Duplication of both *xyl* catabolic operons on TOL plasmid pWW15

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Pseudomonas fluorescens MT15 is the host of the large (250 kbp) TOL plasmid pWW15. We have shown by a combination of hybridization, molecular cloning and enzyme assay that pWW15 carries two distinct regions which share homology with the upper pathway operons (*xylCMABN*) of other TOL plasmids and two distinct regions which are homologous to the *meta* pathway operons (*xylXYZLTEGFJQKIH*) of other TOL plasmids. Both the areas of homology to the upper pathway operons appear to carry all of the structural genes for the three catabolic enzymes of the operon. One of the regions of *meta* pathway operon homology encodes a complete functional pathway, but the second is incomplete and appears to carry only the genes from *xylF* downstream.

Introduction

TOL plasmids encode the catabolism of toluene (or some alkyltoluenes) via the sequential oxidation of the methyl group to produce benzoate (or an alkylbenzoate) and the further catabolism of this to central metabolites by a *meta* cleavage pathway (Worsey & Williams, 1975; Kunz & Chapman, 1981). Whereas most of the molecular biology of the catabolic pathway has been characterized on the 117 kbp plasmid pWW0 from *Pseudomonas putida* mt-2, a number of other plasmids encoding the same catabolic functions have been described (see Assinder & Williams, 1990). There is an increasing amount of evidence which suggests that the genetic organization of the *xyl* catabolic genes found on pWW0, namely single operons for the upper pathway (Harayama *et al.*, 1989) and the *meta* pathway genes (Harayama & Rekik, 1990), is not typical of many of these other plasmids. Chatfield & Williams (1986) showed that newly isolated plasmids contained either two homologous genes or two non-homologous genes for the ring-cleavage dioxygenase, catechol 2,3-dioxygenase (C23O, *xylE*). Plasmid pWW53 falls into the former group, carrying two homologous *xylE* genes as part of a complete duplication of the *meta* pathway operon (Osborne *et al.*, 1988). The

subject of this investigation, pWW15, falls into the latter of the two groups, having two non-homologous genes for C23O (Keil *et al.*, 1985*b*). pWW15 is one of three large, structurally related TOL plasmids, pWW14, pWW15 and pWW20, which have the characteristic property of being strongly counterselected during growth of their natural hosts in benzoate minimal media (Williams & Worsey, 1976). After about 20 generations no strains with the typical TOL phenotype can be detected in the population, which consists either of plasmid-free cells or of cells which have an altered, defective catabolic phenotype (Williams & Worsey, 1976; Worsey & Williams, 1977; Pickup & Williams, 1982; Pickup *et al.*, 1983; Keil & Williams, 1985) and which have suffered large deletions of plasmid DNA. In this paper we report the existence of two upper pathway operons and two *meta* pathway operons on pWW15. One of the *meta* pathway operons is incomplete and lacks the upstream genes.

Methods

Bacterial strains and plasmids and their culture and maintenance. 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*). Strain MT15 was originally classified as *P. putida* but has since been assigned to the closely related species *P. fluorescens* (M. G. Höfle, personal communication).

Plasmid purifications. pWW15 and its large deletion derivatives were prepared by the method of Wheatcroft & Williams (1981). Vectors and recombinant plasmids were extracted from *E. coli* by the cleared lysate procedure (Guerry *et al.*, 1973) followed by caesium chloride/ethidium bromide centrifugation. The method of Holmes & Quigley (1981) was used for rapid screening of plasmid contents of cells.

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Abbreviations: BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; OEH, 2-oxopent-4-enoate hydratase; OD, 4-oxalocrotonate decarboxylase; OT, 4-oxalocrotonate tautomerase; XO, xylene monooxygenase.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Note/reference
<i>P. fluorescens</i> MT15	pWW15	Wild-type TOL plasmid; Williams & Worsey (1976)
<i>P. putida</i> PaW130		Plasmid-free <i>P. putida</i> mt-2; Keil <i>et al.</i> (1985a)
<i>P. putida</i> PaW263	pWW0- <i>xyIA</i>	Franklin & Williams (1980)
<i>P. putida</i> PaW823	pWW0-660	pWW0 plasmid with <i>xyICMAB</i> deletion; Lebens & Williams (1985)
<i>E. coli</i> ED8654		Murray <i>et al.</i> (1977)
	pKT230	Broad host range vector; Bagdasarian <i>et al.</i> (1981)
	pNJ5000	Unstable RP4-derived mobilizing plasmid; Grinter (1983)
<i>Recombinant plasmids with pWW15 fragments cloned into pKT230*</i>		
	pWW15-4019 XC (+)	
	pWW15-4020 HJ (-)	
	pWW15-4024 HF (-)	
	pWW15-4025 HF (+)	
	pWW15-4036 XA (+)	
	pWW15-4050 XB (+)	
	pWW15-4051 XB (-)	
	pWW15-4052 HC (-)	
	pWW15-4053 HC (+)	
	pWW15-4054 HA (+)	

* (+) Orientation transcribing from vector promoter; (-) opposite orientation.

