Physical and Functional Mapping of Two Cointegrate Plasmids Derived from RP4 and TOL Plasmid pDK1

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Cointegrate plasmids were formed *in vivo* between the broad-host-range R-plasmid RP4 and two catabolic plasmids derived from *Pseudomonas putida* HS1. One of these was the wild-type plasmid pDK1 encoding the complete inducible toluene/xylene (TOL) catabolic pathway and one was pDKT1, a deletion derivative of pDK1 selected after growth of HS1 on benzoate and supporting growth on only toluene. The two plasmids formed, pDK2 and pDKT2 respectively, each consisted of a complete RP4 replicon in which was an insert of the parent plasmid DNA respectively 40 and 20 kbp in size. The detailed restriction maps of the two plasmids were determined and many of the catabolic genes were located by subcloning and enzyme assay of recombinant plasmids in *Escherichia coli* and *Pseudomonas* hosts. The insert in pDK2 contained both operons of the catabolic pathway, the 'upper pathway' operon (*xy/CAB*) and the *meta* pathway operon (*xy/DLEGF(I,J,K)H*), and a region identified as having the function of the regulator gene *xy/S*. The insert in pDKT2 contained only the upper pathway operon and the regulatory region. Within each of the three coding regions there was great similarity with the same regions on TOL plasmids pWW0 and pWW53-4 apparent (a) by the same order of the genes, (b) by a similar pattern of restriction sites and (c) by hybridization studies. However, the order and orientations of the three coding regions differed from those previously described for both pWW0 and pWW53-4. The significance of these findings to the evolution of TOL plasmids is discussed.

INTRODUCTION

Strains of *Pseudomonas* isolated from natural environments with the ability to catabolize toluene and methyl-substituted toluenes (xylenes) via benzoate and methylbenzoates (toluates) and the *meta*-cleavage pathway almost always have the catabolic pathway encoded on a plasmid. Many such plasmids have been described (Williams & Worsey, 1976; Yano & Nishi, 1980; Kunz & Chapman, 1981) and have been designated as TOL plasmids, the archetype and best studied of which is pWW0, originally found in *P. putida* mt-2 (Nakazawa & Yokota, 1973; Williams & Murray, 1974).

P. putida HS1 (also called PpC1) was isolated by Kunz & Chapman (1981). During growth on benzoate HS1 spontaneously segregated a unique set of plasmid deletion mutants: one of these, PpCT1, retained the ability to grow on toluene but no longer supported growth on the substituted toluenes and metabolized the benzoate, formed from toluene by the plasmid-determined enzymes, via the chromosomal β -ketoadipate (or *ortho* cleavage) pathway (Kunz & Chapman, 1981).

In this paper we describe the *in vivo* construction of two cointegrate plasmids formed between the broad-host-range R-plasmid RP4 and both pDK1 and pDKT1, the plasmids in wild type *P.*

Abbreviations: BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; C23O, catechol 2,3-oxygenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; 4OT, 4-oxalocrotonate tautomerase.

putida HS1 and its segregant PpCT1 respectively. The two plasmids, designated pDK2 and pDKT2, have been mapped and the DNA derived from the catabolic plasmids has been extensively studied by cloning, subcloning and Southern blotting to locate the majority of the toluene/xylene pathway genes. The resulting physical map shows some major similarities to and significant differences from the isofunctional coding regions on TOL plasmids pWW0 and pWW53-4 (Keil *et al.*, 1987*a*).

METHODS

Bacterial strains and plasmids. The *Escherichia coli* and *Pseudomonas putida* strains and the plasmids used or constructed are listed in Table 1.

Media and culture conditions. Bacteria were grown and maintained as described by Worsey & Williams (1975), Keil *et al.* (1985*a*) and Keil *et al.* (1985*b*). *P. putida* strains carrying the cointegrate plasmids were maintained on minimal agar plates with *m*-toluate (pDK1) or toluene (pDKT1) as carbon sources with added kanamycin (100 µg ml⁻¹). Carbenicillin, donated by Beechams, was added where appropriate at 2 mg ml⁻¹.

Formation of pDK2 and pDKT2. The RP4::pDK1 plasmid pDK2 was constructed by transferring RP4 from *P. putida* AC34(RP4) into HS1 by conjugation on filters. One Cb^rTc^rKm^rMtol⁺ transconjugant, shown to contain both RP4 and pDK1, was then used as a donor in a mating on filters with PaW340 as recipient: selection was made for transconjugants able to grow on *m*-toluate in the presence of streptomycin at 1 mg ml⁻¹ (to select for PaW340 and to counterselect against HS1). All transconjugants examined contained single plasmids; PaW630, which was able to grow on both *m*-xylene and *m*-toluate and contained the smallest of the plasmids examined (pDK2), was used in this study. PaW637, containing the plasmid pDKT2, was formed from strain PpCT1 by essentially the same protocol but using selection for growth on toluene in place of *m*-toluate.

