Molecular Analysis of Regulatory and Structural *xyl* Genes of the TOL Plasmid pWW53-4

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pWW53-4 is a cointegrate between RP4 and the catabolic plasmid pWW53 from *Pseudomonas putida* MT53, which contains 36 kbp of pWW53 DNA inserted close to the *oriV* gene of RP4; it encodes the ability to grow on toluene and the xylenes, characteristic of pWW53, as well as resistance to tetracycline, kanamycin and carbenicillin, characteristic of RP4. A physical map of the 36 kbp insert of pWW53 DNA for 11 restriction enzymes is presented, showing that the relative positions of the two *xyl* operons are different from those on the archetypal TOL plasmid pWW0. The location of the genes for 4-oxalocrotonate decarboxylase (*xylI*) and 4-oxalocrotonate tautomerase (*xylH*) were shown by subcloning and enzyme assay to lie at the distal end of the *meta* pathway operon. Although 2-oxopent-4-enoate hydratase (*xylJ*) and 4-hydroxy-2-oxovalerate aldolase (*xylK*) could be detected on a large cloned *Hind*III fragment, they could not be accurately located on smaller subcloned DNA, but the only credible position for them is between *xylF* and *xylI*. The gene order in the *meta* pathway operon is therefore *xylDLEGF(J,K)IH*. The regulatory genes *xylS* and *xylR* were located close to and downstream of the *meta* pathway operon, and the restriction map of the DNA in this region, as has previously been shown for the two operons carrying the structural genes, shows similarities with the corresponding region on pWW0. Evidence is also presented for the existence of two promoters, termed P3 and P4, internal to the *meta* pathway operon which support low constitutive expression of the structural genes downstream in *Pseudomonas* hosts but not in *E. coli*.

INTRODUCTION

The catabolism of toluene and substituted toluenes in *Pseudomonas* is usually encoded by genes located on extrachromosomal DNA called TOL plasmids (Williams & Worsey, 1976; Kunz & Chapman, 1981; Keil *et al.*, 1985*b*). In order to understand the evolution of TOL genes and their dissemination between replicons under the selection pressures prevailing in the natural environment we are comparing the catabolic genes of different TOL plasmids isolated from strains derived from independent isolations, often from diverse geographical origins.

In previous studies we have demonstrated that the archetype TOL plasmid pWW0 and pWW53-4, a cointegrate derived from a second TOL plasmid pWW53 and the broad host range R plasmid RP4, show considerable similarity in the DNA carrying the structural genes of the catabolic pathway but that this similarity is not found in the DNA outside the *xyl* genes (Keil *et al.*, 1985*b*, 1987). Although the gene order, as far as it was determined, within the *xyl* operons of the two plasmids was found to be identical there was an obvious difference in the position of the two operons relative to each other (Keil *et al.*, 1987).

In this study we have derived a complete restriction map of the pWW53 DNA of the cointegrate pWW53-4 and determined the locations of two of the remaining catabolic genes of the TOL pathway and of regulatory genes *xylR* and *xylS*. We present some results on the mechanism of gene regulation on pWW53 as well as evidence for the existence of non-regulated constitutive *Pseudomonas*-specific promoters upstream of *xylL* and *xylE*.

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METHODS

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

Media, culture conditions and enzyme assays. *P. putida* recombinant strains were grown on solid and liquid minimal medium (Worsey & Williams, 1975) with added streptomycin (150 µg ml⁻¹) or kanamycin (100 µg ml⁻¹) to ensure maintenance of recombinant plasmids; methods of the cultivation of *E. coli* were described by Keil *et al.* (1985a). The enzyme assay procedures for catechol 2,3-oxygenase, 2-hydroxymuconic semialdehyde hydrolase (2-hydroxy-6-oxohepta-2,4-dienoate hydrolase), 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase and 4-hydroxy-2-oxovalerate aldolase were taken from Sala-Trepat & Evans (1971), with the modification of Harayama *et al.* (1984) for the decarboxylase. 2-Oxopent-4-enoate hydratase was assayed according to Collinsworth *et al.* (1973). Benzoate *cis*-glycol dehydrogenase was only assayed where there was no catechol 2,3-oxygenase activity in an NAD⁺-dependent reaction (Reiner, 1972).

