Complementation of Deletion and Insertion Mutants of TOL Plasmid pWW0: Regulatory Implications and Location of *xylC* Gene

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Two spontaneous mutant plasmids of TOL plasmid pWW0 are described. pWW0-660 contains an 18 kbp deletion of the entire *xylCAB* operon and pWW0-673 has a 3.4 kbp insert in the operator–promoter (OP1) region of *xylCAB*. In *Pseudomonas putida* hosts containing either plasmid, *m*-xylene was unable to act as a growth substrate or as an inducer of enzymes of the *xylCAB* or of the *xylDLEGF* operons. Complementation of these strains with a recombinant plasmid pWW0-3006 carrying the *Hind*III region D (HD) of pWW0 restored the Mxy⁺ phenotype and the ability of *m*-xylene to induce the *xylDLEGF* operon. The cloned HD region had no regulatory function but carried the OP1 region, the entire *xylC* structural gene and sufficient of *xylA* to support growth on *m*-xylene. *XylC* was more precisely located on a 1.9 kbp *Sal*I subclone of the HD region. Comparison of the roles of *m*-xylene and *m*-methylbenzyl alcohol as inducers shows they are not equivalent and that whereas *m*-xylene can induce only *xylCAB*, *m*-methylbenzyl alcohol can induce both *xylCAB* and *xylDLEGF*. The restoration of the induction of *xylDLEGF* by *m*-xylene is due to the presence of *xylA* on the recombinant pWW0-3006, converting *m*-xylene to *m*-methylbenzyl alcohol.

INTRODUCTION

The TOL plasmid pWW0 carried by *Pseudomonas putida* mt-2 (PaW1) specifies a set of inducible enzymes that catalyse the catabolism of toluene and *m*- and *p*-xylenes. A model for the regulation of the pathway was proposed by Worsey *et al.* (1978). Genes for the enzymes of the upper part of the pathway form an operon, *xylCAB*, the products of which convert the hydrocarbon substrates to corresponding carboxylic acids. A second operon, *xylDLEGF*, codes for enzymes responsible for further degrading the carboxylic acids to acetaldehyde and pyruvate via *meta* cleavage of catechol derivatives (Fig. 1). It was proposed that expression of the pathway was controlled by two regulatory genes: *xylR* controls the expression of both operons in the presence of *m*-xylene or *m*-methylbenzyl alcohol (which have been used interchangeably as *xylR* coinducers) and *xylS* induces only the lower pathway in the presence of *m*-toluate.

Subsequent work on pWW0 and pTN2, an RP4 cointegrate plasmid carrying the pWW0 catabolic genes, has located most of the genes of the upper and lower pathways by molecular cloning and transposon mutagenesis (Franklin *et al.*, 1981; Inouye *et al.*, 1981*a*; Harayama *et al.*, 1984; Timmis *et al.*, 1985) and demonstrated that *xylR* and *xylS* act as positive regulators (Franklin *et al.*, 1981; Franklin & Williams, 1980; Inouye *et al.*, 1981*b*; Nakazawa *et al.*, 1980). Both regulatory genes have been located and cloned (Franklin *et al.*, 1983; Inouye *et al.*, 1981*b*, 1983). Recombinant plasmids containing *xylR* and *xylS* were used to demonstrate that both are required for expression of the lower pathway when induced with *m*-xylene (Inouye *et al.*, 1983).

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

Fig. 1. Early enzymes of the TOL plasmid degradative pathway. The primary metabolites of the pathway are toluene (R₁ = R₂ = H), *m*-xylene (R₁ = CH₃, R₂ = H) and *p*-xylene (R₁ = H, R₂ = CH₃); *m*-toluate is the carboxylic acid metabolite of *m*-xylene.

It was also shown that *xylS* insertion mutants were unable to express the lower operon in the presence of *m*-methylbenzyl alcohol although the upper pathway was induced normally (Franklin *et al.*, 1983). These results suggest that the regulation of the two pathways is not as straightforward as previously thought. The present work investigated the effect of cloned wild-type DNA on strains carrying derivatives of the TOL plasmid pWW0. Further evidence is presented that the 9.8 kbp *Hind*III D fragment (HD) of pWW0 carries the operator–promoter regions for the upper pathway operon and the entire *xylC* gene, the position of which has been precisely mapped.

