 (coordinates 10.8-13.0 kb)	This study
pWW102-623	2.1 kb <i>Eco</i> RI- <i>Sma</i> I fragment of pWW102-62 in pUC18 (coordinates 10.9-13.0 kb)	This study
pWW102-624	1.0 kb <i>Sal</i> I fragment of pWW102-62 in pUC18 (coordinates 11.1-12.1 kb)	This study
pWW102-63	3.6 kb <i>Sma</i> I fragment of pWW102-6 in pUC18 (coordinates 13.2-16.7 kb)	This study
pVI300	0.87 kb <i>Bal</i> 31- <i>Pst</i> I fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI308	1.02 kb <i>Hpa</i> I- <i>Sac</i> I fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI309	1.02 kb <i>Dde</i> I- <i>Eco</i> RI fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI310	1.13 kb <i>Sal</i> I- <i>Sal</i> I fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI312	1.14 kb <i>Sau</i> I- <i>Bst</i> EII fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI313	1.08 kb <i>Not</i> I- <i>Xho</i> I fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pVI314	0.82 kb <i>Sca</i> I- <i>Pvu</i> II fragment of pVI150 in pMMB66HE	Shingler <i>et al.</i> (1992)
pMT100	1.7 kb <i>Bam</i> HI fragment of pWW0 in pBR322	J.-L.Ramos, CSIC, Granada, Spain
pWW0-7000	3.9 kb <i>Kpn</i> I- <i>Bam</i> HI fragment of pWW0	Williams <i>et al.</i> (1997)
pVI1:00	2.4 kb <i>Eco</i> RV- <i>Eco</i> RV fragment in pBluescript	Bartilson & Shingler (1989)
pWW110-4010	2.2 kb <i>Xho</i> I- <i>Xho</i> I fragment of pWW110 in pUC18	Carrington <i>et al.</i> (1994)
pWW110-6003	2.7 kb <i>Eco</i> RI- <i>Eco</i> RI fragment of pWW110 in pUC18	Carrington <i>et al.</i> (1994)
pWW53-3301	17.5 kb <i>Hind</i> III fragment of pWW53 in pKT230	Keil <i>et al.</i> (1985)

Cloned fragments to be used as DNA probes were extracted according to the method of Girvitz *et al.* (1980) and labelling and detection were carried out by the ECL method (Amersham International).

Nucleotide sequence determinations. Sequences of DNA inserts in pUC18 were determined with a Pharmacia DNA Sequencing kit using the appropriate synthetic oligonucleotide primers. The complete sequences of both strands of the *xyI*/*Q* region were determined from the sequences of 15 individual fragments. Custom-synthesized oligonucleotide primers were used to determine the sequences across the junctions of subfragments from this region. Some sequencing reactions were performed by Alta Biosciences (University of Birmingham) using an ABI automatic sequencer (Applied Biosystems).

Analysis of DNA and protein sequences. Databases were searched for homologous gene sequences using the BLAST programs (Altschul *et al.*, 1990). Manipulation and analysis of the nucleic acid sequences were done with the DNASTAR (Madison, WI, USA) nucleic acid analysis software package.

RESULTS

Strain O₂C₂ and plasmid pWW102

Strain O₂C₂ was isolated from an oil-contaminated soil in the Netherlands by W. A. Duetz (Institut für Biotechnologie, ETH Höggerberg, Zürich, Switzerland) by directly plating supernatants from washed soil onto

Fig. 2. Restriction map of the complete *xyl* catabolic genes of pWW102. The inserts of the major fragments cloned into recombinant plasmids are denoted below the restriction map. The approximate position of the *xyl* genes is shown above the map: the range of the possible positions of each gene as determined from Southern hybridizations is presented in Table 2. The region sequenced is marked by the grey box. B, *Bam*HI; Bg, *Bgl*II; C, *Cl*al; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I.