Enzyme assays. Enzymes were assayed in cell-free extracts using the same procedures as Sala-Trepat & Evans (1971) and Worsey & Williams (1975).

Plasmid and DNA manipulations. Plasmids pDK1, pDKT1, pDK2 and pDKT2 were extracted from *Pseudomonas* hosts according to Wheatcroft & Williams (1981). pDK2, pDKT2, vector plasmids and recombinant plasmids were isolated from *E. coli* hosts by CsCl/ethidium bromide gradient centrifugation. Hydrolyses with restriction endonucleases, DNA ligations and transformation of *E. coli* with plasmid DNA were done by standard procedures (Maniatis *et al.*, 1982). Both *E. coli* and *P. putida* strains were screened for the presence of small plasmids according to Holmes & Quigley (1981). DNA-DNA hybridizations were performed as described by Keil *et al.* (1987*a*) after Southern (1975).

Mobilization of recombinant plasmids into *P. putida*. Recombinant plasmids with inserts in the broad-host-range vector pKT230 were mobilized from *E. coli* into *P. putida* using the unstable RP4 derivative plasmid pNJ5000 as described by Keil *et al.* (1985*b*).

RESULTS

Characterization of cointegrate plasmids pDK2 and pDKT2

Table 2 shows the sizes of DNA fragments obtained by digestion of pDK1, pDKT1, pDK2 and pDKT2 with restriction endonuclease *Hind*III: similar data have been obtained for enzymes *Xho*I, *Eco*RI and *Bam*HI but are not presented here. Analysis of the results showed that pDK2 contained 7 *Hind*III fragments, 15 *Xho*I fragments, 7 *Eco*RI fragments and 5 *Bam*HI fragments identical in size to ones found in the parent pDK1: it also contained novel fragments, not found in pDK1 or RP4, carrying the junctions between RP4 and the pDK1 insertion. The digests of pDKT2 also contained fragments identical in size to those from its parent pDKT1: *Hind*III (4), *Xho*I (5), *Eco*RI (1) and *Bam*HI (2). Most of these were also identical in size to fragments in pDK1. Again novel fragments were found which were not present in either pDKT1 or RP4.

Complete restriction maps for both cointegrate plasmids were constructed using the following methods: (1) single, double and triple digestions with the four endonucleases referred to above; (2) cloning all the *Hind*III fragments, many of the *Xho*I fragments and most of the *Bam*HI fragments; (3) detailed restriction mapping of the cloned DNA using a larger bank of enzymes; and (4) cross-hybridization between cloned DNA by Southern blotting.

The map of pDK2 is shown in Fig. 1, in which the fragments are lettered alphabetically according to their position in the corresponding digests of pDK1. The map shows that about 40 kbp of pDK1 DNA has been inserted into the 13.3 kbp *Kpn*I fragment of RP4, between