Restriction enzyme digestions and electrophoresis. Digestions were carried out according to the manufacturers' instructions and electrophoresis performed according to Wheatcroft & Williams (1981).

Cloning, transformations and DNA hybridizations. These were carried out as described by Keil *et al.* (1985a).

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

Enzyme assays. Cells of *Pseudomonas* were grown in induced and uninduced conditions as described in Keil & Williams (1985) and cells of *E. coli* were grown overnight in Luria broth with addition of an appropriate antibiotic. All enzyme assays were carried out on sonic extracts of cells using previously reported methods: benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH) (Worsey & Williams, 1975); catechol 2,3-dioxygenase (C23O), 2-hydroxymuconic semialdehyde dehydrogenase (HMSD), 2-hydroxymuconic semialdehyde hydrolase (HMSH), 4-oxalocrotonate tautomerase (OT) and 4-oxalocrotonate decarboxylase (OD) (Sala-Trepat & Evans, 1971); 2-oxopent-4-enoate hydratase (OEH) (Collinsworth *et al.*, 1973).

Results

Identification of two upper pathway operons

*Hind*III restriction digests of pWW15 were hybridized with a probe derived from the 7.1 kbp *Hind*III-F fragment of TOL plasmid pWW53, which has been shown to carry some of the genes of the *xyICMABN* (upper pathway) operon (Keil *et al.*, 1987a). Two fragments were found to hybridize strongly with this probe, an 8.5 kbp fragment denoted HF and a 7.2 kbp fragment denoted HJ. When the same probe was used

against *Xho*I digests of pWW15, at least five fragments hybridized including XA, XB and XC. Since the sum of the sizes of these fragments (> 25 kbp) greatly exceeds the size of the probe, the results suggested that there might be multiple areas of homology to *Hind*III-F_{pWW53} on pWW15. We therefore cloned all of the relevant fragments into vector plasmid pKT230 (Table 1). The fragments were digested with 10 different restriction enzymes and the restriction sites mapped: the results are shown in Fig. 1. There are nine sites located in similar positions on both HF and HJ and on the overlapping *Xho*I fragment (Fig. 1). This similarity in the two restriction maps is reflected in the fact that both cloned fragments hybridize against each other in Southern blots. When the maps are compared with that of *Hind*III-F_{pWW53} it is clear that all three have common restriction site configurations: a significant difference is that HJ does not share any similarity with the pWW53 fragment in the region in which the operator-promoter region (OP1) of the latter has been mapped (Keil *et al.*, 1987a).

The relationship between HF and HJ and the co-hybridizing *Xho*I fragments was deduced from two lines of argument.

(a) The cloned fragments were restricted and single and multiple digests were compared for similarly sized fragments.

(b) The presence or absence of the relevant *Hind*III and *Xho*I fragments were examined in a number of the mutants containing large deletions in pWW15 which we had obtained by growth of MT15 on benzoate (Keil & Williams, 1985): for example, in one such plasmid both HF and XB are absent but HJ and XA are present, thus implicating HF and XB as overlapping.

Fig. 1. Restriction maps of two *Hind*III fragments HF and HJ carrying upper pathway genes from plasmid pWW15 compared with the homologous fragment from pWW53. The thick lines represent the two *Hind*III fragments as indicated and the horizontal dotted line is a continuation into the overlapping *Xho*I fragments. HF overlaps to the right with fragment XB which starts at the *Xho*I site at coordinate 6.3 and HJ overlaps to the right with fragment XA which starts at the homologous *Xho*I site also at coordinate 6.3. The positions of *xylCAB* and the operator-promoter (OP1) are as determined by Keil *et al.* (1987a). It is assumed that there is a gene corresponding to *xylM* as has been determined for pWW0 (Harayama *et al.*, 1989). Similar restriction sites in each have been linked with dotted lines. Although the overlapping fragments XA and XB have both been fully mapped, the *Eco*RI and the *Hind*III sites at the right-hand ends of the maps are the only ones which correspond on both regions. Nomenclature for restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I.