minimal agar plates with toluene vapour as the sole carbon source. It has been classified by the National Culture of Industrial and Marine Bacteria as a *Pseudomonas putida* and it produces a blue-white fluorescence on King's medium B (King *et al.*, 1954). It grows on toluene, *m*-xylene, *p*-xylene, benzoate, *m*-toluate (3-methylbenzoate) and *p*-toluate (4-methylbenzoate): spraying colonies grown on any of these substrates with catechol produced the yellow colour characteristic of the presence of induced catechol 2,3-dioxygenase. It therefore has the phenotype characteristic of TOL-plasmid-containing strains (Worsey & Williams, 1975; Williams & Worsey, 1976). A large plasmid, pWW102, can be isolated from cultures and measurement and summation of the sizes of fragments of the plasmid digested with a range of restriction enzymes indicate it is between 220 and 270 kb. Growth through one cycle of benzoate in liquid culture for about 20 generations, which took 5–6 d, produced loss of the characteristic TOL phenotype (growth on toluene, xylenes, toluates) in 100% of the colonies. All retained the ability to grow on benzoate but no longer produced the yellow colour with catechol or contained any plasmid DNA. No reversion to growth on any of the TOL-specific substrates could be detected.

Cloning of the upper pathway operon

To identify restriction fragments carrying genes of the upper pathway operon, plasmid pWW0-7000, which carries *xyl*UW and the 5'-end of *xyl*C from TOL plasmid pWW0 (Table 1; Williams *et al.*, 1997), was used as a hybridization probe against Southern blots of digests of pWW102. Three fragments reacted positively: an 8.6 kb *Hind*III fragment and two *Eco*RI fragments of 8.2 and 8.7 kb. The appropriate areas were excised from agarose gels, the DNA was extracted and ligated into pUC18, and transformants were checked for hybridization against pWW0-7000 by dot-blotting. Two plasmids with an 8.6 kb *Hind*III insert in opposite orientations

(pWW102-7 and pWW102-8) and two with an 8.2 kb *Eco*RI insert in opposite orientations (pWW102-9 and pWW102-10) were identified. When the inserts of pWW102-7 and pWW102-9 were mapped for several restriction sites (Fig. 2), comparison of the maps indicated an overlap of about 5.5 kb which was confirmed by cross-hybridization (data not shown).

Functional analysis of upper pathway genes

E. coli containing each of pWW102-7 and pWW102-9 produced pink colonies which turned blue on LB agar plates and produced insoluble blue particles in LB. This reaction is diagnostic for the conversion of tryptophan, present in the medium, to indigo and can be effected by the presence of cloned *xyl*MA, encoding xylene mono-oxygenase (Mermoud *et al.*, 1986; Keil *et al.*, 1987). Two enzymes of the upper pathway operon *xyl*UWCMABN were identified by direct assay. Significant activity of benzyl alcohol dehydrogenase (XylB) was demonstrated in the strain containing pWW102-9 and of benzaldehyde dehydrogenase (XylC) in strains carrying either pWW102-7 or pWW102-9 (data not presented).

Location of genes of the upper pathway operon

Southern hybridization of digests of pWW102-7 and pWW102-9 located *xyl*UW, *xyl*C, *xyl*A and *xyl*B in the positions shown (Table 2). This location of *xyl*B appears to conflict with the fact that XylB activity was expressed only from pWW102-9 and not from pWW102-7. It must be that the gene extends beyond the end of pWW102-7 (at coordinate 25.2 kb) but insufficiently far to hybridize at detectable levels beyond the *Hind*III site. No probes were used for *xyl*M or for *xyl*N.

Cloning of the meta pathway operon

Two plasmids carrying *meta* pathway genes were identified from a library of plasmid fragments inserted

Table 2. Summary of Southern blot hybridization of gene-specific probes against digests of plasmids containing fragments from pWW102 DNA