Table 1. Bacterial strains and plasmids

Strain	Plasmid	Notes and references
<i>Escherichia coli</i> strains		
CA60		<i>hsdR hsdM recBC</i> derivative of C600
C600	pNJ5000	Grinter (1983)
C600	pKT230	Bagdasarian <i>et al.</i> (1981)
C600	pBR325	Prentki & Kirsch (1982)
ED8654		<i>hsdR hsdM⁺ trpR met⁻</i>
<i>Pseudomonas putida</i> strains		
HS1	pDK1	Kunz & Chapman (1981)
PpCT1	pDKT1	Kunz & Chapman (1981)
AC34	RP4	Ade ⁻ Cb ⁺ Km ⁺ Tc ^r (gift of C. J. Duggleby)
PaW95		Plasmid-free mutant in chromosomal benzoate <i>cis</i> -diol dehydrogenase (Keil <i>et al.</i> , 1985 <i>b</i>)
PaW130		Rif ^r plasmid-free derivative of <i>P. putida</i> mt-2 (PaW1) (Keil <i>et al.</i> , 1985 <i>a</i>)
PaW263	pWW0- <i>xyIA</i>	Franklin & Williams (1980)
PaW340		Trp ⁻ Str ^r plasmid-free derivative of PaW1 (Jeenes & Williams, 1982)
PaW630	pDK2	RP4::pDK1 plasmid in PaW340 (this study)
PaW637	pDKT2	RP4::pDKT1 plasmid in PaW340 (this study)
PaW823	pWW0-660	pWW0 plasmid with <i>xyICAB</i> deletion (Lebens & Williams, 1985)
Recombinant plasmids		
	pDK1-3014	2.1 kbp <i>Xho</i> I fragment X of pDK1 in pKT230
	pDK1-3042	4.4 kbp <i>Xho</i> I fragment I of pDK1 in pKT230
	pDK1-3059	7.6 kbp <i>Hind</i> III fragment G of pDK1 in pKT230
	pDK1-3060	6.1 kbp <i>Sma</i> I/ <i>Hind</i> III part of pDK1-3059 generated by deletion of 1.9 kbp <i>Sma</i> I fragment (one site on vector)
	pDK2-3064	3.9 kbp <i>Bam</i> HI fragment D of pDK2 in pBR325
	pDK2-3067	15.5 kbp <i>Hind</i> III fragment A of pDK2 in pBR325
	pDK2-3085	Internal 4.6 kbp <i>Sma</i> I subfragment of pDK2-3067 in pKT230
	pDK2-3087	15.5 kbp <i>Hind</i> III insert of pDK2-3067 recloned into pKT230
	pDK2-3090	6.8 kbp <i>Bam</i> HI fragment C of pDK2 in pBR325
	pDK2-3091	<i>Bam</i> HI insert of pDK2-3064 recloned into the unique (vector) <i>Bam</i> HI site of pDK2-3087
	pDK2-3092	Internal 2.6 kbp <i>Xho</i> I fragment of pDK2-3090 in pKT230
	pDK2-3095	Internal 600 bp <i>Bgl</i> II fragment deleted from pDK2-3064
	pDK2-3098	Internal 3.0 kbp <i>Sst</i> I fragment of pDK2-3087 in pKT230
	pDK2-3100	The deleted <i>Bam</i> HI insert of pDK2-3095 cloned into the unique (vector) site of pDK2-3087
	pDK2-3107	Internal 2.8 kbp <i>Sma</i> I fragment of pDK2-3087 in pKT230
	pDK1-3109	2.9 kbp <i>Xho</i> I fragment R of pDK1 in pKT230
	pDK1-3110	Internal 4.1 kbp <i>Pst</i> I fragment of pDK1-3059 in pBR325
	pDK2-3111	3.7 kbp <i>Hind</i> III fragment from pDK2-3090 in pKT230 (contains 300 bp of pBR325)
	pDK2-3113	Internal 4.3 kbp <i>Sst</i> I fragment of pDK2-3067 in pKT230
	pDK2-3114	Internal 3.8 kbp <i>Sst</i> I fragment of pDK2-3090 in pKT230
	pDK1-3117	2.7 kbp <i>Sma</i> I/ <i>Bam</i> HI part of pDK1-3060 generated by deletion of internal 4.3 kbp <i>Bam</i> HI fragment (one site on vector)
	pDK1-3119	9.5 kbp <i>Sma</i> I/ <i>Bam</i> HI fragment formed when <i>Bam</i> HI fragment C is cloned into unique <i>Bam</i> HI site of pDK1-3117

coordinates 11 and 14 as measured clockwise from its single *Eco*RI site (Lanka *et al.*, 1983): as far as can be ascertained there has been no deletion of RP4 DNA in the cointegration.

The map of pDKT2 is shown in Fig. 2. The fragments are labelled according to the same convention as for pDK2, apart from *Hind*III-Kn and *Xho*I-Dn, which are both novel fragments found in pDKT1 but not in pDK1. It is presumed that these are fragments formed originally in the deletion event which gave rise to the formation of pDKT1 from pDK1 during the benzoate selection (Kunz & Chapman, 1981). In pDKT2 about 20 kbp of pDKT1 DNA has been incorporated into the apparently complete RP4 replicon, but the site of insertion in this case is in the 1.8 kbp *Kpn*I fragment of RP4 at about coordinate 38 from its *Eco*RI site.

A more detailed restriction map of the two TOL plasmid inserts in the cointegrates is shown in Fig. 3, together with the major subclones used in the determination of the catabolic gene locations.

Fig. 1. Restriction map of pDK2. The fragments are labelled with letters corresponding to their positions in the digests of pDK1 (as Table 2). nf refers to novel fragments containing DNA from both pDK1 and RP4. The shaded area represents the complete RP4 replicon. The scale is in kbp.

Fig. 2. Restriction map of pDKT2. The fragments are labelled with letters corresponding to their positions in the digests of pDKT1 (as Table 2). nf refers to novel fragments containing DNA from both RP4 and pDKT1. The shaded area represents the complete RP4 replicon. The scale is in kbp.