Substrates for enzyme assays. The enzymic conversion of DL-allylglycine to 2-oxopent-4-enoate was done as described by Collinsworth *et al.* (1973); 4-hydroxy-2-oxovalerate was produced from 2-oxo-3-carboxyethyl-4-methyl-γ-lactone by mild hydrolysis in 10 mM-NaOH (Bayly *et al.*, 1966); *Alcaligenes eutrophus* mutant B9 was used for the microbial accumulation of *cis*-1,2-dihydro-1,2-dihydroxybenzoate (benzoate *cis*-glycol) from benzoate (Reiner & Hegeman, 1971). 4-Oxalocrotonate was a gift from W. C. Evans, University College of North Wales, Bangor, UK.

Plasmid purification and DNA manipulation experiments. Restriction endonuclease hydrolyses and DNA ligations, as well as the transformation of *E. coli* strains, were done according to standard procedures (Maniatis *et al.*, 1982). *E. coli* and *P. putida* strains were screened for the presence of recombinant DNA by the method of Holmes & Quigley (1981) and vector and cloned DNA were purified by CsCl-ethidium bromide equilibrium centrifugation.

Mobilization of recombinant plasmids into *P. putida*. Using the unstable RP4 derivative pNJ5000 (Grinter, 1983), all pKT230-derived recombinant plasmids were mobilized into plasmid-free *P. putida* strains PaW130 and PaW95 by plate mating as described by Keil *et al.* (1985b).

RESULTS

Physical map of pWW53-4

The RP4::pWW53-4 cointegrate plasmid has been shown to carry all the genes necessary for the inducible catabolism of *m*-xylene (Keil *et al.*, 1985b). The *Bam*HI fragments common to pWW53 and pWW53-4, BC (11.9 kbp), BD (10.4 kbp), BE (6.5 kbp) and BF (3.6 kbp), together with the smaller of the two novel fragments generated by the cointegration with RP4 were cloned into pBR325 and subject to detailed mapping using 11 hexanucleotide-cutting restriction enzymes (Fig. 1). The site of integration of the 36 kbp insert from pWW53 DNA into RP4 is about 11 kbp clockwise from the *Eco*RI site on the circular map of RP4 close to *ori*V (Lanka *et al.*, 1983).

Location of xylH and xylI structural genes

The activities both of 4-oxalocrotonate tautomerase (*xylH*) and 4-oxalocrotonate decarboxylase (*xylI*) were detected in strains carrying plasmid pWW53-3301 which has the 17.5 kbp *Hind*III fragment HA cloned in pKT230 (Keil *et al.*, 1985b; Table 2). The specific activities in *P. putida* were much higher than in *E. coli* and were increased by more than 10-fold after induction with *m*-toluate (Table 2). *xylH* was located more accurately downstream of the *Xho*I site at coordinate 19.7 and the *Sma*I site at coordinate 20.0, since both pWW53-3508 and pWW53-3703 expressed 4-oxalocrotonate tautomerase activity in *E. coli* and *P. putida*, although activity in cells containing pWW53-3703 was much lower (Table 2). 4-Oxalocrotonate decarboxylase was expressed from pWW53-3506 and pWW53-3709 but not from pWW53-3807 (Table 2), indicating that *xylI* is located upstream of *xylH*, between the *Sma*I site at coordinate 19.0 and the *Xho*I site at 19.7.

The two remaining enzymes of the catabolic pathway 2-oxopent-4-enoate hydratase (*xylJ*) and 4-hydroxy-2-oxovalerate aldolase (*xylK*) were expressed and were inducible by *m*-toluate from plasmid pWW53-3301 although the activity of the aldolase, which cannot be measured in *E. coli* because of its high NADH oxidase activity, was low even in *Pseudomonas* (Table 2). None of the four subclones derived from HA which span the 3 kbp gap between *xylF* and *xylI*