Evidence is also presented that sufficient of the *xylA* gene resides on HD to allow expression of xylene oxidase. The phenotypes of the complemented and uncomplemented strains carrying mutant plasmids suggested that, contrary to the currently held model, *m*-xylene is unable to induce the lower pathway operon, even when both *xylR* and *xylS* are functional, and that the product of the upper pathway responsible for induction of the lower pathway is *m*-methylbenzyl alcohol.

METHODS

Bacterial strains and plasmids. The *Pseudomonas putida* strains used in this study are shown in Table 1 with their plasmid complements and relevant phenotypes. The *Escherichia coli* strains were all derived from strain PM191, a *recA* derivative of strain C600, which was used as recipient in all transformations.

The different mutant derivatives of the TOL plasmid pWW0 were originally isolated and mapped by Jeenes (1982).

The vectors used for cloning pWW0 DNA were the broad host-range plasmid pKT230 (Bagdasarian *et al.*, 1981), which has a number of unique restriction endonuclease recognition sites in both the kanamycin and streptomycin resistance genes, and pBR325 (Bolivar, 1978) which carries genes for resistance to ampicillin, tetracycline and chloramphenicol.

Plasmid purification. The TOL plasmid and its derivatives were extracted using the sucrose gradient method of Wheatcroft & Williams (1981). Vector and recombinant plasmids were extracted from *E. coli* strains by a cleared lysate procedure followed by CsCl gradient centrifugation (Guerry *et al.*, 1973).

Recombinant plasmids were rapidly extracted from strains of both *E. coli* and *Pseudomonas* by the method of Holmes & Quigley (1981).

Restriction endonuclease digestion of DNA and agarose gel electrophoresis. All restriction endonuclease digestions were done in O'Farrell T4 DNA polymerase buffer (Maniatis *et al.*, 1982). All restriction endonucleases were obtained from BRL or NBL Enzymes.

Digested DNA was analysed by agarose gel electrophoresis as previously described (Jeenes *et al.*, 1982). Specific DNA fragments were eluted from agarose gels by the method of Girvitz *et al.* (1980).

Cloning of specific DNA fragments from pWW0 and its derivatives. The HD fragment from pWW0 was obtained from a shotgun cloning experiment in which total pWW0 DNA was digested with *Hind*III and ligated into the

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Plasmid phenotype*	Notes and references
<i>Pseudomonas putida</i> mt-2 (PaW1)	pWW0	Mxy ⁺ Mtol ⁺	
PaW130	—	—	Plasmid-free derivative of PaW1, Rif ^r
PaW660	pWW0-660	Mxy ⁻ Mtol ⁺	Jeenes (1982)
PaW673	pWW0-673	Mxy ⁻ Mtol ⁺	Jeenes (1982)
PaW790	pWW0-3006	Sm ^r Km ^s	This study
PaW791	pWW0-3024	Sm ^r Km ^s	This study
PaW796	pWW0-673, pWW0-3006	Mxy ⁺ Mtol ⁺	This study
PaW820	pWW0-660, pWW0-3006	Mxy ⁺ Mtol ⁺	This study
PaW825	pWW0-660, pWW0-3024	Mxy ⁺ Mtol ⁺	This study
<i>Escherichia coli</i> PM191	—	—	Thi ⁻ Th ^r Leu ⁻ RecA; N. J. Grinter (Searle, High Wycombe, UK)
	pWW0-3006	Sm ^r Km ^s	HD in pKT230, this study
	pWW0-3024	Sm ^r Km ^s	<i>Sma</i> I/ <i>Hind</i> III subclone of HD; this study
	pWW0-3001	Sm ^r Km ^s	XF in pKT230; this study
	pWW0-3002	Sm ^r Km ^s	XG in pKT230; this study
	pWW0-3010	Sm ^r Km ^s	XF fragment with 3.4 kbp insert from pWW0-673 in pKT230; this study
	pWW0-3026	Sm ^r Km ^s	<i>Sma</i> I/ <i>Bam</i> HI subclone of HD in pKT230; this study
	pWW0-3027	Ap ^r Cm ^r Tc ^s	<i>Sal</i> I subclone of XG in pBR325; this study
	pWW0-3028	Ap ^r Cm ^r Tc ^s	<i>Sal</i> I subclone of XG in pBR325, opposite orientation to pWW0-3027
	pWW0-3029	Sm ^r Km ^s	<i>Xho</i> I/ <i>Bam</i> HI subclone of XG in pKT230; this study

