A comparison of the multiple alleles of *xyIS* carried by TOL plasmids pWW53 and pDK1 and its implications for their evolutionary relationship

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Both of the independently isolated TOL plasmids pWW53 and pDK1 contain multiple regions homologous to the *xyIS* regulatory gene of the archetypal TOL plasmid pWW0. The three homologues on pWW53 vary in the extent of their homology to *xyIS*_{pWW0}. *xyIS*_{1pWW53} is 99% identical to *xyIS*_{pWW0} and is located relative to the single copy of *xyIR*_{pWW53} in exactly the same way as *xyIS* and *xyIR* on pWW0. The DNA sequence of *xyIS*_{3pWW53} is 87% identical to the *xyIS*_{pWW0} sequence within the coding region but the non-coding DNA upstream is not homologous. There is a frame-shift change at the end of the coding region which causes the C terminus of *XylS*_{3pWW53} to be extended by an additional 10 amino acids relative to *XylS*_{pWW0}. *xyIS*_{2pWW53} is anomalous and appears to encode a truncated pseudogene lacking the first 525 bases found in the other *xyIS* genes. Evidence is presented to show that both *xyIS*_{1pWW53} and *xyIS*_{3pWW53} act as regulators of *meta* pathway operons. Plasmid pDK1 carries two homologues of *xyIS*. *xyIS*_{1pDK1} is functional and is a hybrid gene: its 5' end and the upstream sequences are highly homologous to both *xyIS*_{1pWW53} and *xyIS*_{pWW0}, whereas its 3' end is identical to *xyIS*_{3pWW53}. The sequence of *xyIS*_{2pDK1} is identical to that of the anomalous truncated *xyIS*_{2pWW53}. Comparison of the organization and the restriction maps of the *xyI* catabolic operons on pDK1 and pWW53, together with the nucleotide sequences presented here, indicates that the catabolic DNA on pDK1 has derived from a replicon on which the *xyI* genes are organized similarly to pWW53 and that a genetic rearrangement has taken place involving a reciprocal recombination internal to two of its *xyIS* homologues.

Introduction

The molecular biology of the TOL pathway for the catabolism of toluene and the xylenes has been characterized in greatest detail on the 117 kbp plasmid pWW0

from *Pseudomonas putida* mt-2. However, a number of other plasmids encoding the same catabolic functions have been reported (see Assinder & Williams, 1990) and the gene organization on two, pWW53 (Keil *et al.*, 1985a, 1987a, b; Osborne *et al.*, 1988) and pDK1 (Shaw & Williams, 1988) has been described in some detail. This evidence suggests that the genetic organization of the *xyI* catabolic genes found on pWW0 (Franklin *et al.*, 1981), namely single operons for the upper pathway (Harayama *et al.*, 1989), and the *meta* pathway genes (Harayama & Rekik, 1990), is not typical of other TOL plasmids. Not only are the *xyI* operons arranged in different orders and orientations but there is increasing evidence that many carry more than one copy either of whole operons or of individual genes (Keil *et al.*, 1985b; Chatfield & Williams, 1986; O'Donnell & Williams, 1991). In particular we have shown that the 107 kbp plasmid pWW53 carries two homologous, but distinguishable, direct repeats of the *meta* pathway operon *xyIXYZLTFGFJQKIH* both of which are functional (Osborne *et al.*, 1988). In this paper we show the

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers L02356 (*xyIS*_{1pWW53}), L02643 (*xyIS*_{2pWW53}), L02357 (*xyIS*_{3pWW53}), L02358 (*xyIS*_{1pDK1}) and L02642 (*xyIS*_{2pDK1}).

existence on two TOL plasmids of multiple copies of the *xylS* regulatory gene, responsible for the activation of the *meta* pathway operon by benzoate or methylbenzoates (toluates). Consideration of the nucleotide sequences, together with the overall *xyl* gene organization, suggests that plasmid pDK1, which was isolated by Kunz & Chapman (1981) in Minneapolis-St Paul, USA, appears to have derived from a major recombinational rearrangement from a plasmid similar in its *xyl* gene organization to pWW53 which was independently isolated from the geographically distant location of Bangor, North Wales, UK.

Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Table 1. Bacteria were grown and maintained according to Worsey & Williams (1975) and Keil *et al.* (1985*a, b*).