Probe	Gene(s) carried	Source of probe genes*	<i>xyl</i> homologue	Coordinates (kb)†
pWW110-6003	<i>bphX</i>	pWW110	<i>xylX</i>	2.7–3.9
pVI1300	<i>dmpQ</i>	pVI150	<i>xylT</i>	5.9–7.0
pWW110-4010	<i>bph(T)E</i>	pWW110	<i>xyl(T)E</i>	5.9–8.0
pVI:00	<i>dmpBC</i>	pVI150	<i>xylEG</i>	6.9–9.3
pVI1308	<i>dmpD</i>	pVI150	<i>xylF</i>	8.0–9.3
pVI1309	<i>dmpE</i>	pVI150	<i>xylJ</i>	10.2–10.9
pVI1310	<i>dmpF</i>	pVI150	<i>xylQ</i>	10.9–12.2
pVI1312	<i>dmpG</i>	pVI150	<i>xylK</i>	12.1–13.1
pVI1313	<i>dmpH</i>	pVI150	<i>xylI</i>	12.4–13.3
pVI1314	<i>dmpJ</i>	pVI150	<i>xylH</i>	13.3–14.8
PCR <i>xylH</i> ‡	<i>xylH</i>	pWW102	<i>xylH</i>	
pMT100	<i>xylS</i>	pWW0	<i>xylS</i>	13.0–15.2
pWW53-3340§	<i>xylR</i>	pWW53	<i>xylR</i>	15.2–16.6
pWW0-7000	<i>xylUW(C)</i>	pWW0	<i>xylU(C)</i>	19.1–20.7
PCR <i>xylC</i> ‡	<i>xylC</i>	pWW0	<i>xylC</i>	19.1–23.1
PCR <i>xylA</i> ‡	<i>xylA</i>	pWW0	<i>xylA</i>	23.1–25.2
PCR <i>xylB</i> ‡	<i>xylB</i>	pWW0	<i>xylB</i>	23.1–25.2

* Sources of probe DNA are the biphenyl catabolic plasmid pWW110 (Carrington *et al.*, 1994), TOL plasmids pWW0 (this study), pWW53 (Keil *et al.*, 1985) and pWW102 (this study), and phenol catabolic plasmid pVI150 (Shingler *et al.*, 1992); all pVI plasmids were prepared and supplied by V. Shingler, Umeå University, Sweden.

† The coordinates are presented as the minimum span of the gene(s) as deduced from the hybridization obtained and the restriction map presented in Fig. 2. Because of the location of available restriction sites, they represent a region potentially larger than the size of the gene itself but within which it is substantially located.

‡ Specific gene probes prepared by PCR using primers derived from the published gene sequences.

§ 1.8 kb *Bgl*III–*Hind*III fragment of pWW53-3301 extracted from gel and used directly to probe.

into pUC18 by using the ability of strains carrying a cloned catechol 2,3-dioxygenase to convert catechol to its yellow product, 2-hydroxymuconic semialdehyde. These contained large overlapping inserts, a 17 kb *Hind*III fragment (pWW102-3) and an 11.6 kb *Bam*HI fragment (pWW102-6) (Table 1). Both plasmids were completely mapped using 14 restriction enzymes (Fig. 2).

Functional analysis of *meta* pathway genes

Confirmation of the presence of *meta* pathway genes on pWW102-3 and pWW102-6 was obtained by assaying three representative enzymes, catechol 2,3-dioxygenase (*XylE*), 2-hydroxymuconic-semialdehyde hydrolase (*XylF*) and 2-hydroxymuconic-semialdehyde dehydrogenase (*XylG*) (data not shown).

Location of the genes of the *meta* pathway operon

The locations of *xyl* genes on the inserts were determined by hybridizations of Southern blots of restriction digests of plasmid pWW102-3 and three plasmids subcloned from pWW102-6 (pWW102-61, pWW102-62 and pWW102-63, carrying a 4.0 kb *Bam*HI–*Sac*I, a 3.1 kb

*Sac*I–*Sma*I and a 3.6 kb *Sma*I fragment, respectively) (Table 1). Three sources of *meta* pathway genes, all from *Pseudomonas* catabolic plasmids, were used to prepare gene-specific probes: (1) cloned fragments of the *meta* pathway genes of the phenol catabolic plasmid pVI150 carrying parts or complete genes from *dmpB* (for catechol 2,3-dioxygenase) downstream (Shingler *et al.*, 1992); (2) for the genes for conversion of benzoate to catechol, fragments carrying *bph* genes from the biphenyl catabolic plasmid pWW110 (Carrington *et al.*, 1994); (3) a 0.7 kb PCR-generated fragment of *xylH* genes from the archetypal TOL plasmid pWW0. The hybridization results showed that the gene order of the *meta* pathway of pWW102 is *xylXYZLTEGFQKIJH* (Fig. 2), identical to the lower pathway operon on TOL plasmid pWW0 (Harayama & Rekik, 1990, 1993).