* Mxy⁺ and Mtol⁺ denote the ability to grow on *m*-xylene and *m*-toluate, respectively, as sole source of carbon and energy; Sm^r, Km^r, Cm^r and Tc^r denote resistance to streptomycin, kanamycin, carbenicillin and tetracycline, respectively.

*Hind*III site of pKT230. Ligations were done with T4 DNA ligase obtained from BRL under conditions recommended by the suppliers. Ligated DNA was transformed into CaCl₂-treated cells of *E. coli* strain PM191 and transformants were selected on nutrient agar plates containing streptomycin (15 µg ml⁻¹). Colonies containing recombinant plasmids were detected by their sensitivity to kanamycin (15 µg ml⁻¹) which was determined by replica picking of single colonies.

Strains containing recombinant plasmids were screened by small-scale rapid plasmid extraction followed by restriction endonuclease analysis in which inserts were compared with *Hind*III digests of pWW0 DNA. In this way the plasmid pWW0-3006 was isolated which contained the HD fragment of pWW0 in pKT230.

An identical procedure was used to obtain the *Xho*I digestion fragments XF and XG of pWW0 in the recombinant plasmids pWW0-3001 and pWW0-3002 respectively.

The *Xho*I fragment from the mutant plasmid pWW0-673 which contained insert DNA was cloned into pKT230 after electroelution from agarose gels. The various subclones were produced by restriction of the appropriate recombinant DNA with the required enzymes and religation either directly or in the presence of an alternative vector. The fragments contained in the different recombinant plasmids generated in this study are shown in Fig. 2.

Matings and complementation analysis. All conjugation experiments were done non-quantitatively by patching donor and recipient bacteria onto a nutrient agar plate and incubating at 30 °C overnight in the case of *Pseudomonas* strains or at 37 °C for 4 h in the case of *E. coli*. Cells from the mixed colony were then suspended in 0.5 ml 0.1 M-sodium phosphate buffer pH 7.5 and dilutions were spread onto appropriate selective agar plates. Using RP4, the recombinant plasmids pWW0-3006 and pWW0-3024 were mobilized into PaW130, a plasmid-free Rif^r derivative of PaW1. The resulting transconjugant strains were PaW790 and PaW791 which were subsequently used as recipients for the mutant TOL plasmid pWW0-660 (Table 1).

Fig. 2. Restriction map of pWW0 and its derivative plasmids. The expanded region in the lower half shows a detailed map of the HD region and the subclones generated during this study. The open rectangles show the pWW0 DNA, the solid line pKT230 and the broken line pBR325; ● marks the kanamycin promoter of pKT230 reading towards the inserts and ◊ marks the tetracycline promoter of pBR325 in the same orientation. Restriction enzyme sites are denoted: B, *Bam*HI; Bs, *Bst*EII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sma*I; X, *Xho*I; Xb, *Xba*I.

Enzyme assays and preparation of cell extracts. Enzymes assayed as being representative of the upper pathway were benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH). Representative enzymes of the lower pathway were catechol 2,3-oxygenase (C23O), 2-hydroxymuconic semialdehyde hydrolase (HMSH) and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD). Cells were grown up in the presence of appropriate inducers and cell extracts were prepared according to previously described procedures (Jeenes *et al.*, 1982).

Enzymes were assayed by previously described methods (Sala-Trepat & Evans, 1971; Williams & Murray, 1974; Worsey & Williams, 1975). All assays were done at least twice on different batches of cells, and the values quoted are the mean. The variation was within $\pm 20\%$ of the quoted value.

Xylene oxidase was assayed by measurement of the stimulation of oxygen uptake in a Clark oxygen electrode in response to addition of *m*-xylene.