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Notes and references
<i>E. coli</i> CA60		<i>hsdR hsdM recBC</i> derivative of strain C600
<i>E. coli</i> C600	pNJ5000	Grinter (1983)
<i>E. coli</i> C600	pKT230	Bagdasarian <i>et al.</i> (1981)
<i>E. coli</i> C600	pBR325	Prentki & Kirsch (1982)
<i>E. coli</i> C600	pUC12	Messing (1983)
<i>P. putida</i> PaW130		Keil <i>et al.</i> (1985 <i>b</i>)
<i>P. putida</i> PaW95		Keil <i>et al.</i> (1985 <i>b</i>)
<i>P. putida</i> MT53	pWW53	Keil <i>et al.</i> (1985 <i>b</i>)
	pWW53-4	Keil <i>et al.</i> (1985 <i>b</i>)
	pWW53-3301	17.5 kbp <i>Hind</i> III fragment HA of pWW53 cloned into pKT230 (Keil <i>et al.</i> , 1985 <i>b</i>)
	pWW53-3311	HA in reverse orientation in pKT230 (Keil <i>et al.</i> , 1985 <i>b</i>)
	pWW53-3019	1.5 kbp <i>Sma</i> I/ <i>Hind</i> III subfragment of <i>Hind</i> III fragment HF (Keil <i>et al.</i> , 1987)
	pWW53-3020	5.8 kbp <i>Sma</i> I/ <i>Hind</i> III fragment carrying OP1 and <i>xylCA</i> (Keil <i>et al.</i> , 1987)
	pWW53-3300	17.5 kbp <i>Hind</i> III fragment HA cloned into unique <i>Hind</i> III site of pWW53-3019
	pWW53-3403	Keil <i>et al.</i> (1985 <i>b</i>)
	pWW53-3404	12.6 kbp <i>Bam</i> HI fragment BC of pWW53 cloned into unique <i>Bam</i> HI site of pWW53-3403
	pWW53-3407	12.0 kbp <i>Bam</i> HI/ <i>Hind</i> III subfragment of HA, internal 6.1 kbp <i>Bam</i> HI fragment deleted from pWW53-3311
	pWW53-3409	Internal 600 bp <i>Bgl</i> II fragment deleted from pWW53-3407
	pWW53-3331	The two <i>Bam</i> HI/ <i>Hind</i> III fragments from pWW53-3403 (Keil <i>et al.</i> , 1985 <i>b</i>) and pWW53-3409 ligated into <i>Hind</i> III restricted pKT230
	pWW53-3320	16.9 kbp <i>Hind</i> III fragment of pWW53-3331 cloned into unique <i>Hind</i> III site of pWW53-3019
	pWW53-3912	12.6 kbp <i>Bam</i> HI fragment BC of pWW53 cloned into pBR325
	pWW53-3921	12.6 kbp <i>Bam</i> HI fragment BC of pWW53 cloned into pKT230
	pWW53-3812	5.5 kbp <i>Pst</i> I fragment of pWW53-3801 (Keil <i>et al.</i> , 1985 <i>b</i>) cloned into pUC12
	pWW53-3823	5.5 kbp <i>Sma</i> I/ <i>Hind</i> III fragment of pWW53-3812 cloned into pKT230
	pWW53-3825	Internal 3.6 kbp <i>Xba</i> I fragment deleted from pWW53-3823
	pWW53-3555	Keil <i>et al.</i> (1985 <i>b</i>)
	pWW53-3556	Reverse orientation of insert pWW53-3555 in pKT230
	pWW53-3510	Keil <i>et al.</i> (1985 <i>b</i>)
	pWW53-3506	1.6 kbp <i>Xho</i> I fragment of HA cloned into pKT230
	pWW53-3508	3.9 kbp <i>Xho</i> I fragment of HA cloned into pKT230
	pWW53-3703	3.4 kbp <i>Sma</i> I fragment of HA cloned into pKT230
	pWW53-3707	1.5 kbp <i>Sma</i> I fragment of HA cloned into pKT230
	pWW53-3709	1.0 kbp <i>Sma</i> I fragment of HA cloned into pKT230
	pWW53-3806	3.1 kbp <i>Pst</i> I fragment of HA cloned into pBR325
	pWW53-3807	3.5 kbp <i>Pst</i> I fragment of HA cloned into pBR325
	pWW53-3029	3.4 kbp <i>Sma</i> I fragment of HA cloned into unique <i>Sma</i> I site of pWW53-3020

(pWW53-3510, pWW53-3506, pWW53-3707 and pWW53-3806) expressed detectable activities of either enzyme, although all are inserted in the correct orientation for expression from the vector promoter (Table 2).