Table 2. Expression of activities of upper pathway enzymes in *E. coli* ED8654 carrying cloned pWW15 fragments

Plasmid	Cloned fragment	Specific activity [munits (mg protein) ⁻¹]	
		BADH	BZDH
pWW15-4025	HF(+)	1	16
pWW15-4020	HJ(-)	<1	11
pWW15-4036	XA(+)	490	1
pWW15-4050	XB(+)	650	<1

Determination of upper pathway enzyme activities expressed

The recombinant plasmids carrying the fragments HF, HJ, XA and XB were assayed in *E. coli* hosts for the *xylB* and *xylC* gene products, benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH) respectively (Table 2). The results clearly show the presence of BZDH, but not BADH, on HF and HJ and correspondingly the presence of BADH, but not BZDH, on XA and XB. This shows that the right-hand end of each *Hind*III fragment must lie within or upstream of

xylB whereas the left-hand end of each *Xho*I fragment must lie upstream of the gene. This is what was expected from the comparison with the pWW53 map (Fig. 1).

The *xylMA* gene product, xylene monooxygenase (XO), cannot be assayed in cell extracts but can be determined either by complementation of a XO-negative mutant (Keil *et al.*, 1987a) or by production of indigo from indole or tryptophan (Mermod *et al.*, 1986; Keil *et al.*, 1987a). Transfer of the recombinant plasmid pWW15-4025 carrying HF into a *P. putida* host and growth in the presence of indole caused accumulation of indigo indicating the presence of *xylMA*. However, this

Fig. 2. Comparison of the restriction maps of the two fragments carrying *meta* pathway operon genes from pWW15 (HA and HC) with the corresponding fragment carrying the *meta* operon 1 genes from pWW53 (Keil *et al.*, 1985a). Restriction sites in similar location on the pWW53 fragment and HA are linked with dotted lines. The presence of *xylT* and *xylQ* homologues on pWW53 have not been demonstrated but are inferred from the results of Harayama & Rekik (1990) with pWW0. Restriction sites are denoted as in Fig. 1.

did not work with the corresponding plasmid pWW15-4020 carrying HJ. We presume that this was because it was oriented in the opposite direction relative to the vector promoter and did not express sufficiently high levels of activity: the production of indigo by XO is far weaker than the corresponding reaction carried out by cloned dioxygenases (Ensley *et al.*, 1983). However, the presence of at least one of the two genes of *xylMA* on HJ was indicated by complementation by pWW15-4020 of PaW263, a XO-negative mutant of *P. putida* mt-2 (Keil *et al.*, 1987a), enabling it to grow on *m*-xylene.

Identification of two *meta* pathway operons

During experiments in which we were attempting to map regions of pWW15, many restriction fragments were cloned and hybridized against single and multiple restriction digests of the plasmid. The largest *Hind*III fragment HA (18 kbp), when used as a probe in this way, was found to hybridize both with itself and with a second *Hind*III fragment HC (11.4 kbp). This latter fragment was then cloned into pKT230 (Table 1) and both HA and HC were mapped for restriction sites (Fig. 2). Comparison of the two maps shows a small degree of similarity around coordinates 10–14 kbp where there are four common sites, but no other similarities.

A comparison of the restriction maps with the published map of the *meta* pathway operon of pWW53 (operon 1) shows that the complete operon on HA has considerable similarity with 22 common sites. There are

also 11 sites in common with the homologous operon on pWW0 (not shown). Alignment of the restriction sites between HA and pWW53 (Fig. 2) can only be optimized if it is assumed that there are at least two insertions/deletions on HA relative to pWW53, one of which is particularly apparent at the beginning of the operon. Thus the close *Xba*I–*Sst*I–*Pst*I sites at about coordinate 2 on operon 1 of pWW53 are found at about 3.4 on HA of pWW15. This may be the result of about 1.4 kbp of DNA in HA which is not present in pWW53. However, the pWW53 and the pWW15(HA) operons are both on virtually identically sized fragments (17.4–17.5 kbp) and the extra DNA in the latter may be compensated for by a corresponding deletion somewhere later in the operon (cf. the close alignment of the two *Bgl*II sites on each around coordinate 15–16).

Determination of *meta* pathway activities expressed

Both recombinant plasmids were mobilized into plasmid-free PaW130 in order to assess their function and the enzyme activities expressed. PaW130(pWW15-4045), containing HA, was able to grow on *m*-toluate, suggesting that it might contain a complete *meta* pathway operon but PaW130(pWW15-4053), with cloned HC, was unable to grow on *m*-toluate. The two PaW130-derived strains were assayed for *meta* pathway activities in the absence and presence of *m*-toluate or *m*-xylene as inducers (Table 3).