Location of regulator genes *xylS* and *xylR*

Using probes prepared from *xylS* and *xylR* from TOL plasmids pWW0 and pWW53 (Table 2), the homologues from pWW102 were located between coordinates 13.0 and 15.2 kb and between 15.2 and 16.6 kb, respectively (Fig. 2).

Linkage between two catabolic operons

Cross-hybridization of Southern blots of pWW102-6, containing a *Bam*HI insert, and pWW102-7, containing a *Hind*III insert, showed that they had in common a 1 kb *Hind*III–*Bam*HI fragment at their termini (data not shown). The two fragments are therefore adjacent and overlapping and, together with the other overlapping fragments, span a contiguous 28 kb of pWW102 DNA (Fig. 2).

Nucleotide sequence of the *xylQ* gene

From the hybridization results, *xylQ* (for ADA) was shown to be located between the *Kpn*I and *Sal*I sites at coordinates 10.0 and 12.2 kb (Fig. 2) and on the subclone pWW102-62 (Table 1). Three subclones of this region were made carrying as inserts a 2.2 kb *Kpn*I–*Sma*I fragment (pWW102-622), a 2.1 kb *Eco*RI–*Sma*I fragment (pWW102-623) and a 1 kb *Sal*I–*Sal*I fragment (pWW102-624) (Table 1). The nucleotide sequence on both strands of 1611 bp was determined from these plasmids. The ORF of *xylQ* (921 bp) encodes a protein with a predicted molecular mass of 33 kDa. Upstream of *xylQ* is the 3'-end of an ORF of 82 codons, giving 25 amino acids which showed 96.4% homology with the C-terminus of NahI from pWW60-22 (Platt *et al.*, 1995), and is therefore identified as *xylJ*, the gene for 2-oxopent-4-enoate hydratase. Downstream of *xylQ* is the 5'-end of an ORF of 140 codons giving a protein sequence of 134 residues which is 75.2% identical with the N-terminus of NahM, and is thereby identified as *xylH*, the gene for 4-hydroxy-2-oxovalerate aldolase.

Enzyme assay and electrophoretic analysis of expressed ADA proteins

The 3.1 kb *Sac*I–*Sma*I insert of pWW102-62 was excised and re-ligated into expression vector pTrc99a to form plasmid pWW102-62E. After induction with IPTG, both *E. coli* JM109(pWW102-62) and *E. coli* JM109-(pWW102-62E) expressed high ADA activities [400–500 mU (mg protein)⁻¹] compared with induced cells of *P. putida* O₂C₂, where the activity was about 12 mU (mg protein)⁻¹. Extracts were also submitted to SDS-PAGE and showed the presence of a strongly expressed protein with a molecular mass of 35 kDa, in fair agreement with that predicted from the deduced amino acid sequence of the corresponding ADA protein (data not presented).

DISCUSSION

The large plasmid pWW102 of *P. putida* strain O₂C₂ appears to confer the phenotypic properties of other TOL plasmids. It too is counterselected by growth on benzoate minimal medium (Williams & Murray, 1974; Williams & Worsey, 1976), and the plasmid is totally lost after just one cycle of 20 generations. High frequencies of loss have been noted before but those plasmids which exhibited it appeared to segregate deletion mutants in which some of the TOL-encoded phenotypes were retained (Pickup & Williams, 1982;

Keil & Williams, 1985; Osborne *et al.*, 1988; O'Donnell & Williams, 1991). pWW102 is the first example where the high frequency of loss is associated with complete loss of the plasmid.