Location of xylR and xylS regulatory genes

The insert in pWW53-3301 contains both the operator-promoter region for the *meta* pathway operon (OP2) and a functional regulatory gene *xylS* (Keil *et al.*, 1985*b*); whereas OP2 was mapped around coordinate 7.8, *xylS* was only roughly located at the distal end of the operon. To map it accurately a deletion derivative from pWW53-3311 was constructed by removing the 0.6 kbp *Bgl*II fragment from coordinates 21.2 to 21.8. The resulting plasmid pWW53-3331 was mobilized into plasmid-free *P. putida* PaW130 and assayed for induction of catechol-2,3-oxygenase, 2-hydroxymuconic semialdehyde hydrolase and 2-hydroxymuconic semialdehyde

Table 2. Specific activities of meta pathway enzymes in *E. coli* and *P. putida* carrying plasmidsEnzyme activities are expressed as milliunits of activity (mg protein)⁻¹. ND, Not determined.

Plasmid	Host	Inducer	4-Oxalocrotonate tautomerase	4-Oxalocrotonate decarboxylase	2-Oxopent-4-enoate hydratase	4-Hydroxy-2-oxo valerate aldolase
pWW53	<i>P. putida</i>	<i>m</i> -Toluate	40 000	300	6 000	2
		None	4 500	25	800	ND
None	<i>P. putida</i>	None	< 50	< 1	< 10	0.4
pWW53-3301	<i>E. coli</i>	None	570	4	34	ND
	<i>P. putida</i>	<i>m</i> -Toluate	350 000	210	17 000	2
		None	32 000	20	1 270	0.2
pWW53-3508	<i>E. coli</i>	None	30 000	< 0.1	< 10	ND
	<i>P. putida</i>	None	10 500	< 0.1	< 10	0.1
pWW53-3703	<i>E. coli</i>	None	100	< 0.1	< 10	ND
	<i>P. putida</i>	None	2 400	< 0.1	< 10	0.1
pWW53-3807	<i>E. coli</i>	None	800	< 0.1	< 10	ND
pWW53-3806	<i>E. coli</i>	None	< 50	< 0.1	< 10	ND
pWW53-3506	<i>E. coli</i>	None	< 50	6	< 10	ND
	<i>P. putida</i>	None	< 50	9	< 10	0.1
pWW53-3709	<i>E. coli</i>	None	< 50	7	< 10	ND
	<i>P. putida</i>	None	< 50	22	< 10	0.1

dehydrogenase in the presence and absence of *m*-toluate and *m*-methylbenzyl alcohol (Table 3): the strong induction by *m*-toluate and the weaker induction by *m*-methylbenzyl alcohol with pWW53-3311 was not found with pWW53-3331, showing that *xylS* spans one or both *Bgl*II sites.

xylR is the regulatory gene which mediates the induction by *m*-xylene and *m*-methylbenzyl alcohol of the upper pathway operon (*xylCAB*). Because of the similarity of the restriction maps of the region which carries *xylS* and *xylR* on pWW0 and the pWW53-4 DNA around coordinates 20 to 24 (Fig. 2) we attempted to show the presence of *xylR* in an analogous position to that on pWW0 by constructing two strains carrying the early part of the *xylCAB* operon (OP1 and *xylCA*) on plasmid pWW53-3020 together with part of the putative *xylSR* region. Plasmid pWW53-3029 contains a 3.5 kbp *Sma*I fragment, which should contain *xylS* but probably not *xylR*, inserted into the unique *Sma*I site of plasmid pWW53-3020: there was, as expected, no significant induction of benzaldehyde dehydrogenase (*xylC* product) from this plasmid by *m*-methylbenzyl alcohol (Table 4). However, in an *E. coli* strain carrying pWW53-3020 and a second recombinant plasmid pWW53-3912, which extends to the *Bam*HI site at coordinate 24.3, *m*-methylbenzyl alcohol caused a threefold induction of benzaldehyde dehydrogenase activity