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Notes and reference
<i>Pseudomonas</i> strains		
PaW130		Keil <i>et al.</i> (1985 <i>b</i>)
MT53	pWW53	Keil <i>et al.</i> (1985 <i>a</i>)
MT53-1	pWW53-1	Osborne <i>et al.</i> (1988)
MT53-2	pWW53-2	Osborne <i>et al.</i> (1988)
HS1	pDK1	Wild type isolate (Kunz & Chapman, 1981)
PpCM1	pDKM1	Benzoate-selected segregant of HS1 (Kunz & Chapman, 1981)
Vector plasmids		
	pUC18/pUC19	Yanisch-Perron <i>et al.</i> (1985)
	pKT230	Bagdasarian <i>et al.</i> (1981)
	pFBI.1	Pharmacia
	pBR325	Prentki & Kirsch (1982)
Recombinant plasmids from pWW53		
	pWW53-3002	Osborne <i>et al.</i> (1988)
	pWW53-3004	Osborne <i>et al.</i> (1988)
	pWW53-3508	Keil <i>et al.</i> (1987 <i>b</i>)
	pWW53-3010	6.7 kbp <i>HindIII</i> -H of pWW53 in pKT230
	pWW53-1013	14.0 kbp <i>KpnI</i> -C of pWW53 in pKT230
	pWW53-1201	5.3 kbp <i>SmaI</i> - <i>KpnI</i> fragment obtained by <i>SmaI</i> digestion of pWW53-1013
	pWW53-1001	3.2 kbp <i>BamHI</i> fragment of pWW53-3010 cloned into pFBI.1 (<i>xylS</i> _{pWW53})
	pWW53-1002	3.2 kbp <i>BamHI</i> fragment of pWW53-1001 cloned into pWW53-3002
	pWW53-1003	3.2 kbp <i>BamHI</i> fragment of pWW53-1001 cloned into pWW53-3004
Recombinant plasmids from pDK1		
	pDK2-3351	1.35 kbp <i>HindIII</i> -S in pUC18
	pDK2-3352	<i>HindIII</i> -S in pUC18 in opposite orientation to 3351
	pDK2-3353	1.1 kbp <i>HindIII</i> -U in pUC18
	pDK2-3376	1.45 kbp <i>XhoI</i> -C' in pUC18
	pDK2-3377	<i>XhoI</i> -C' in pUC18 in opposite orientation to 3376
	pDK2-3364	3.9 kbp <i>BamHI</i> -D in pUC18
	pDK2-3365	<i>BamHI</i> -D in pUC18 in opposite orientation to 3364

Enzyme assays. Induction by *m*-toluate was achieved by growing *P. putida* cells in liquid minimal medium with 5 mM-*m*-toluate as sole source of carbon and energy. Cells were harvested after 24 h, cell extracts prepared and enzymes assayed as described previously (Assinder & Williams, 1988).

DNA manipulations. Vector and recombinant plasmid DNA isolation, construction of recombinant DNA, *Escherichia coli* transformation, nick-translation to radiolabel DNA with ³²P and DNA-DNA hybridizations were by standard procedures (Sambrook *et al.*, 1989). pWW53, pDK1 and their derivatives were extracted according to Wheatcroft & Williams (1981).

DNA subcloning strategies and sequencing. Sequencing was performed on both DNA strands using the double-stranded dideoxy chain-termination procedure (Sanger *et al.*, 1977) either with M13 forward and reverse primers, or with custom-synthesized oligonucleotide primers. Nested deletions of clones for sequencing were obtained using S1 nuclease and exonuclease III (Henikoff, 1984) using a Pharmacia kit.

(i) Sequencing of *xylS*_{pWW53}. The 3.9 kbp *XhoI* fragment of pWW53 contained in pWW53-3508 (Keil *et al.*, 1987*b*) was used as a source of *xylS* for DNA sequencing. Contiguous 0.6 kbp *BglII* and 1.2 kbp *BglII*-*SmaI* fragments were subcloned into pUC18 in both orientations.

(ii) Sequencing of *xylS*_{pWW53}. The 14.0 kbp *KpnI* fragment KC of pWW53 was cloned into pUC19 giving pWW53-1013. Internal *SmaI* fragments were excised by digestion with *SmaI* and religation to generate pWW53-1201. This carries the *xylS*_{pWW53} gene on a 5.3 kbp *SmaI*-*KpnI* fragment.

(iii) Sequencing of *xylS*_{pWW53}. The 3.2 kbp *BamHI* fragment carrying *xylS*_{pWW53} in pWW53-1001 was subcloned by transferring the 2.0 kbp *BamHI*-*BglII* fragment into the *BamHI* site of pUC19 in both orientations.

(iv) Sequencing of *xylS*_{pDK1}. Determination of the sequence of *xylS* started with 3.9 kbp *BamHI*-D. This was cloned into pUC19 in both orientations to give plasmids pDK2-3364 and pDK2-3365. A small (0.7 kbp) *SmaI* fragment was deleted from pDK2-3364 between the *SmaI* site in the multicloning site of the vector and the internal *SmaI* site in the insert. Similarly a *PstI* fragment was excised from pDK2-3365 using the multicloning site and an internal site in the insert leaving a 2.4 kbp *PstI*-*BamHI* insert.

(v) Sequence of *xylS*_{pDK1}. The sequence of *xylS* was initiated from three fragments cloned into pUC18, the adjacent 1.35 kbp *HindIII*-S (pDK2-3351 and pDK2-3352) and 1.1 kbp *HindIII*-U fragments (pDK2-3353), and the overlapping *XhoI*-C' fragment (pDK2-3376 and pDK2-3377).

Results

Identification of three *xylS* homologues on pWW53

A 300 bp fragment internal to the *xylS*_{pWW0} gene (gift of F. C. H. Franklin, University of Birmingham, UK) was radiolabelled and hybridized to pWW53 DNA cut with a range of restriction endonucleases. Two regions carrying putative *xylS* homologues were identified in addition to that already shown to exist at the end of operon 1 (to be referred to as *xylS*_{pWW53}) (Keil *et al.*, 1987*b*; Osborne *et al.*, 1988). One of these regions, denoted *xylS*_{pWW53}, was at the downstream end of