Location of genes of xylCAB operon

The results from assaying the enzymes of the 'upper pathway' encoded by recombinant plasmids carrying cloned genes are shown in Table 3.

Benzaldehyde dehydrogenase (BZDH, *xylC*) can be detected in strains of *E. coli* containing plasmids pDK1-3042, pDK1-3059, pDK1-3060 and pDK1-3110, but not in strains carrying pDK1-3117 or pDK2-3090, and can therefore be located between coordinates 6 and 9.4.

Table 2. Sizes of DNA fragments obtained by digestion of pDK1, pDK2, pDKT1 and pDKT2 with restriction endonuclease HindIII

pDK1		pDK2		pDKT1		pDKT2	
Fragment	Size (kbp)	Fragment	Size (kbp)	Fragment	Size (kbp)	Fragment	Size (kbp)
						RP4n1*	> 60
		RP4n2*	≈ 38				
		RP4n3*	≈ 28				
A	15.5	A	15.5				
B	14.5			B	14.5		
C	12.6						
D	9.8			D	9.8		
E	9.8			E	9.8		
F	9.1			F	9.1		
G	7.6	G	7.6	G	7.6	G	7.6
H	7.6	H	7.6				
I	6.8			I	6.8		
J	6.0						
K	5.5			Kn†	5.1	Kn†	5.1
				Ln†	5.1		
L	3.9					RP4n4*	3.85
M	3.3						
N	2.8						
O	2.1						
P	1.9						
Q	1.7						
R	1.5						
S	1.3	S	1.3				
T	1.25	T	1.25	T	1.25	T	1.25
U	1.1	U	1.1				
V‡	0.55	V‡	0.55	V‡	0.55	V‡	0.55

* Novel fragments containing both RP4 and TOL plasmid DNA.

† Novel fragments found in pDKT1 but not in pDK1.

‡ Fragments visualized only after subcloning plasmids.

Benzy alcohol dehydrogenase (BADH, *xyiB*) activity is expressed from pDK2-3090, pDK2-3092 and pDK2-3114 but not from pDK1-3059, and can thus be placed between coordinates 3.4 and 5.5.

Xylene oxygenase (*xyiA*), which cannot be assayed in cell extracts, was located by complementation of PaW263, a mutant of PaW1 (*P. putida* mt-2) with a nonfunctional *xyiA* but with normal activities of the subsequent enzymes of the TOL pathway (Franklin & Williams, 1980). When mobilized into PaW263, pDK1-3059, pDK1-3060 and pDK1-3111 complemented it for growth on *m*-xylene, enabling the gene to be positioned between coordinates 4.7 and 8.1.

The location of the operator-promoter for the operon (OP1) was first found by Southern blotting using cloned DNA from the OP1 region of pWW0 as a probe. Confirmation of the role of this region as a functional necessity for inducible expression of the operon was obtained in PaW823. This strain contains pWW0-660, a deletion derivative of pWW0, which lacks the complete *xyiCAB* operon but which has both regulatory genes *xyiS* and *xyiR* intact (Lebens & Williams, 1985): as a result it will not grow on *m*-xylene. When pDK1-3059 and pDK1-3060 were mobilized into PaW823, BZDH activity was inducible by *m*-xylene but no inducible activity was found when pDK1-3042 was used. OP1 is therefore located between coordinates 10.3 and 10.8. The presence of pDK1-3059 and pDK1-3060 in PaW823 enables it to grow on *m*-xylene, but only very poorly due to the incomplete *xyiB* gene on the two recombinants, which is only poorly compensated for by the low isofunctional BADH activity that is chromosomally encoded. However, the construction of the subclone pDK1-3119, containing the complete

Fig. 3. Detailed physical maps showing the locations of restriction sites and catabolic genes on the complete 20 kbp insert of pDKT1 DNA in the cointegrate plasmid pDKT2 and on the 40 kbp insert of pDK1 DNA on the cointegrate plasmid pDK2. The various subclones constructed for this study are shown below the maps. The small arrows show the direction of transcription from the vector promoters in the recombinant plasmids carrying the cloned fragments. The abbreviations for restriction enzyme sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. OP1 and OP2 are the operator-promoter regions at the start of each operon; the locations of OP2 and *xyI*R are only tentatively located on the basis of the similarity in restriction sites and homology to the OP2 and *xyI*R regions of pWW53-4 and pWW0 (Keil *et al.*, 1985b). The right-hand column indicates the genes which have been definitely located on the corresponding subclones. The vertical dotted lines indicate the area where pDK2 and pDKT2 diverge, presumably as a result of the deletion which gave rise to the formation of pDKT1 from pDK1.