Fig. 1. Restriction map of the complete 36 kbp insert of pWW53 DNA in the cointegrate plasmid pWW53-4. The hatched boxes at either end represent RP4 DNA at the termini of the pWW53 insert. The complete pathway for toluene catabolism is shown above the restriction map. The arrows are directed from the positions of the corresponding structural genes (black bars), determined in this and previous studies (Keil *et al.*, 1985b, 1987), to the corresponding enzymic activities. The various subclones constructed in this study are shown below the map. The small arrows below the subcloned fragments show the directions of transcription from the vector promoters (see Table 2). 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. Two alternative locations for a single *Sal*I site which have not been distinguished are indicated by brackets. OP1 and OP2 are the operator-promoter regions at the start of each operon; P3 and P4 are the internal promoters identified in this study. ORF is a small open reading frame at the start of the *xylCAB* operon which we have sequenced on pWW0 (M. R. Lebens & P. A. Williams, unpublished results) and which shows considerable homology with the corresponding region on pWW53-4. The brackets around *xylS* and *xylR* for some of the plasmids signify that we have been unable to show experimentally that the regulator genes are on those plasmids because OP2 is not present and therefore regulated transcription of the structural genes is not possible; however, the comparison with other recombinant plasmids confirms that the regulator genes are present on those plasmids.

Table 3. *Effect of different inducers on specific activities of enzymes of meta pathway in E. coli and P. putida carrying plasmids**P. putida* host was PaW130. Enzyme activities are expressed as milliunits (mg protein)⁻¹.

Plasmid	Host	Inducer	Catechol 2,3-oxygenase	2-Hydroxymuconic semialdehyde hydrolase	2-Hydroxymuconic semialdehyde dehydrogenase
pWW53	<i>P. putida</i>	None	96	11	10
		<i>m</i> -Toluate	3450	220	77
		<i>m</i> -Xylene	1970	280	140
pWW53-3300	<i>E. coli</i>	None	4	0.4	0.05
		<i>m</i> -Toluate	7	0.6	0.1
		<i>m</i> -Methylbenzyl alcohol	26	1.4	0.15
	<i>P. putida</i>	None	800	70	18
		<i>m</i> -Toluate	4700	430	120
		<i>m</i> -Methylbenzyl alcohol	6000	480	120
		<i>m</i> -Xylene	4000	350	85
	<i>E. coli</i>	None	150	4	1
		<i>m</i> -Toluate	235	9	1.5
		None	925	80	22
		<i>m</i> -Toluate	9900	390	210
		<i>m</i> -Methylbenzyl alcohol	9900	290	100
pWW53-3320	<i>E. coli</i>	<i>m</i> -Xylene	8300	390	140
		None	18	0.5	0.1
		<i>m</i> -Toluate	25	1	0.1
	<i>P. putida</i>	None	590	57	16
		<i>m</i> -Toluate	910	98	28
		<i>m</i> -Methylbenzyl alcohol	350	26	5
		<i>m</i> -Xylene	620	34	11
	<i>E. coli</i>	None	15	0.5	0.1
		<i>m</i> -Toluate	29	1	0.2
		<i>m</i> -Methylbenzyl alcohol	12	0.5	0.1
		None	400	55	15
		<i>m</i> -Toluate	4400	600	170
pWW53-3311	<i>P. putida</i>	<i>m</i> -Methylbenzyl alcohol	1660	90	25
		<i>m</i> -Xylene	650	30	11
		None	31	1	0.2
	<i>E. coli</i>	<i>m</i> -Toluate	25	1	0.2
		None	685	120	30
		<i>m</i> -Toluate	600	90	25
		<i>m</i> -Methylbenzyl alcohol	720	70	20
	<i>P. putida</i>	None	290	27	7
		<i>m</i> -Toluate	270	35	8
		<i>m</i> -Methylbenzyl alcohol	280	29	7
		<i>m</i> -Xylene	295	21	6
		None	3	0.2	<0.1
pWW53-3407	<i>E. coli</i>	<i>m</i> -Toluate	2	0.2	<0.1
		None	370	28	8
		<i>m</i> -Toluate	370	41	8
	<i>P. putida</i>	None	510	48	11
		<i>m</i> -Toluate	510	47	11
		None	510	47	11
		<i>m</i> -Toluate	510	47	11
	<i>P. putida</i>	None	510	47	11
		<i>m</i> -Toluate	510	47	11
		None	510	47	11
		<i>m</i> -Toluate	510	47	11
		None	510	47	11