Fig. 1. Restriction maps of the areas containing the *xyl* genes of TOL plasmids pDK1 and pWW53. The maps are aligned to highlight the structural relationships between the two plasmids. The restriction map for *Xho*I and *Hind*III for pDK1 is taken from Shaw & Williams (1988) and represents the pDK1 insert in the RP4:pDK1 cointegrate pDK2. The restriction map for pWW53 has not been published previously in this form but some of the data is from Osborne *et al.* (1988). The two maps have been aligned on the two upper pathway operons (*xylCMABN*; upper). The extent of homology around this region is shown by the two bold arrows. The single *Hind*III site in this region which is present on pWW53 but not on pDK1 is shown as a dashed line. The second region of homology extends between *xylS3* (*S3*) past *xylS2* (*S2*), including *meta* pathway operon 2 (*meta 2*) on pWW53 and from *xylS1* (*S1*) and past *xylS2*, including the *meta* pathway operon 1 (*meta 1*) on pDK1 and is denoted by the bold dashed arrows: there are no differences in restriction sites for nine enzymes within this region. The three *xylS* homologues which are proposed as being involved in the rearrangement of catabolic DNA from a pWW53-like plasmid to give pDK1 are shown as cross hatched boxes: *xylS1*_{pWW53} and *xylS3*_{pWW53} appear to have recombined to give the hybrid *xylS1*_{pDK1}. The two *xylS2* homologues which have been shown to have identical sequences are shown as black boxes. The two *meta* pathway operons which are indistinguishable by restriction mapping and presumably have very similar, if not identical sequences, are shown as shaded boxes. The directions of transcription of the various transcription units are shown as light arrows. Relevant restriction fragments mentioned in the text are lettered. The extent of the deletions in pWW53 which have occurred in the formation of the plasmids pWW53-1 and pWW53-2 are shown above the pWW53 map. R, *xylR*.

operon 2, although not on the large fragment *Hind*III-B (HB) which carries the operon (Fig. 1). A third region of homology (*xylS3*_{pWW53}) was identified between the two *meta* pathway operons (Fig. 1).

A 1.5 kbp *Bgl*II–*Sma*I fragment of pWW53 *Hind*III-A (HA), shown by Keil *et al.* (1987b) to carry part of *xylR*_{pWW53} was used as a probe against digests of DNA from pWW53, pWW53-1 and pWW53-2. It hybridized only to the region adjacent to *xylS1*_{pWW53} at the downstream end of operon 1 on pWW53, implying that *xylR* is present in a single copy on pWW53. As confirmation of this, no hybridization was observed to the deletion derivatives of pWW53, pWW53-1 and pWW53-2, since the downstream end of operon 1 is deleted in these plasmids (Fig. 1).

Functional analysis of *xylS3*_{pWW53}

The ability of *xylS3*_{pWW53} to act as a regulator of *meta* pathway operons was examined using the following strategy (Fig. 2). *Hind*III fragment H from pWW53 which carries *xylS3* (Fig. 1) was cloned into pKT230 (pWW53-3010). Using the unique vector *Bam*HI site, the 3.2 kbp *Bam*HI fragment was subcloned into pFBI.1 giving pWW53-1001. The *Bam*HI fragment from pWW53-1001 was cloned into pWW53-3002 (consisting of pWW53 HB cloned into the *Hind*III site of pKT230; Osborne *et al.*, 1988) to give plasmid pWW53-1002. This construct allowed us to study the interaction between *xylS3*_{pWW53} and the promoter of *meta* pathway operon 2, *Pm2*_{pWW53}. It was not possible to make an exactly

Fig. 2. Cloning strategy for the functional analysis of $xylS3_{pWW53}$. The sizes of the vectors are indicated within the circles. The location of $xylS3_{pWW53}$ is shown as a black box. The location of pWW53 meta operon 2 is shown as a hatched box. H, HindIII; B, BamHI. The origins of the plasmids and accurate sizes of the cloned inserts are given in Table 1.

Table 2. Specific activities of meta pathway enzymes in PaW130 carrying the $xylS3_{pWW53}$ gene and either the hybrid meta pathway operon from pWW53-2 (HA') or meta operon 2 from pWW53 (HB)

$xylS3$	meta operon	<i>m</i> -Toluate	Specific activity*		
			C23O	HMSD	HMSH
—	HA'	—	13	5	2
—	HA'	+	36	17	12
+	HA'	—	5	4	2
+	HA'	+	139	105	113
—	HB	—	7	4	1
—	HB	+	17	7	8
+	HB	—	5	5	2
+	HB	+	183	130	151

* Specific activities are expressed as milliunits (mg protein)⁻¹ and represent the mean of three replicates. C23O, catechol 2,3-oxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase.

equivalent construct for the promoter of meta pathway operon 1, PmI_{pWW53} , since HA contains a site for BamHI. As an alternative, the BamHI fragment from pWW53-1001 was cloned into pWW53-3004 (consisting of the

novel HindIII fragment HA' of pWW53-2 cloned into pKT230; Osborne *et al.*, 1988) to give plasmid pWW53-1003. This novel HindIII fragment of pWW53-2 contains a hybrid meta operon with the upstream region of meta operon 1 fused through spontaneous recombination to the downstream region of operon 2 (see Fig. 1) (Osborne *et al.*, 1988). This construct allowed us to study the interaction between $xylS3_{pWW53}$ and PmI_{pWW53} .

Plasmids pWW53-3002, pWW53-3004, pWW53-1002 and pWW53-1003 were individually mated into the plasmid-free *P. putida* strain PaW130 and representative meta pathway enzymes were assayed after growth in the presence and absence of *m*-toluate as inducer (Table 2). Induction was observed only in the presence of both the $xylS3_{pWW53}$ gene and *m*-toluate.