The organization of the *xyl* operons on pWW102 is different from any described before. However, within each of the two catabolic operons and the two adjacent but divergently expressed regulatory genes, we can detect no difference from any of the previously reported plasmids. Given that (a) our analysis of gene location has a degree of imprecision due to the location of restriction sites available, (b) we did not use gene-specific probes for all 24 of the genes (probes for *xylY,Z,L,N* were absent) and (c) we did not determine the directions of transcription, there is nothing which would make us doubt that the internal operonic organization differed from that in pWW0. The organization of the operons is novel and, compared with the archetype pWW0, the two catabolic operons are reversed in order, *xylUWCMABN* being located downstream of *xylXYZLTEGFJQKIH*. pWW102 does bear considerable similarity to pWW53 in that all its *xyl* genes are in the same orientation and proximity as are the pWW53 *meta* pathway operon 1, the regulatory pair *xylSR* and the single upper pathway operon. We have acquired no evidence during this study of pWW102 to suggest that pWW102 has any of the *xyl* gene duplications found on pWW53 (Osborne *et al.*, 1988) or on pWW15 (O'Donnell & Williams, 1991).

Sequencing of *xylQ* confirmed that the gene encoding ADA is within the *meta* pathway operon of pWW102 in the place indicated by DNA–DNA hybridization. On either side of *xylQ* are partial reading frames confirming the gene order is *xylJQK*, just as found on the TOL plasmid pWW0 (Harayama & Rekik, 1990). This group of three genes is found in the same order in a number of pathways which involve extradiol cleavage of the aromatic ring. In the *xyl meta* pathway operon of TOL plasmids, the *dmp* operon of phenol catabolic plasmid pVI150 (Shingler *et al.*, 1992) and the second *nah* operon of naphthalene catabolic plasmid pWW60-22 (Platt *et al.*, 1995) it is located towards the end of a conserved gene cluster (homologous to *xylTEGFJQKIH*) encoding the conversion of catechol to central metabolites: in each case, however, the first genes of the operon differ and encode the conversion of a chemically stable compound (benzoate, phenol and salicylate, respectively) to catechol. The same group of homologues of these three genes is also found in the same order (a) as the terminal three genes in the adjacent cumate (*cmtFHG*) (Eaton, 1996) and toluene (*todGIH*) (Lau *et al.*, 1994) operons of *P. putida* F1, (b) in several biphenyl (*bph*) 'upper' operons, where they are involved in the catabolism of the 2-hydroxypenta-2,4-dienoate formed as the product after cleavage of the first ring of the biphenyl (Kikuchi *et al.*, 1994; Hofer *et al.*, 1994; Kimura *et al.*, 1997; GenBank accession no. D85853) and (c) in the pathway for cumene (isopropylbenzene) catabolism (*cumFGH*) (Habe *et al.*, 1996). In all these latter examples the three genes are found with adjacent

Fig. 3. Comparisons of amino acid sequences of ADA proteins from *Pseudomonas* catabolic pathways. The sequences were obtained from GenBank with accession numbers: XylQ (plasmid pWW102), this work; NahO, U13232; CumG, D63377; BphJ (strain KF707), D85853; BphJ (strain LB400), X76500; CmtH, U24215; BphG (strain KKS102), D16407; TodI, U09250; XylQ (plasmid pDK1), R. J. Benjamin, personal communication; XylQ (plasmid pWW0), M94186; DmpF, X60835. (a) Alignments produced by CLUSTAL W 1.7 (Thompson *et al.*, 1994), using the BLOSUM series of amino acid weight matrices with gap penalty 10 and gap extension penalty 0.1. Residues conserved in all sequences are marked with an asterisk. (b) Dendrogram drawn from the CLUSTAL alignment using the built in neighbour-joining function (Saitou & Nei, 1987). The numerical values are the bootstrap values with 1000 trials. The scale represents percentage divergence.

genes either unrelated or only distantly related to those which surround them in the *xyl*, *nah* and *dmp* operons.