Table 3. *Specific activities of the enzymes of the 'upper pathway' operon in P. putida and E. coli*Enzyme activities are expressed as milliunits (mg protein)⁻¹.

Plasmid	Host*	Inducer	BADH	BZDH	<i>xylA</i> complementation†
None	<i>E. coli</i>	None	3	<1	ND
pDK1	HS1	<i>m</i> -Xylene	322	39	ND
		None	13	4	ND
pDK2	PaW630	<i>m</i> -Xylene	127	33	ND
		None	13	5	ND
pDKT1	PpCT1	Toluene	460	85	ND
		None	17	4	ND
pDKT2	PaW637	Toluene	138	92	ND
		None	23	5	ND
pWW0-660	<i>P. putida</i>	<i>m</i> -Xylene	15	9	ND
		None	13	7	ND
pDK1-3042	<i>E. coli</i>	None	4	13	ND
	<i>P. putida</i>	<i>m</i> -Xylene	19	10	—
		None	12	6	ND
pDK1-3059	<i>E. coli</i>	None	4	9	ND
	<i>P. putida</i>	<i>m</i> -Xylene	10	125	+
		None	14	18	ND
pDK1-3060	<i>E. coli</i>	None	4	16	ND
	<i>P. putida</i>	<i>m</i> -Xylene	11	144	+
		None	11	9	ND
pDK2-3090	<i>E. coli</i>	None	450	<1	ND
pDK2-3092	<i>E. coli</i>	None	644	<1	ND
pDK1-3110	<i>E. coli</i>	None	4	11	ND
pDK1-3111	<i>E. coli</i>	None	4	<1	ND
	<i>P. putida</i>	<i>m</i> -Xylene	14	8	+
		None	13	6	ND
pDK2-3114	<i>E. coli</i>	None	190	<1	ND
pDK1-3117	<i>E. coli</i>	None	4	<1	ND
pDK2-3119	<i>E. coli</i>	None	246	15	ND
	<i>P. putida</i>	<i>m</i> -Xylene	443	132	ND
		None	47	10	ND

ND, Not determined.

* *E. coli* C600 or *P. putida* PaW823 unless indicated.† Determined by acquisition by PaW263 of ability to grow on *m*-xylene.

xylCAB operon together with OP1, and its mobilization into PaW823 produced a strain which grew well on *m*-xylene and contained high induced levels of BADH and BZDH (Table 3).

Location of genes of the meta pathway operon

Recombinant plasmid pDK2-3087, with the 15.5 kbp insert of *Hind*III fragment A, conferred the ability to grow on *m*-toluate upon plasmid-free *P. putida*, thus showing that it carried the entire *meta*-cleavage pathway from catechol to pyruvate and acetaldehyde and possibly also from benzoate (or *m*-toluate) to catechol (or 3-methylcatechol), although this latter transformation could be carried out by the isofunctional, chromosomally-coded enzymes.

Table 4 summarizes the results of assays of *meta* pathway enzymes in strains carrying cloned inserts of pDK2 DNA.

Catechol 2,3-oxygenase (C23O, *xylE*) activity was located on a small *Xho*I fragment delineated by coordinates 25 and 26.8 (pDK1-3014). Both 2-hydroxymuconic semialdehyde hydrolase (HMSH, *xylF*) and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD, *xylG*) were located between coordinates 26 and 29 on plasmid pDK2-3098. However, only HMSD activity was expressed from the recombinant with the *Sma*I insert, pDK2-3107, showing that *xylF* is downstream of *xylG* and extends past coordinate 28.6.

4-Oxalocrotonate tautomerase (4OT, *xylH*) activity was detected in strains carrying pDK1-3109, which has an *Xho*I insert which maps between coordinates 32.2 and 35.2.

Table 4. *Specific activities of the enzymes of the meta pathway operon in strains of E. coli and P. putida*Enzyme activities are expressed as milliunits (mg protein)⁻¹.