Table 3. Expression of activities of meta pathway enzymes in *P. putida* PaW130 carrying cloned pWW15 fragments

Plasmid	Inducer	Specific activity [munits (mg protein) ⁻¹]					
		C23O	HMSD	HMSH	OT	OD	OEH
pWW15-4054	None	50	5	5	19 300	120	480
HA(+)	<i>m</i> -Toluate	380	50	150	205 000	820	8 180
pWW15-4053	None	<1	<1	12	1470	30	310
HC(+)	<i>m</i> -Toluate	<1	<1	11	ND	ND	ND

ND, Not determined.

The plasmid carrying cloned HA expressed detectable levels of all six activities assayed, in the absence of inducers: the presence of *m*-toluate, but not *m*-xylene, caused significant induction of all the activities. These results are exactly analogous to the results obtained previously with cloned *meta* pathway operon 1 from pWW53 (Keil *et al.*, 1985a) and indicate that, in addition to the structural genes for the enzymes, the operon also has a gene, functionally equivalent to the regulatory gene *xylS* and an operator-promoter (OP2) upstream of the operon equivalent to OP2. This is not unexpected since at the downstream end of the fragment there are two *Bgl*II sites characteristic of those found in equivalent positions on pWW0 (Spooner *et al.*, 1986) and pWW53

operon 1 (Keil *et al.*, 1987b) and associated with the region of *xylSR*. We also detected C23O activity on the small Xh subclone of HA (data not shown), confirming the earlier results of Keil *et al.* (1985b) which showed *xylE* on that small (2.1 kbp) fragment.

On the other hand, the recombinant plasmid carrying HC caused significant expression of only four of the six enzymes assayed (HMSH, 4OT, 4OD and 2OEH) and no detectable C23O or HMSD activity (Table 3). HC therefore appears to contain only part of a *meta* pathway operon and, in line with the published order of *xylXYZLTEGFJQKIHSR* (Harayama & Rekik, 1990) would seem to be lacking the upstream end of the operon and the early genes *xylXYZLTEG*.

Fig. 3. Demonstration of co-linearity of DNA on pWW15 fragments HA and HC with the DNA of *meta* pathway operon 1 from pWW53. The pWW53 fragments used as radioactive probes are designated by hatched boxes and are numbered 1 to 6. The area on pWW15 hybridizing to each probe was determined by Southern blots of the pWW15 fragments digested both with single and multiple enzymes. The area hybridizing on each is denoted by open boxes also numbered 1 to 6: each represents the maximum area of homology given the particular digestions used. Restriction sites are denoted as in Fig. 1.

An additional experiment was carried out to confirm the colinearity of the DNA on the two pWW15 fragments and the well-characterized pWW53 operon 1 (Keil *et al.*, 1985a). A sequence of subcloned fragments from the pWW53 operon were used as probes against different restriction digests of both pWW15 fragments. Six probes were used covering the genes *xylXYZL* (probe 1), *xylFJ* (probe 2), *xylJK* (probe 3), *xylI* (probe 4), *xylHS* (probe 5) and *xylR* (probe 6); the results are presented in Fig. 3. The results confirm (a) the co-linearity of the DNA in each operon, (b) the absence of the early enzymes from HC and (c) the apparent existence of extra DNA present in HA which does not hybridize with probe 1. As has been described above, *xylSR* appears to lie downstream of the operon on HA and is characterized by the presence of the two adjacent *Bgl*II sites at around coordinate 16. However, it is notable that the *xylR* probe (probe 6) hybridizes with a region of DNA upstream of the partial operon on HC. The hybridization on the Southern blot was strong suggesting that this is not a non-specific reaction and indicates that some major rearrangement may have taken place during the genesis or past history of the DNA of this region. We have no evidence that this area of homology carries a functional *xylR* gene.

Discussion

The evidence presented here further confirms our earlier results showing that many TOL plasmids carry duplications of catabolic DNA (Keil *et al.*, 1985b; Chatfield & Williams, 1986; Osborne *et al.*, 1988) and extends the scope of the duplications revealed. The duplications previously reported involved only the genes of the *meta* pathway, in particular C23O, whereas here we report the first identification of a duplicated upper pathway operon. The results show that pWW15 carries two functional upper pathway operons, a complete *meta* pathway operon, a homologous but incomplete *meta* pathway operon and also the previously reported additional gene for a C23O (Keil *et al.*, 1985b) which is non-homologous to any of the *xylE* genes found on *xyl meta* pathway operons. Evidence not presented here indicates that the duplicated operons are considerable distances apart on pWW15. HF and HA are on one side of the large region lost in the deletion mutants whereas HJ and HC are on the opposite side: thus each operon is on the opposite side of the plasmid from its duplicate copy.