Protein was determined by the Biuret method.

RESULTS

Characterization of pWW0-660 and pWW0-673 and the mapping of the HD region of pWW0

The mutant plasmids pWW0-660 and pWW0-673 both arose spontaneously during studies in which pWW0 was transferred into the chlorobenzoate-degrading strain *Pseudomonas* sp. B13 (Jeenes, 1982). When transferred back into a plasmid-free derivative of *P. putida* mt-2, both produced a similar phenotype and biochemical characteristics: host strains were Mxy⁻ Mtol⁺, expressed none of the catabolic enzymes when induced with *m*-xylene but expressed the lower operon enzymes when induced with *m*-toluate (Jeenes, 1982). Restriction analysis of their plasmids showed distinct differences. pWW0-660 contained a deletion of about 18 kbp which covered all the upper operon genes but did not affect the lower operon or either of the regulatory genes *xy*/R or *xy*/S. pWW0-673 on the other hand contained a 3.4 kbp insertion in the *Hind*III D fragment (HD). The location of this was accurately mapped by restriction analysis of cloned restriction fragments of pWW0 and of the *Xho*I fragment from pWW0-673 containing the insert (Fig. 3). The map of the HD fragment of pWW0 was constructed by restriction analysis of cloned HD and *Xho*I F and G fragments (Fig. 2).

According to the mapping data of Inouye *et al.* (1983, 1984), the insert in pWW0-673 is in the operator-promoter region (OP1) of the upper operon. As far as the present data allow, it is

Fig. 3. *SmaI/HindIII* subclone of the HD region of pWW0 showing the location of the insertion in pWW0-673, the position of OP1 and the *xyIC* gene.

identical in nature and location to the inserts in the previously described plasmids pWW0-2001 and pWW0-2023 (Jeenes & Williams, 1982). The origin of the insert has been discussed elsewhere by Lehrbach *et al.* (1983).

Complementation of mutant plasmids with recombinant plasmids pWW0-3006 and pWW0-3024

When pWW0-660 was mated into recipient strains carrying pWW0-3006 (carrying HD cloned into pKT230; Fig. 2) and pWW0-3024 (*SmaI/HindIII* subclone of HD in pKT230; Fig. 2) both of the respective transconjugants were able to grow at the expense of *m*-xylene, although by comparison with PaW1 the growth was very slow. The co-existence of pWW0-660 with each recombinant plasmid was confirmed by isolation of each plasmid separately. Whereas only the large TOL plasmid was isolated by the sucrose gradient method, only the small recombinant plasmids were isolated by the small-scale rapid extraction. Thus it was demonstrated that both plasmids were maintained as independent replicons. Since they shared no sequence homology the possibility of recombination did not arise.

Enzyme assays of PaW820 (pWW0-660, pWW0-3006) showed that the only fully complemented upper operon enzyme was BZDH (*xyIC* gene product): its activity was comparable to wild-type PaW1 and was inducible by *m*-xylene, whereas BADH (*xyIB*) levels remained low and were not induced. These results confirmed that both *xyIC* and the operator-promoter region (OP1) were located on HD but that *xyIB* was not. Furthermore, since PaW825 (pWW0-660, pWW0-3024) showed the same phenotype and induction patterns (data not shown) as PaW820, these functions could be more precisely located on the 5.5 kbp *SmaI/HindIII* fragment in pWW0-3024.

The uptake of O₂ was stimulated by *m*-xylene to a low but detectable level in PaW820 but not in strains carrying either pWW0-660 or pWW0-3006 alone, suggesting a low degree of complementation of the xylene oxidase activity (data not presented).

Enzymes of the lower operon were not induced by *m*-xylene in strains containing only pWW0-660 but were induced in PaW820 (Table 2), indicating the presence of a gene on HD which affects the expression of the lower pathway in the presence of *m*-xylene.

Similar results were obtained when the insertion mutant plasmid pWW0-673 was complemented with pWW0-3006 (Table 2), showing that the insertion in OP1 affected the induction by *m*-xylene of both operons, and that this effect was complemented by pWW0-3006: induction of the lower operon enzymes by *m*-toluate was as with PaW1.