Fig. 2. Comparison of the restriction maps of the *xylSR* regions on pWW0 (Inouye *et al.*, 1983; Spooner *et al.*, 1986) and on pWW53-4. The restriction enzyme abbreviations are as in Fig. 1.

Table 4. Specific activities of benzaldehyde dehydrogenase in *E. coli* carrying cloned genes

Plasmid(s)	Host	Inducer	Specific activity [milliunits (mg protein) ⁻¹]
pWW53-3029	<i>E. coli</i>	None	9
		<i>m</i> -Methylbenzyl alcohol	11
pWW53-3020	<i>E. coli</i>	None	40
+ pWW53-3912		<i>m</i> -Methylbenzyl alcohol	130

(Table 4); in this experiment, which could only be carried out in an *E. coli* host, we were not able to test induction by *m*-xylene because of the deleterious effect of the hydrocarbon on the cells of this host. The *xylR* gene must therefore span the *Sma*I site at 23.3 but not the *Bam*HI site at 24.3.

xylR has been reported also to mediate the induction of the *meta* pathway operon (*xylDLEGF*...) in the presence of *m*-xylene and/or *m*-methylbenzyl alcohol as coinducers (Worsey *et al.*, 1978; Franklin *et al.*, 1983; Inouye *et al.*, 1983). Since *m*-xylene does not induce *meta* pathway enzymes in *Pseudomonas* strains containing pWW53-3311 (Table 3), the regulatory model would predict that the insert in pWW53-3311 (up to the *Hind*III site at 23.6) does not carry a functional *xylR* gene. Because of the similarity in the patterns of restriction sites in the vicinity of *xylSR* on plasmids pWW0 and pWW53-4 (Fig. 2), we constructed two plasmids pWW53-3300 and pWW53-3404 with extensions of 1.5 and 0.6 kbp respectively beyond the right hand *Hind*III site of the insert in pWW53-3311 (Table 1, Fig. 1) to test the effect of *xylR* on the induction of the *meta* pathway enzymes. In *P. putida* both *m*-xylene and *m*-methylbenzyl alcohol did cause significant induction (between 5- and 11-fold) of the enzymes assayed (Table 3) as predicted by the regulatory model. This confirms that *xylR* is upstream of the *Bam*HI site at coordinate 24.3 and that the *Hind*III site at coordinate 23.7 lies in the gene. A *Bgl*/II deletion derivative of pWW53-3300, pWW53-3320, equivalent to pWW53-3331 described earlier, was also constructed. In this case neither *m*-xylene nor *m*-methylbenzyl alcohol acted as inducers (Table 3) indicating that *xylR* extends upstream of the *Bgl*/II site at coordinate 21.8. These results also show that a functional *xylR* gene is necessary for the effective induction of the *xylDLEGF* operon by *m*-xylene and *m*-methylbenzyl alcohol. Furthermore *m*-xylene itself does act as a good inducer of this operon without its further metabolism, in agreement with earlier results obtained with pWW0 (Inouye *et al.*, 1983) but in conflict with a recent paper from our laboratory (Lebens & Williams, 1985).

Identification of internal promoters P3 and P4

Recombinant plasmid pWW53-3407 was found to confer the ability to grow on *m*-toluate to plasmid-free *Pseudomonas*, albeit more slowly than did pWW53-3311. Since the insert of pWW53 DNA was in the orientation such that initiation of transcription could not start from the vector promoter, enzyme activities in *E. coli* were very low, but in *P. putida* they were equal

Table 5. *Specific activities of enzymes in E. coli and P. putida carrying plasmids with putative internal promoters*

P. putida host was PaW95; enzyme activities are expressed as milliunits (mg protein)⁻¹. ND, Not determined. The specific activities for 2-hydroxymuconic semialdehyde hydrolase were <0.1 in each case.