Identification of two *xylS* homologues on pDK1

In our earlier paper on pDK1 (Shaw & Williams, 1988) we demonstrated that at the upstream end of the upper pathway operon *xylCMABN* of pDK1 there were two adjacent BglII sites as found on pWW0 in the centre of the divergently transcribed *xylRS* gene pair (Spooner *et al.*, 1986). These sites were located on a 3.9 kbp BamHI fragment D (BD) and the overlapping 2.8 kbp XhoI fragment S (XS) (around coordinates 25–27, Fig. 1). Both fragments hybridized with the $xylS_{pWW0}$ -specific probe (see above) and, when BD was inserted into a recombinant plasmid containing the cloned pDK1 meta pathway operon, the meta pathway enzymes were inducible by *m*-toluate (Shaw & Williams, 1988). BD appears therefore to carry a functional *xylS* gene which we have termed $xylS1_{pDK1}$.

Plasmid pDKM1 is a spontaneous deletion derivative of pDK1 (Kunz & Chapman, 1981) which carries an inducible meta pathway and supports growth on *m*- and *p*-toluates but has lost the upper pathway operon as part of its deletion. Restriction digests showed that the fragments around the $xylS1_{pDK1}$ gene were not present on pDKM1 (results not shown) having been deleted with the adjacent upper pathway operon and its *xylR* gene (see Fig. 1). However, the $xylS_{pWW0}$ -specific probe did hybridize to Southern blots of digested pDKM1 at positions corresponding to the 3.8 kbp XhoI fragment J (XJ) and the overlapping 1.35 kbp HindIII-fragment S (HS); we have termed this region of homology $xylS2_{pDK1}$.

Sequence determination of the pWW53 and pDK1 *xylS* genes

For clarity only the DNA sequences corresponding to the ORF of $xylS_{pWW0}$ plus a short upstream region are

-24 -12

(a) CTTAAAAAGAACGTCTTCGTTCTGCTTGGCGTTATTTTGGCTTGGAAAAGTGGTCACTGA 60
 (b) ::::::::::::::T::::::::::::::::::A::::::::::::::::::C::::::::::::::::::
 (c) ccggctcggtcgcgataaccgcgacatgatccagctcattaaggagttcgacgacatggg
 (d) tatattattctacgcagccgatttgaccgggcactatggtgcttcgatggcggggctgg
 (e) ::::::::::::::::::::A:

TTGCAAAAAGGATGGCGCAACGTGGCAATGGGGGTAAACCGTATACGCATCACGTGAGA 120
 ::::::::::::::::::::
 cgtggccatccgatttttggatgacggcatcagcacagagggaaacctgggcaaaatggg
 tcattggctgaccgttgatcggtcgcgtcatggcttgggcatattgcgagtatcaatgg
 ::::::::::::::C::::::::::::::::::

TGCATTTTCATCGACTTGGCGCCTTTCTACATCACACCAAGCAGCCCACATTAAAATAAG 180
 ::::::::::::::C::::::::::::::::::T::::::::::::::::::
 ggtgaccatcctgtcagccgtggctcaggccgaacgcctgcggatactggaacggaccaa
 agtgtgcgaggtgggcagcctaggccttgagttcctcgtaaccacata:AA::T:::::
 ::::::::::::::C::::::::::::::::::

xylS--→

AGAACCGTGAACATATGGATTTTGTCTATTGAACGAGAAAAGTCAGATCTTCGTCCACGC 240
 ::::::::::::::G::ATG::::::::::::C::::::::::::::::::T:::
 cgaaggctcgttttgaagcaaaggccaaggcgtgaagtttggccgcaaacctacgggtgga
 ::C:::::::::GT:ATG::::G:C::::G::::::::::::G:::::::::A:T:::
 ::::::::::::::G::ATG::::::::::::

CGAGCCCTATGCAGTCTCCGATTATGTTAACCAGTATGTCGGTACGCACTCTATTGCGCT 300
 ::::::::::::::C::::::::::::::::::
 caaagccgaggtattcacgctccacggtcagggtatcagtgcgatggaaattgccaaacg
 ::C::::::::CTTC:::::::::C::C:::::::::AC:::::::::T:::::::::G:::::::::
 ::::::::::::::

GCCCAAGGGCGGGCGCCCGGCAGGCAGGCTGCACCACAGAATCTTCGGATGCCTCGACCT 360
 ::::::::::::::::::::G::::::::::::::::::
 actgaagattggctcgctcgacggctctacaaagtgctggcatcataagttcatctgacaga
 ::::::A:T:::T:::::A::C::C::A:::::C:CT:::CA:::T::T:::
 ::::::::::::::

GTGTCGAATCAGCTACGGCGGTAGCGTGAGGGTAATCTCGCCTGGATTAGAGACCTGTTA 420
 ::::::::::::::::::::G::::::::::::::::::G::::::::::::::::::
 cgcgctgctgtcagccccgcccattgcttagcgctactttaaaatcccttttctgaggcgccc
 ::::::::::::::T::::::::::::::::::A::G:C::::A::C::G::A::::C:::
 ::::::::::::::