Comparison of m-xylene and m-methylbenzyl alcohol as inducers of the lower operon

The inability of *m*-xylene to induce the *xyIDLEGF* operon in pWW0-660 in spite of the functional presence of both *xyIR* (as indicated by the induction of BZDH activity on HD) and *xyIS* on the plasmid is in conflict with the present model for the regulation of the two operons (Worsey *et al.*, 1978). This also applies to pWW0-673 which carries an insert in or close to the OP1 region of the plasmid, and led to the incorrect conclusion for the indistinguishable plasmid pWW0-2001 that the insert was in the *xyIR* gene which was consequently wrongly located

Table 2. *Enzyme activities in complemented and parental strains of P. putida*

Enzyme activities are expressed as milliunits of activity per mg protein; ND, not determined.

Strain	Plasmid	Inducer	BADH	BZDH	C23O	HMSH	HMSD
PaW1	pWW0	<i>m</i> -Xylene	580	480	15 600	1000	120
		<i>m</i> -Toluate	15	60	3 700	325	115
		None	16	8	130	8	25
PaW790	pWW0-3006	<i>m</i> -Xylene	44	12	< 1	ND	ND
		None	38	14	< 1	ND	ND
PaW660	pWW0-660	<i>m</i> -Xylene	58	30	130	38	20
		<i>m</i> -Toluate	50	25	10 000	250	130
		None	45	11	170	10	19
PaW820	pWW0-660, pWW0-3006	<i>m</i> -Xylene	45	1100	18 000	370	150
		None	55	45	150	5	16
PaW673	pWW0-673	<i>m</i> -Xylene	15	25	170	5	20
		<i>m</i> -Toluate	20	95	7 700	220	100
		None	20	10	230	9	11
PaW796	pWW0-673, pWW0-3006	<i>m</i> -Xylene	40	380	9 100	510	240
		None	12	10	170	10	15

Table 3. *Comparison of the induction of lower operon enzymes by m-xylene and m-methylbenzyl alcohol*

Enzyme activities are expressed as milliunits of activity per mg protein.

Strain	Plasmid	Inducer	C23O	HMSH	HMSD
PaW660	pWW0-660	<i>m</i> -Methylbenzyl alcohol	8 900	570	180
		<i>m</i> -Xylene	200	38	20
PaW673	pWW0-673	<i>m</i> -Methylbenzyl alcohol	23 300	640	160
		<i>m</i> -Xylene	170	5	20

upstream of the *xy/CAB* operon (Jeenes & Williams, 1982; Lehrbach *et al.*, 1983). It had been reported to us that similar insertion mutants expressed the genes of the lower operon normally when induced with *m*-methylbenzyl alcohol (K. Timmis, personal communication). This led us to compare the roles of *m*-xylene and *m*-methylbenzyl alcohol as inducers in PaW660 and PaW673. The results (Table 3) show conclusively that their roles as inducers are not equivalent as previously assumed and that whereas *m*-xylene is unable to induce the *xy/DLEGF* operon, *m*-methylbenzyl alcohol can.

Location of xy/C on fragment HD

The *Sma*I/*Hind*III fragment from pWW0-3024 was subcloned to locate the BZDH (*xy/C*) gene more precisely. The structures of the subclones used are shown in Fig. 2. The plasmids were all maintained in *E. coli* and assays were done with cell-free extracts made from these strains.

The XG fragment (in pWW0-3002) expressed low levels of BZDH activity [40 munits (mg protein)⁻¹] despite being in the wrong orientation to allow expression from the kanamycin promoter of the pKT230 vector. It was concluded from this observation that there was a weak promoter upstream of the *xy/C* gene on the XG fragment. None of the subclones in which the XG fragment was cut at the *Bam*HI site (pWW0-3026, pWW0-3029) expressed BZDH activity [< 1 munit (mg protein)⁻¹], suggesting that the *Bam*HI site lay within the structural gene. However, the *Sal*I subclone of XG, pWW0-3027, exhibited high levels of BZDH activity [400 munits (mg protein)⁻¹]. It was shown that these high levels of expression were due to the tetracycline promoter of the pBR325 vector since reversal of the orientation of the fragment in pWW0-3028 resulted in complete loss of activity. These results show that the entire *xy/C* gene is located on a single 1.9 kbp *Sal*I fragment within XG. The end of the gene lies in the 100 bases

between the *Bam*HI site and the *Sal*I site close to the unique *Xba*I site on XG. The start of the gene has not yet been located precisely but lies downstream of the *Sal*I sites at the left-hand end of XG and therefore at least 1 kbp away from the site identified as OP1. These results are summarized in Fig. 3.