Plasmid	Host	Benzoate <i>cis</i> -glycol dehydrogenase	Catechol 2,3-oxygenase	2-Hydroxymuconic semialdehyde dehydrogenase
pWW53-3556	<i>E. coli</i>	ND	<0.1	<0.1
	<i>P. putida</i>	ND	95	<0.1
pWW53-3812	<i>E. coli</i>	ND	235	0.2
pWW53-3823	<i>E. coli</i>	ND	1	0.1
	<i>P. putida</i>	ND	505	5
pWW53-3825	<i>E. coli</i>	<1	<1	<0.1
	<i>P. putida</i>	54	<1	<0.1

to the uninduced levels expressed from pWW53-3311 (Table 3). These results suggested that there was a promoter sequence, specific to *Pseudomonas*, downstream of the *Bam*HI site at coordinate 11.6. The function of this promoter activity was not affected by the presence of *xylS* and *xylR* and the addition of any inducer (*m*-xylene, *m*-methylbenzyl alcohol, *m*-toluate or benzoate) as is shown by the assays in *Pseudomonas* containing pWW53-3291 and pWW53-3409 (Table 3). Further confirmation was obtained from pWW53-3556 with the 2.1 kbp *Xho*I insert carrying *xylE*, inserted in the reverse orientation to the vector promoter. Catechol 2,3-oxygenase activity was low in *E. coli* but high in *P. putida* and benzoate or *m*-toluate caused no induction (Table 5).

Evidence for a second internal constitutive promoter was obtained from the *xylL*-specific plasmid pWW53-3825. The insert is also orientated away from the vector promoter, and the benzoate *cis*-glycol dehydrogenase activity was expressed constitutively in *Pseudomonas* but not in *E. coli* (Table 5). These results suggested that upstream of both *xylL* and *xylE* there are *Pseudomonas*-specific promoter sequences (which we have termed P3 and P4 respectively), which allow low constitutive expression of the *xyl* genes downstream of them. Presumably this expression, combined with the chromosomal *ben* genes (for conversion of benzoates to catechols) must account for the ability of pWW53-3407 to support growth on *m*-toluate.

DISCUSSION

This paper presents new data on the cointegrate TOL plasmid pWW53-4, describing the locations of two more of the *meta* pathway genes (*xylIH*), of the two regulatory genes (*xylSR*) and of two internal promoters (P3, P4) within the *meta* pathway operon. It also presents a detailed restriction map of the entire 36 kbp insert derived from pWW53.

The only two structural genes we have not been able to position are *xylJ* and *xylK*. Although expressed from the large *Hind*III insert in pWW53-3311 we could not detect either activity on any of the smaller subcloned fragments. We assume that there may be *E. coli*-specific terminator sequences downstream of *xylF*, which block expression when cloned in *E. coli* vectors such as pBR325, and that the restriction sites available for cloning in pKT230 (which would allow expression in *P. putida*) unfortunately lie within the genes. However, the only credible position for the genes is in the 3 kbp gap between *xylF* and *xylI*.

The totality of data we have obtained about pWW53-4 (Keil *et al.*, 1985*b*, 1987; this paper) has shown that the DNA encoding the *xyl* genes shows a remarkable similarity with the corresponding DNA of the archetypal TOL plasmid pWW0 and its RP4 cointegrate pTN2 (Nakazawa *et al.*, 1978). Within each operon, the order of the genes is identical and there is a considerable conservation of restriction sites. This also applies to the regulatory *xylSR* region (Fig. 2), where the two *Bgl*II sites at coordinates 21.2 and 21.8 are in an analogous position in the