TCATCTGCAAATAATACTCAAAGGCCATTGCCTGTGGCGTGGCCATGGCCAGGAGCACTA 480
 ::::::::::::::A::::::::::::::::::
 C::C::::GC::T:G::::G::T:::::::::T:::::::::A::::TT:::::::::
 ::C::::GG::C:G::::G::::::::::::T:::::::::T:::::::::
 ::::::::::::::T::::::::::::::::::

Fig. 3 (for legend see page 563)

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TTTTGCGCCGGGCGAACTATTGCTGCTCAATCCGGATGACCAAGCCGACCTGACCTATTC 540
:::
:::A:::G::A:::C:::GC::G::G:::T:::
:::T:::T:::A:::G::G:::T:::
:::T:::
AGAAGATTGCGAGAAATTTATCGTTAAATTGCCCTCAGTGGTCCTTGATCGGGCATGCAG 600
:::
G::G::G:::C::T::C:::G::TA::A:::A:C:::A
G::G:::T:::C:::C:::A:::C:::
:::
TGACAACAATTGGCACAAGCCGAGGGAGGGTATCCGTTTCGCCGCGCGACACAATCTCCA 660
:::G:::
:::GCT:::G:::T:GA:::C::C:C:::T:GC::G::
:::G:G:T:::T:::C::A::A:::C::G::
:::
GCAACTCGATGGCTTTATCAATCTACTCGGGTTAGTTTGTGACGAAGCGGAACATACAAA 720
:::
:::G::TA:::C:::A:::AC:G:::ACAT
:::G::T::C:G:::G:::
:::
GTCGATGCCTCGGGTCCAAGAGCACTATGCGGGGATCATCGCTTCCAAGCTGCTCGAAAT 780
:::
G:::A:CTT::GCG:::CA::AA:::A:::G:C
T:::G::AT:::A:::T::A:::G::
:::
GCTGGGCAGCAATGTCAGCCGTGAAATTTTCAGCAAAGGTAACCCGTCTTTCGAGCGAGT 840
:::
:::CA:::CA::GGC::GT:G:::C:::A:G:::
:::A:::C::G:CC:::G:::TG:::C:::
:::
CGTTCAATTCATTGAGGAGAATCTCAAACGGAATATCAGCCTTGAGCGGTTAGCGGAGCT 900
:::
:::G:::A::G:::G:::A:::
:::G:::C:::A::GC:::AC:G:::
:::
GGCGATGATGAGTCCACGCTCGCTCTACAATTTGTTTCGAGAAGCATGCCGGCACCACGCC 960
:::
:::C:::C:::C:::CCA:::A:::
:::C:::C:::C:::T:::
:::C:::C:::C:::T:::

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Fig. 3 *continued* (for legend see facing page)

Fig. 3. Comparison of the nucleotide sequence of (a) the known ORF of *xyIS_{pWW0}* with the aligned sequences of regions spanning (b) *xyIS_{I_{pWW53}}*, (c) *xyIS_{2_{pWW53}}* and *xyIS_{2_{pDK1}}* (both identical), (d) *xyIS_{3_{pWW53}}* and (e) *xyIS_{I_{pDK1}}*. The sequences were optimally aligned using DNASIS (Pharmacia). Colons indicate identity with the pWW0 sequence. Extended regions of non-homology with pWW0 are given in lower case. Gaps introduced into the sequences to achieve optimal alignment are indicated by ^. The positions of the -12, -24 promoter for *xyIS_{pWW0}* (Inouye *et al.*, 1986) are underlined. The sequences of (b) and (e) are virtually identical to (a) in the region upstream of the ORF, whereas those of (c) and (d) are non-homologous. A region showing homology to the -12, -24 promoter consensus in (d) is underlined (bases 29-42) although its functional significance has not been established. The transcriptional start for *xyIS_{pWW0}* is indicated by a horizontal arrow (Ramos *et al.*, 1987). The proposed ORFs are as follows (putative start and stop codons are italicized): (a) 194-1159 (Inouye *et al.*, 1986), (b) 194-1159, (c) 719-1192, (d) 194-1189 and (e) 194-1189. An in-frame stop codon (334-346) in the region of (c) which is non-homologous to (a) is underlined.

In the case of *xyIS*_{1p_{DK1}}, 1679 bp were sequenced, with a putative ORF of 331 amino acids ($M_r = 37924$). The sequence of *xyIS*_{2p_{DK1}} was identical to that of *xyIS*_{2p_{WWS3}} and therefore encodes a possible truncated XylS-like protein of 157 amino acids ($M_r = 17894$).

Regulation of meta pathway operons on pWW53

The arrangement of catabolic genes on TOL plasmid pWW53 is clearly more complex than that on pWW0 (Franklin *et al.*, 1981). In addition to the large duplication of the *meta* pathway operon identified previously (Osborne *et al.*, 1988), there exist three homologues of *xylS*. Two of these (*xylS1* and *xylS3*) have definitively been shown to encode positive regulator proteins for the *meta* pathway genes. The expression of the *meta* pathway enzymes must involve interactions between these regulatory proteins and the operator-promoter regions of the two *meta* pathway operons. Although both *meta* pathway operons are functional, there are major differences in the specific activities of their encoded enzymes: the specific activities encoded by operon 1 are approximately 10-fold higher than those on operon 2, even when both operons are cloned on