Plasmid	Host*	Inducer	C23O	HMSD	HMSH	4OT
None	<i>E. coli</i>	None	<0.1	<0.1	<0.1	<100
pDK1	HS1	<i>m</i> -Toluuate	160	39	57	110000
		None	1.3	0.9	0.8	400
pDK2	PaW630	<i>m</i> -Toluuate	125	25	39	25700
		None	1.2	0.3	0.5	ND
pDK1-3014	<i>E. coli</i>	None	40	<0.1	<0.1	ND
	<i>P. putida</i>	None	5	<0.1	<0.1	ND
pDK2-3087	<i>E. coli</i>	None	2	0.5	0.8	ND
	<i>P. putida</i>	<i>m</i> -Toluuate	56	24	12	ND
		None	21	11	7	ND
pDK2-3091	<i>E. coli</i>	None	1	0.5	1.4	ND
	<i>P. putida</i>	<i>m</i> -Toluuate	238	98	106	143000
		None	28	11	10	7000
pDK2-3098	<i>E. coli</i>	None	<0.1	1.3	2.5	ND
	<i>P. putida</i>	None	<0.1	1.5	1.2	ND
pDK2-3100	<i>E. coli</i>	None	2	0.5	0.1	ND
	<i>P. putida</i>	<i>m</i> -Toluuate	57	25	21	ND
		None	25	12	12	ND
pDK2-3107	<i>E. coli</i>	None	<0.1	3.5	<0.1	ND
pDK1-3109	<i>E. coli</i>	None	ND	ND	ND	2300
	<i>P. putida</i>	None	ND	ND	ND	7300

ND, Not determined.

* *E. coli* CA60 or ED8654 or *P. putida* PaW130 unless indicated.

The first activities of the operon, benzoate oxygenase (*xyiD*) and benzoate *cis*-diol dehydrogenase (*xyiL*), which together convert benzoate to catechol, were shown to reside on *Hind*III-A and were more precisely positioned as follows. *E. coli* containing either pDK2-3085 or pDK2-3113, plasmids with overlapping inserts (Fig. 3) were streaked onto Isosensitest agar (Oxoid) containing 5 mM-*m*-toluuate. In the case of only the former plasmid, the colonies and surrounding agar developed a dark brown colour due to accumulation of 3-methylcatechol and its subsequent autoxidation; it follows that both *xyiD* and *xyiL* are between the *Sma*I sites at coordinates 21.1 and 25.8. The presence or absence of *xyiL* was tested by mobilizing both plasmids separately into PaW95, a plasmid-free mutant of PaW1 which has a mutation in the chromosomal benzoate *cis*-diol dehydrogenase but has a functional chromosomal benzoate oxygenase and does not grow on benzoate. Both pDK2-3085 and pDK2-3113 restored the ability of PaW95 to grow on benzoate, showing the presence of *xyiL* on both. The order of the two genes in this area of the plasmid must be *xyiDL*.

Identification of regulatory gene *xyiS*

As can be seen in Fig. 3, the point at which the TOL insert in pDKT2 diverges from that of pDK2 is beyond the two *Bgl*II sites at 13.8 and 14.4 respectively but before the *Xho*I site in pDK2 at 15.1. Two similar adjacent *Bgl*II sites occur in the *xyiSR* region of both pWW0 and pWW53-4 (Keil *et al.*, 1985b). A Southern blot, using as the probe a small subclone of pWW0 DNA internal to its *xyiS* gene (a gift of F. C. H. Franklin) hybridized to the *Bam*HI subclone pDK2-3064 as well as to the *Hind*III-Kn fragment of pDKT2. A construction was made by recloning the *Bam*HI insert of pDK2-3064 into the unique *Bam*HI site which is in the vector part of plasmid pDK2-3087, forming plasmid pDK2-3091. A second such construct was made by first deleting the 600 kbp between the two *Bgl*II sites in the insert of pDK2-3064 thus forming pDK2-3095: the shortened insert was then cut out with *Bam*HI and religated into the same single *Bam*HI site in pDK2-3087 to form pDK2-3100: the rationale for making this deletion is that if the two *Bgl*II sites are in the same positions as in the *xyiSR* region of pWW0 then its

deletion removes the complete intergenic region together with part of each coding region for the two regulatory proteins, thus inactivating them (Spooner *et al.*, 1986).

All three plasmids, pDK2-3087, carrying the start of the *meta* pathway operon, pDK2-3091, with the additional insert of putative *xyIS* DNA, and pDK2-3100, with the possible deletion in the putative regulatory genes, were mobilized into plasmid-free PaW130 and assays were done to detect induction of C23O, HMSH and HMSD in the presence and absence of *m*-toluate, the inducer which acts in combination with the *xyIS* gene product (Worsey *et al.*, 1978; Inouye *et al.*, 1981). There was an induction by *m*-toluate of 8- to 10-fold in the case of pDK2-3091 but not with the other plasmids, showing that there is a *xyIS* gene which spans one or both of the two *Bg*/II sites in pDK2-3064. Subsequent initial studies not presented here have shown that the gene products of *xyIH*, *xyII* and *xyIJ* are also induced by *m*-toluate from pDK2-3091.