It is remarkable that pWW15 remains a stable plasmid under most conditions of growth: we have not detected any deletion derivatives during normal maintenance of the host strain *P. fluorescens* MT15 on non-selective

carbon sources such as succinate, acetate or nutrient broth. However, this stability must be finely balanced since there is a very rapid counterselection against the native plasmid during growth on benzoate-minimal medium (Williams & Worsey, 1976; Keil & Williams, 1985; Stephens & Dalton, 1987, 1989). Such counterselection applies to all TOL plasmids and the reasons have been the subject of much debate (see Assinder & Williams, 1990). Our interpretation has been that there must be a low frequency of spontaneous formation of segregants and that growth on benzoate simply amplifies their presence as a result of their faster growth rate (Williams *et al.*, 1988). In two instances, the segregants formed are the result of recombination between regions of homology on the plasmid. These are the 1.4 kbp direct repeats on pWW0 (Meulien *et al.*, 1981), which result in the loss of the entire intervening catabolic DNA (Bayley *et al.*, 1977), and the two directly repeated duplicate *meta* pathway operons on pWW53, which recombine to form a hybrid operon with the deletion of the intervening upper pathway operon (Osborne *et al.*, 1988). It is likely that the greater degree of duplication on pWW15 may well contribute to the high rate of loss of the wild-type phenotype during benzoate selection of strain MT15 and that the unusual nature of the mutant phenotypes results from differences in the regulation of the repeated elements.

On a more general level, the potential instability of these plasmids, as is revealed by growth on benzoate, suggests that there is a counteracting selection pressure which ensures the perpetuation of the duplications under normal growth conditions in the laboratory. Gene duplication appears to be a widespread phenomenon amongst TOL plasmids and plasmids with duplicated operons may also be at a selective advantage in the natural environment over those with only single copies. Certainly gene duplications *per se* are not stable. We have reported instances where we have been able to induce tandem multiplication of the catabolic genes under the right selective pressures using some of the deletion derivatives of pWW15 (Keil & Williams, 1985). These mutants are, as expected, highly unstable under non-selective conditions and rapidly lose the multiple gene copies.

If gene duplications are potentially so unstable and yet appear to be so prevalent in new isolates from the environment, what is their selective advantage? Several testable hypotheses can be made.

(a) Heteromultimeric enzymes with advantageous properties are formed between the gene products of the duplicate copies of some of the genes.

(b) The regulation of the duplicate operons/genes results in their being expressed under different environmental conditions and those conditions fluctuate such

that there is intermittent selection for maintenance of both.

(c) Gene conversion occurs between the duplicate copies to ensure a high level of repair of mutations, as has been shown to occur between the homologous *cat* and *pca* genes of the β -ketoadipate pathway (Doten *et al.*, 1987).

The existence of duplications also poses the question as to how they arose in the first place. Did they result from tandem duplications within a plasmid and subsequent mutational divergence or from a recombination event occurring between two different catabolic plasmids co-existing transiently within the same cell? There may not be a single answer to this question and both mechanisms may operate. On pWW15 there is some similarity in the restriction maps of the two *meta* pathway regions covering a region of about 4 kbp and four restriction sites (*Sal*I to *Sma*I, coordinates 10–14, Fig. 2) which is not found on the other TOL plasmid operons which have been mapped. This could be taken to indicate a common origin by internal duplication. On the other hand, if gene conversion were to occur between duplicate copies, there would be a degree of genetic homogenization between them which would introduce similarities and mask initial differences.

There are more questions posed by this phenomenon than current data can answer. There have been some reports of duplications on other catabolic plasmids (Negoro *et al.*, 1984; Kaphammer *et al.*, 1990) and it seems unlikely that there is a feature unique to toluene-xylene catabolism which isolates it in this respect from other plasmid-encoded catabolic pathways. It would, however, seem to be prudent to investigate the generality of this situation in strains which have been recently isolated from nature rather than strains which have been subcultured in laboratories for long periods. Thus the archetypal TOL plasmid pWW0, with its single copies of the two catabolic operons, may be atypical of wild-type plasmids in the environment for this very reason. It was originally isolated nearly 40 years ago (T. Nakazawa, personal communication) and has subsequently been continuously subcultured. It is possible that its present genetic organization is significantly different from when it was first isolated.

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