XylS _{pWW0}	MDFCLLNEKSQIFVHAEPYAVSDYVNQYVGTHSIRLPKGGRPAGRLHHRI	(50)
XylS1 _{pWW53}	:::R::	
XylS3 _{pWW53}	::CR::::::R::N:D::F::::::::H::S:C::::::S:C:EAS:N:ST	
XylS1 _{pDK1}	::	
	FGCLDLCRISYGGSVRVISPGLETCTYHLQIILKGHCLWRGHGQEHYFAPG	(100)
	::G::	
	::S::::::::::::::::T::::::::VL::::::::Y:L::::S::	
	::	
	ELLLNPDQADLTYSCEKFIKLVSVVLDRACDNNWHKPREGIRFA	(150)
	::	
	:::I:::R::::::::::::::::::::::::::::::::::::ESY:::S:::T	
	::	
	ARHNLQQLDGFINLLGLVCDEAEHTKSMPRVQEHYAGIIASKLLEMLGSN	(200)
	::	
	T::::::::::::::::::::::::::::N::::Y:T:::T:::S::	
	::	
	VSREIFSKGNPSFERVVQFIEENLKRNISLERLAELAMMSPRSLYNLFEK	(250)
	::	
	:::T:E:C::::::::::::D::QS::::::::L::::::::T:::	
	::	
	HAGTTPKNYIRNRKLESIRACLNDPSANVRSITEIALDYGFLHLGRFAEN	(300)
	::	
	::::::::::::::::::::::C::R:S::N::::V::M::::F:T:::	
	::::::::::::::::::::::C::R:S::N::::V::M::::F:T:::	
	YRSAFGELPSDTRLRQCKKEVA	(321)
	::::::::::::::::::::::::::::::	(321)
	:::T::::::::::::RR:MKWLDPEESLPPLP	(331)
	:::T::::::::::::RR:MKWLDPEESLPPLP	(331)

Fig. 4. Comparison of the deduced amino acid sequences of the XylS proteins from pWW53 and pDK1 with the published sequences of the proteins from pWW0 (Inouye *et al.*, 1986; Spooner *et al.*, 1986). Colons indicate identity with the homologous pWW0 protein.

equivalent *Hind*III fragments downstream of an identical vector promoter (Osborne *et al.*, 1988). It has been suggested that the lower activities of the operon 2

enzymes reflect intrinsic differences in the kinetic properties of the enzymes, rather than a difference in the expression of the two operons (Williams *et al.*, 1990).

DNA sequencing of the two operon promoters, *Pm1*_{pWW53} and *Pm2*_{pWW53}, as part of this study (data not shown) supports this view by demonstrating that both promoters are very similar and both highly homologous to *Pm*_{pWW0}.

Functional analysis of the *pWW53* and *pDK1* *xylS* genes

The regulatory function of *xylS*_{pWW53} has been demonstrated previously by the inducibility of the *meta* pathway enzymes in a *P. putida* strain carrying HA cloned into pKT230 upon which it is the only homologue (Keil *et al.*, 1985a). The activity of *xylS*_{pWW53} was demonstrated in this study by the inducibility of *meta* pathway enzymes in strains carrying this gene in conjunction with either HB of pWW53 or the novel *Hind*III fragment HA' from pWW53-2 (Table 2). The data are consistent with *xylS*_{pWW53} encoding a functional gene product which can interact with the promoters of both *meta* pathway operons.

The regulatory function of *xylS*_{pDK1} has been demonstrated in an earlier study (Shaw & Williams, 1988).

It is impossible to state with any certainty whether the *xylS2* genes from either plasmid can act as functional activators. In both cases there is very indirect evidence which points to possible functionality. This comes from deleted plasmids generated *in vivo* in which the *xylS2* genes are the only homologues present. In the case of plasmid pWW53 the deleted derivatives are pWW53-1 and pWW53-2 (see Fig. 1; Osborne *et al.*, 1988), and for pDK1 the deletion is in plasmid pDKM1 (Kunz & Chapman, 1981). In *P. putida* strains carrying these plasmids the *meta* pathway genes are induced by benzoate or toluates (Osborne *et al.*, 1988; Kunz & Chapman, 1981). However, we have as yet been unable to demonstrate that either of the cloned *xylS2* genes can function as benzoate- or toluate-stimulated activators in an expression system. Furthermore, a recent paper has demonstrated that a gene in the *P. putida* chromosome, which is probably *benR*, can substitute for *xylS* in such an expression system although only with unsubstituted benzoate as inducer (Jeffrey *et al.*, 1992). It is possible that the inducibility of the *meta* pathway genes on pWW53-1, pWW53-2 and pDKM1 could be due to this chromosomal regulator gene.

Sequence analysis of the *pWW53* and *pDK1* *xylS* genes

There is striking homology between *xylS*_{pWW53} and *xylS*_{pWW0} at both the DNA (99%) (Fig. 3) and amino acid (99%) sequence level (Fig. 4).

For *xylS*_{pWW53}, there is a high level of DNA (87%)

and amino acid (83%) sequence homology with *xylS*_{pWW0} within the coding portion of the gene, but there is significant divergence in the 5' leader sequence (Fig. 3) and downstream of the ORF (data not shown). The upstream breakpoint in homology starts only about 23 bases upstream of the presumed translational start codon. There is a frame-shift mutation towards the end of the coding region (base 1146) which causes the C terminus of the *XylS*_{pWW53} protein to be elongated by an additional 10 amino acid residues relative to *XylS*_{pWW0}. The deduced amino acid sequence of *XylS*_{pDK1} is the same length (331 amino acids) as *XylS*_{pWW53} and has the same C-terminal extension.