xylSR region of pWW0 (Franklin *et al.*, 1983; Inouye *et al.*, 1983; Spooner *et al.*, 1986). The main difference between the *xyl* genes of pWW53-4 and pWW0 is in the relative positions of the two operons. On both plasmids the two operons are transcribed in the same direction, but on pWW0 the upstream operon is *xylCAB*, whereas on pWW53-4 it is *xylDLEGF(J,K)IH*: on both plasmids, the *xylSR* region is just downstream of the *meta* pathway operon. The catabolic DNA of both plasmids must therefore have a common ancestral origin. A simple transposition (or recombination) of the *xyl* genes between replicons is insufficient to explain the different relative positions of the operons although this difference could have occurred by a single additional translocation of one of the operons. The evolutionary relationship between pWW0 and pWW53 is, however, more complex since we have recently cloned a second distinct, but homologous, *xylDLEGF(J,K)IH* operon from pWW53, which obviously has not been incorporated in this cointegrate pWW53-4 (D. A. Osborne, H. Keil & P. A. Williams, unpublished results) so the results from pWW53-4 represent only a part of the *xyl* gene organization on the native pWW53 plasmid.

Although the basic model for the regulation of the *xyl* operons has been generally accepted (Worsey *et al.*, 1978; Franklin *et al.*, 1983) there has been uncertainty about some of the details. Although there is no doubt about the role of *xylR* as the regulatory protein involved in induction of *xylCAB* by *m*-xylene, the mechanism by which it is involved in the induction of the *xylDLEGF(J,K)IH* operon has not been definitively elucidated. Recent investigations in our laboratory into some spontaneous deletion and insertion mutants of pWW0 indicated that *m*-xylene is unable to induce the *meta* pathway operon via *xylR* (possibly with *xylS* also playing some role), but that its conversion to *m*-methylbenzyl alcohol was a prerequisite for the induction (Lebens & Williams, 1985). We have now looked at the *meta* pathway induction on pWW53-4 by generating specific deletion mutants within the *xylSR* region on cloned DNA and by analysing the resulting induction pattern in *P. putida*. The results confirm that *xylS* alone is sufficient for the induction of *xylDLEGF(J,K)IH* by *m*-toluate, and that *m*-methylbenzyl alcohol can partially replace *m*-toluate in the *xylS*-mediated induction, giving about 30% of the specific activities: this presumably is the result of low inducer specificity of the *xylS* protein. Full induction of the *meta* pathway operon with *m*-methylbenzyl alcohol and induction with *m*-xylene was only obtained with an extension of the DNA to include a functional *xylR* gene. Because of the particular configuration of restriction sites, we were unable to generate a cloned fragment which was *xylR*⁺*xylS*⁻, and cannot therefore offer any evidence concerning the possible role of a functional *xylS* in the *xylR*-mediated induction of the *meta* pathway as has been suggested (Franklin *et al.*, 1983). The above discussion on the regulation assumes that results applicable to pWW0 also apply to pWW53-4 and vice versa: when we started this comparative study, this obviously could not be assumed but in the light of the demonstrated similarity between the two, we now consider it to be a valid assumption.

The presence of two internal promoter sequences, P3 and P4, which allow low constitutive expression of the *xyl* genes downstream raises interesting questions about the possible evolution of the catabolic pathway. Since (a) these promoters can allow expression of the *meta* pathway enzymes in the absence of OP2, and (b) the *xylDL* genes can be complemented by the isofunctional chromosomal *ben* genes (for conversion of benzoate to catechol), we speculate that the operon originally evolved as an unregulated pathway for the degradation of catechols, and that the additional biochemical activities (*xylDL* for converting benzoates to catechols) were acquired by recruiting genes (such as the *ben* genes): this could be tested by hybridization and sequence analysis of the *xylDL* region with the chromosomal *ben* genes.

Such a modular acquisition of functional elements would also explain the similarities between the *meta* pathway operon of TOL plasmids and the corresponding operon of NAH plasmids (Cane & Williams, 1986; S. J. Assinder & P. A. Williams, unpublished results): in the naphthalene pathway the first gene of the corresponding operon is *nahG* (for salicylate hydroxylase) but the subsequent genes carry identical functions to *xylEGFJKIH*. In this case an already evolved sequence for catechol catabolism could have recruited this different initial gene to produce the naphthalene *meta* pathway operon.

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