A model for the evolution of the *Pseudomonas meta* pathway operons which has been proposed involves the recombination of pre-evolved genetic modules to give

the gene arrangements seen in modern strains (Cane & Williams, 1986; Harayama *et al.*, 1987; Assinder & Williams, 1988; van der Meer *et al.*, 1992; Williams & Sayers, 1994). In the case of the *xyl meta* pathway operon, we can see evidence for three such 'modules'. The first and largest is the homologous sequence

equivalent to *xylT*–*xylH* found in the *meta* pathway operons for catabolism of toluene/xylene (Harayama & Rekik, 1993; Keil *et al.*, 1985), phenols (Shingler *et al.*, 1992), salicylate (and therefore naphthalene) (Assinder & Williams, 1988) and biphenyl (Carrington *et al.*, 1994). The second is located within these operons as the 'submodule' equivalent of *xylJQK* found also in other catabolic operons (see above). The third is upstream within the same operon, the *xylXYZL* genes for conversion of benzoate to catechol, homologues of which turn up as *benABCD* in the chromosomal β -ketoadipate pathway in both *Acinetobacter calcoaceticus* and *P. putida* (Neidle *et al.*, 1991, 1992).

If all *xyl meta* pathway operons, for example, share a common ancestor which arose by a modular assembly, then it would be expected that the genes and proteins found in all modern *xyl* pathways would be more closely related to each other than to those in any other pathway, such as a *nah* pathway, which would have arisen by an independent assembly and subsequently divergently radiated. However, alignment of the amino acid sequences of *Pseudomonas* ADA proteins (Fig. 3) shows this expectation not to be the case. Thus XylQ of pWW102 is more closely related to NahO of pWW60-22 than to the other XylQ proteins of TOL plasmids pWW0 and pDK1. Other comparisons of genes within the *Pseudomonas meta* pathway operons have shown a similar result.

In a detailed comparison of the nucleotide sequences of the *meta* pathways of pWW0, pVI150 and NAH7, Harayama & Rekik (1993) showed that the 5'-end of the 'catechol module' on pWW0 was more homologous to the isofunctional *nah* genes of NAH7, whereas its 3'-end was closer to the *dmp* genes of pVI150. They proposed that the *xyl* operon was the result of recombination between ancestral *nah* and *dmp* operons. Analysis of the nucleotide sequence of the *xylE* gene on TOL plasmid pDK1 showed that it had an apparent discontinuity in its homology and that its 5'-end was more homologous to *nahH* from NAH7 whereas the 3'-end was closer to *xylE* of TOL plasmid pWW0 (Benjamin *et al.*, 1991). Similarly the *xylS* regulatory gene on TOL plasmid pDK1 is also clearly a chimera of two different *xylS* homologues, one of each of which is found on another TOL plasmid, pWW53 (Assinder *et al.*, 1993). These results suggest that recombination between two sequences, not necessarily encoding the catabolism of the same original substrate, may give rise to hybrid or mosaic patterns within individual genes or within operons. Such an event has been recorded between the duplicate *meta* pathway operons of TOL plasmid pWW53 (Osborne *et al.*, 1988).

The ADA proteins fall into two clearly differentiated clusters (Fig. 3). This is very apparent in some areas where there are strong identities within the two sub-clusters but not between them. These are (a) residues 257–274, where all the proteins in the cluster containing XylQ from pWW102 contain the same deletion of 18 residues, and (b) three locations where the two clusters differ by identical small deletions: at positions 29, 98–99

and 129–130 in the alignment of Fig. 3(a). Exactly the same pattern of clustering is found when the protein sequences of the upstream genes (XylJ homologues) and of the downstream genes (XylK homologues) are compared (data not shown), which shows that in all these strains the *xylJQK* homologues have the same pattern of ancestry. However, there are no clear lineages within any of this cluster of three genes which directly correlate with their metabolic function (Fig. 3b). Thus the pWW102 ADA is closest to the homologous sequence on the naphthalene plasmid pWW60-22 but is in the opposite subcluster to the two TOL plasmid proteins from pWW0 and pDK1. In a parallel manner, two of the *bph* ADA proteins are close to the pWW0 XylQ, whereas another *bph* protein and TodI, the genes for which are very similar in arrangement to *bph* genes, are in the other subcluster and closely related to the pWW102 protein. There are two possible explanations: either the gene organizations we see conserved in these pathways have evolved separately on more than one occasion or, more likely in our opinion, recombination between homologous genes in these operons has resulted in mixing of the sequences between different metabolic pathways in a manner equivalent to gene conversion, thus obscuring their origin.

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