The DNA upstream of *xylS*_{pWW53} and *xylS*_{pDK1} is highly homologous to that of *xylS*_{pWW0} to the extent that it is possible to identify the -12, -24 promoter (Fig. 3) and a number of the other regulatory elements which have been demonstrated or proposed for *xylS*_{pWW0} (data not shown). Within the non-homologous upstream region of *xylS*_{pWW53}, there is a sequence showing homology to the -12, -24 promoter of *xylS*_{pWW0} (Fig. 3) but no evidence has been obtained as yet to demonstrate its functional significance.

The two *xylS2* sequences are identical and both present the same problem of interpretation. There is 82% identity between the *xylS2* region and the pWW0 ORF downstream of base 420 but no homology upstream (Fig. 3). The possibility that *xylS2* encodes a single *XylS* protein with a divergent N terminus is ruled out by the presence of an in-frame TAA stop codon (bases 344–346) (Fig. 3). In the region that is homologous to *xylS*_{pWW0}, there is an ORF which runs from bases 719 to 1192 with a potential ribosome-binding site (RBS) centred 10 bp upstream of the ATG which shows good homology with other *Pseudomonas* RBS sequences (Spooner *et al.*, 1986). This ORF would give rise to a protein of 157 amino acids homologous to the C-terminal portion of *XylS*_{pWW0} but lacking its N-terminal half. However, if this does encode a truncated version of *XylS*, it is surprising that there is still such marked homology with the *xylS*_{pWW0} sequence upstream of its start codon. It might be expected that any similarity in DNA sequence would have been lost outside of the shortened coding region, unless the divergence of *xylS2* from *xylS*_{pWW0} was very recent in evolutionary terms. Analysis of both *xylS2* sequences upstream of their regions of homology to the pWW0 *xylS* ORF has shown the presence in each case of a complete ORF with a high degree of homology to a number of recombinase and transposon resolvase genes (Assinder *et al.*, 1992). At present the most probable explanation is that the two *xylS2* homologues represent the residues of complete *xylS* genes into which a transposon or insertion sequence has been inserted recently.

The presence of *xylR* genes on *pDK1* and *pWW53*

Hybridization results have suggested that both plasmids (Keil *et al.*, 1987*b*; Shaw & Williams, 1988) contain only one region that is homologous to *xylR*_{pWW0}. In both plasmids this region is adjacent to their *xylS1* genes. This is confirmed by the sequences upstream of both *xylS1*_{pWW53} and *xylS1*_{pDK1} (data not shown). In both there is the start of an ORF which is highly homologous to the start of *xylR*_{pWW0} and similarly reads in the opposite direction. No such homology is found upstream of the other *xylS* homologues.

The relationship between *pDK1* and *pWW53*

The major point of interest concerning the two *xylS* homologues on *pDK1* comes when we compare them with the three homologues from *pWW53*, taking into account the genetic organization and restriction maps of the two plasmids (Fig. 1). The plasmids share two large areas which have identical or virtually identical restriction maps. The *meta* pathway operon (*xylXYZLTEGFJQKIH*) of *pDK1* has been cloned on a *Hind*III fragment (HA, Fig. 1) measured at 15.5 kbp (Shaw & Williams, 1988). One of the two *meta* pathway operons on *pWW53*, referred to as *meta* pathway operon 2, is found on *Hind*III fragment HB, measured at 15.6 kbp (Osborne *et al.*, 1988). The two fragments have in common 48 restriction sites for eight different enzymes. Furthermore, the identity of the two restriction maps extends about 6 kbp upstream of the fragment carrying the operon and about 5 kbp downstream, taking in a further 25 restriction sites (Fig. 1). Within a total length of about 26 kbp we have found no difference in the restriction fragmentation of the two plasmids. This paper has shown the identity at the nucleotide level for the *xylS2* regions which are internal to these long indistinguishable regions on the two plasmids (Fig. 3).

The second region of homology shared by *pWW53* and *pDK1* covers the 7 kbp of their two upper pathway operons (*xylCMABN*) together with about 2 kbp downstream and 4 kbp upstream, which includes the *xylR* genes on each plasmid. Within these regions they share 33 common sites for nine restriction enzymes. In this case, however, there is a single difference as *pWW53* has one additional *Hind*III site towards the end of the operon (Fig. 1) which is not present on *pDK1*. It could be that this homology extends further downstream of the operons but we have not mapped *pDK1* any further in that direction.

The two homologous regions shared by *pDK1* and *pWW53* differ in their relative orientations on the two plasmids and on *pDK1* they are contiguous whereas on *pWW53* they are separated (Fig. 1). This can be explained when the sequence of *xylS1*_{pDK1} (Fig. 3) is examined. The