It is worth noting that in *P. putida* the recombinant plasmid pDK2-3091, with the complete regulated *meta* pathway operon, supported a noticeably faster rate of growth on *m*-toluate than did pDK2-3087, which did not have the additional *xyIS* insert. This is doubtless due to the higher specific activities of the enzymes expressed (Table 4).

Attempts to locate the regulatory gene xyIR

We have tried to do similar experiments to show that the region around the two *Bg*/II sites also carries a functional *xyIR* gene which in combination with *m*-xylene can initiate transcription of the *xy/CAB* operon from OP1. Attempts to construct a single recombinant plasmid carrying the insert of pDK2-3064 (the putative *xy/SR* region) and that of pDK1-3119 (the complete *xy/CAB* operon) have not been successful. However, Southern blotting has shown that the pDK2-3064 insert displays strong homology with the *xyIR* region of pWW53-4 and enzyme assay demonstrates that toluene and *m*-xylene induce both BADH and BZDH in strains containing pDK2 and pDKT2 (Table 3), showing that a regulator gene must reside somewhere on the TOL DNA inserts of those plasmids. The localization of *xyIR* will require further investigation.

DISCUSSION

Pseudomonas putida HS1 containing the TOL plasmid pDK1 was originally isolated from soil in Minnesota, USA (Kunz & Chapman, 1981), geographically remote from the sources of the other two carefully mapped TOL plasmid-containing strains, *P. putida* mt-2, isolated in Japan in the 1950s and containing the archetype pWW0, and *P. putida* MT53, host to pWW53, isolated in North Wales. Yet the physical organization of the catabolic genes on pDK1 reinforces the features which have already been shown by the comparison between pWW0 and the RP4::pWW53 cointegrate plasmid pWW53-4 (Keil *et al.*, 1987b).

Firstly, the pathway genes on all three plasmids are organized in three separate units, the 'upper pathway' operon *xy/CAB*, the *meta* pathway operon, *xyDLEGF(J,I,K)H* and a regulatory cluster of two genes *xy/SR*. Within each of the three units, the gene order is absolutely conserved, as far as can be ascertained, given that all the *meta* pathway genes have not been completely localized on pWW53-4 or pDK1. In all three plasmids the identity of gene order within the three units is reinforced by a high degree of conservation of restriction sites in what appear to be the same positions. This latter can only be a rough estimate because restriction mapping is obviously not sufficiently accurate to be able to determine whether two sites for the same enzyme on two separate strands of DNA are in identical positions on both. However, there is a remarkable coincidence in the order of the cutting sites for the enzymes used for the three TOL plasmids. Thus within the coding regions for the *meta* pathway operon, 15 of the 29 sites found on pDK2 (and therefore by inference on pDK1) are in apparently the same position on pWW0 and 16 are shared with pWW53. The similarity between the *xy/CAB* operons of pDK1 and pWW53-4 is even more remarkable: all of the 20 restriction sites found on pDK1 are also found on the corresponding region of pWW53, and there is but one additional site on pWW53 not found on pDK1.

The strong homology implied by the same gene order and shared restriction sites is borne out by hybridization, and results not presented here show that within the three units on the three

plasmids there is very strong colinearity in homology: in fact in the early stages of this work we used Southern blotting with already characterized fragments of pWW0 or pWW53 as a means of facilitating our identification of coding regions of pDK1.

The similarity of the genes is complemented by the apparent similarity in the regulation. Not only are both the OP1 region, upstream of the *xyiCAB* operon, and the region identified as *xyiS* highly conserved but there is also homology on pDK1 to parts of pWW53-4 and pWW0 which have been shown to have OP2 and *xyiR* functions on those plasmids, but which we have not yet been able to show have such a function on pDK1. Additionally there is a degree of functional cross-reaction between the regulatory elements on the three plasmids. Thus in this work and in our study of pWW53 (Keil *et al.*, 1987*a*) we have demonstrated that the product of the regulatory gene *xyiR* on pWW0 can cause induction downstream of the OP1 operator-promoter regions on pWW53 and pDK1.

There is therefore considerable evidence for a high degree of conservation of the catabolic genes on these three plasmids in spite of their very diverse geographical origins, but it is also clear that the conservation exists only within the coding regions and there is no evidence from either restriction site analysis or from hybridization studies that the homology continues into the non-catabolic regions of the plasmids.

What is perhaps most surprising about the comparison of the three plasmid-encoded pathways is that the relative positions of the three modules which made up the TOL DNA on each are totally different. If the relevant DNA is oriented with the *xyiCAB* operon transcribed from left to right the three plasmids read as follows, with the arrows denoting the directions of transcription: pWW0 (→*CAB*)(→*DLEGFJKIH*)(*SR*); pWW53-4 (→*DLEGF(J,K)IH*)(*SR*)(→*CAB*); pDK1 (*H(J,K,IFGELD)*)(*S(R?)*)(→*CAB*) (Fig. 4). It is clear therefore that the acquisition of the complete set of TOL genes by the three replicons has not been the straightforward recombinational transfer of a continuous sequence of DNA.