Fig. 5. Comparison of the nucleotide sequence of *xyIS1*_{pDK1} and the flanking DNA with the homologous DNA of (a) *xyISpWW0*, (b) *xyIS1*_{pWW53} and (c) *xyIS3*_{pWW53}. The sequences were optimally aligned using DNASIS (Pharmacia). The number of mismatches between the two sequences was determined within a 60 bp window of the *xyIS1*_{pDK1} sequence. Successive bar lines represent the windows displaced along the sequence by 30 bp and the bar is drawn at a position on the corresponding to the mid-point of the 60 bp window. The numbering on the x-axis corresponds to the numbering of the bases in the Genbank file for *xyIS1*_{pDK1}, which extends 440 bases upstream of the beginning of the sequence as presented in Fig. 3(d) and 633 bases upstream of its ORF. All three comparisons extend to base 1650, 24 bp after the end of the ORF. The arrows represent the point at which it was possible to start the comparison because of the length of the sequence information available on the other strands: (b) starts at base 121, and (c) at base 361. The locations of *xyIRpWW0* (R) and *xyISpWW0* (S) and their intergenic region is shown at the top of (a), and the direction of transcription is indicated by the horizontal arrows. The compared sequence for (a) is taken from Spooner *et al.* (1986).

gene is located at the boundary of the two regions of homology which *pDK1* shares with *pWW53* (Fig. 1). Comparison of its nucleotide sequence with those of *xyIS1*_{pWW53} and *xyIS3*_{pWW53} reveals that it appears to be a hybrid of the two (Fig. 5). From the first common nucleotide sequenced up to approximately base 1340 (as numbered on Fig. 5) the sequences of *xyIS1*_{pDK1} and *xyIS1*_{pWW53} are highly homologous. Within the coding

region there is an even distribution of base differences of around 0–3 bases per 60 nucleotides. In the intergenic region between *xyIR* and *xyIS* there is a higher level of difference which is due almost entirely to two separate insertions of 8 bp which are found only in the *xyIRS1*_{pWW53} sequence. In fact the sequence of pDK1 DNA in this region and for most of the coding region of *xyIS1* is even closer to that of *xyIS*_{pWW0} DNA (Fig. 5a) than that of *xyIS1*_{pWW53} (Fig. 5b).

Over the same region (up to about base 1340) there is a much-lower degree of homology between the *xyIS1*_{pDK1} DNA and the DNA around *xyIS3*_{pWW53} (Fig. 5c). Upstream of the *XyIS* ORFs there is no significant homology with base differences of around 35 per 60 bases: this agrees with the apparent absence of a *xyIR* gene upstream of *xyIS3*_{pWW53}. Within the ORFs there are approximately 5–10 differences per 60 bases, showing a substantially lower degree of homology than found when the comparison is with the coding sequences of *xyIS1*_{pWW53} and *xyIS1*_{pDK1} (Fig. 5b). The 5' end of the *xyIS1*_{pDK1} ORF is therefore very similar to both *xyIS*_{pWW0} and *xyIS1*_{pWW53} (but marginally closer to the former) and is significantly different to *xyIS3*_{pWW53}. However, from about base 1340 the level of homology with both *xyIS1*_{pWW53} and *xyIS*_{pWW0} drops (Fig. 5a, b) to less than 90% until at the end of the ORF and into the short stretch of the downstream regions which have been sequenced it becomes insignificant. Over this same region, *xyIS3*_{pWW53} and *xyIS1*_{pDK1} are identical (Fig. 5c).

The hybrid nature of the *xyIS1*_{pDK1} gene (*xyIS1*_{pWW53}:*xyIS3*_{pWW53}) exactly parallels its location relative to the two large regions of homology which are shared by pDK1 and pWW53. Whereas the 5' end of the gene is adjacent to a *xyIR* gene and is upstream of the upper pathway operon, in exactly the same way as *xyIS1*_{pWW53}, its 3' end is located identically to *xyIS3*_{pWW53}, both genes being upstream of regions carrying indistinguishable *meta* pathway operons.

Both the hybrid nature of *xyIS1*_{pDK1} and its location can be explained if pDK1 is derived from a plasmid similar to pWW53. A single reciprocal recombination event between two copies of *xyIS* on pWW53 (in this case *xyIS1* and *xyIS3*) in the vicinity of base 900 (Fig. 3) (or codon 232, Fig. 4) would produce a gene with the structure of *xyIS1*_{pDK1} and would also cause a substitution of a *meta* pathway operon like (*xyIXYZLTEGFJQKIH*)₂ in place of and in the opposite orientation to (*xyIXYZLTEGFJQKIH*)₁, giving exactly the gene arrangement found on pDK1 (Fig. 1).

Two possible events could lead to the structure of pDK1. The recombination could occur either within a plasmid like pWW53 or between two TOL plasmids co-existing within the same strain. In either case, one ancestor of pDK1 would require more than one copy of a *xyIS* gene

to give the structure found. It had occurred to us that such a recombination event might have taken place in our laboratory since both strains were being studied on adjacent benches: this would make a weaker case for the occurrence of such an event being relevant to evolution in the wild. However, one argument can be brought to bear against the formation of pDK1 being artefactual. The 5' end of *xyIS1*_{pDK1} is unique and is not identical to the 5' ends of either *xyIS1*_{pWW53} or *xyIS*_{pWW0} (Figs 4 and 5). Furthermore, the entire sequence of the *meta* pathway operon and the *xyIRS1* region of pDK1 has been independently determined and the two sequences are identical (R. C. Benjamin, personal communication).

Duplications appear to be a common feature of the catabolic genes of TOL plasmids and possible reasons that they might confer a selective advantage on a plasmid have been discussed elsewhere (O'Donnell & Williams, 1991). Whether or not there is any selective advantage, the results in this paper illustrate that a consequence of the presence of such duplications is that they can serve as points at which recombination can occur to give rise to both new hybrid alleles of the genes and major rearrangements of DNA.

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