It has been shown that 56 kbp of pWW0 encompassing the entire regulated TOL region can move from its plasmid location into the chromosome (Jeenes & Williams, 1982) and can then recombine with other plasmid replicons, thus forming novel TOL plasmids. This observation is in remarkable coincidence with the finding that *P. putida* MW1000 is the only well-authenticated example of a soil isolate carrying the toluene/xylene catabolic route which has the genes located, not in a plasmid, but on the chromosome (Sinclair *et al.*, 1986) and the evidence from Southern hybridization is that the catabolic DNA of this strain is identical in its digestion by restriction enzymes to that of pWW0. These two findings, taken together with the very strong recent evidence that the TOL genes of pWW0 reside on a 56 kbp transposon designated Tn4561 (Tsuda & Iino, 1987), might suggest that different TOL plasmids arose by the transposition of that 56 kbp element between replicons in much the same way as many of the R-plasmids have evolved by acquisition of recognizable transposable elements such as Tn1.

The differences between the gene organization on pDK1 and pWW53 and that on pWW0 seem to suggest a less simple situation. If they did acquire the TOL genes via a transposon such as Tn4561 found on pWW0, then there has been considerable rearrangement of the operons and the regulatory genes subsequently, and probably a loss of the transposition function, since there is no apparent similarity in the DNA flanking the catabolic region where the transposition genes on pWW0 have been shown to reside (Tsuda & Iino, 1987). An alternative hypothesis might be that the pWW53 and pDK1 replicons had acquired the TOL operons at different times and from different locations. This is a plausible possibility since either operon could exist singly as a functional metabolic unit of selective advantage to its host cell. This is well demonstrated by this study in particular since pDKT1 and pDKT2 are both plasmids formed *in vivo* which carry only the upper pathway operon and confer the ability to convert toluene to benzoate which is then further metabolized by the chromosomal route. The further acquisition by such a plasmid, carrying the upper pathway operon, of a second operon encoding the *meta* pathway sequence would be selectively advantageous to hosts since it would make available the methyl-substituted analogues as growth substrates. It could be argued that the spontaneous dissociation of pDK1 upon benzoate selection (Kunz & Chapman, 1981) is perhaps a reflection of a modular construction in the first place.

Fig. 4. Comparison of the locations of the *xyl* genes on TOL plasmids pDK1, pWW0 and pWW53-4. Transcription of the operons and genes is indicated by the arrows. The positions of the genes on pWW0 are taken from a number of references including Harayama *et al.* (1984) and Lebens & Williams (1985) and that of pWW53-4 from Keil *et al.* (1987b). ORF, open reading frame.

Furthermore, recent results indicate that the *meta* pathway operons which form the second regulons of the pathways for naphthalene dissimilation on plasmids NAH7 (Harayama *et al.*, 1987) and pWW60-1 (Assinder & Williams, 1988) share a common ancestry with the same sequence on TOL plasmids and that the naphthalene pathway might also have evolved by the separate evolution of its two operons followed by their coacquisition by a vector plasmid.

Two other features of pDK1 are shared with pWW0 and pWW53. The first is its segregation of deletion mutants during growth on benzoate, although in each case the nature of the deletion and the resulting phenotypes of the hosts are unique to each plasmid. In the case of pDK1 the point at which the deletion gives rise to loss of the *meta* pathway genes in the formation of pDKT1 is close to the regulatory gene *xylS* at coordinate 14.8 ± 0.3 (Fig. 3). The second property common to the TOL plasmids pDK1, pWW53 and pWW0 is the ease with which they form cointegrate plasmids with RP4 (Nakazawa *et al.*, 1978; Keil *et al.*, 1985b). Some of the RP4::pWW0 cointegrates such as pTN2 probably contain the 56 kbp transposed into the R-plasmid, but others involve stretches of DNA of different lengths (Lehrbach *et al.*, 1982). In the case of pWW53 and pDK1, and its derivatives, we appear to get a wide variety of cointegrate plasmids which contain inserts of TOL DNA of differing sizes in a complete RP4 (Pickup, 1984; unpublished results). In the case of the two plasmids described here the integration sites in RP4 are not identical but both share what appears to be a common terminus of the pDK1 DNA. Whether this is fortuitous or whether it represents the location of a recombinational hotspot on pDK1 will have to be left to further investigations.

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