DISCUSSION

The present work has demonstrated that cloned wild-type DNA from the HD region is able to complement the deletion of the entire upper operon on pWW0-660 and restore the ability to grow on *m*-xylene, albeit slowly. The only gene product which was fully complemented was *xy*/C and this gene has been subsequently subcloned and located on a 1.9 kbp *Sal*I fragment. Interestingly, although Timmis *et al.* (1985) have determined the gene order of the operon to be *xy*/CAB, *xy*/C is not the product of the open reading-frame found directly after OP1, which Inouye *et al.* (1984) initially proposed was *xy*/A and Mermod *et al.* (1984) identified as *xy*/C; the function of this initial putative polypeptide which is terminated before *xy*/C is at present under investigation.

The ability of the complemented strains to grow on *m*-xylene requires a functional *xy*/A gene and this must also be present on HD since on pWW0-660 the gene is entirely deleted and there is no corresponding chromosomal function. The absence of BADH (*xy*/B) agrees with the map location determined by Inouye *et al.* (1981*a*) and Timmis *et al.* (1985) to be downstream of the right-hand end of HD. Presumably the ability of the complemented strains to grow on *m*-xylene in the absence of *xy*/B requires the participation of the chromosomal BADH gene. This may be the cause of their slow growth on *m*-xylene. Alternatively it may be that the complete genetic information for *xy*/A expression is not present on HD, which would also account for the low stimulation of O₂ uptake by *m*-xylene in the complemented strains PaW820 and PaW825. However, we would not wish too much emphasis to be put on conclusions drawn from what is an unsatisfactory method of assaying direct enzyme attack on a hydrocarbon which is likely to have multiple and unrelated effects on cellular respiration.

The most unexpected result from the complementation experiments was that HD restored the induction of the lower pathway operon by growth in the presence of *m*-xylene. Since the generally accepted model for regulation proposed that *m*-xylene could act with the product of *xy*/R to induce the *xy*/DLEGF operon (Worsey *et al.*, 1978), the assumption was made that the insertion in pWW0-2001 (indistinguishable from that in pWW0-673) was in the *xy*/R gene, which was therefore assumed to be upstream of the *xy*/CAB operon (Jeenes & Williams, 1982; Lehrbach *et al.*, 1983). This was shown to be incorrect by the later mapping of *xy*/R at least 35 kbp distant from the insertion site (Franklin *et al.*, 1983). The inability of *m*-xylene to induce *xy*/DLEGF on the deleted plasmid pWW0-660 reported here casts additional serious doubt on the model. It could be argued that pWW0-660 and pWW0-673 carry additional regulatory mutations but this seems unlikely since both are here shown to carry fully functional *xy*/S and *xy*/R genes. *Xy*/S functions normally since *m*-toluate acts as both inducer of *xy*/DLEGF and a growth substrate for host strains of both plasmids. Furthermore, *xy*/R must also be present and functional since *xy*/C is induced by *m*-xylene when HD is present *in trans* on a recombinant plasmid. The inability of *m*-xylene to induce *xy*/DLEGF in the presence of a functional *xy*/R must represent a natural feature of the regulation.

The restoration of *m*-xylene induction of the lower operon by HD must therefore be due to a gene product encoded on HD. We propose that this gene is in fact *xy*/A, the presence of which on HD has been demonstrated by the restoration of the Mxy⁺ phenotype by complementation. This can convert *m*-xylene to *m*-methylbenzyl alcohol and, as we have shown conclusively with PaW660 and PaW673, *m*-methylbenzyl alcohol is not equivalent to *m*-xylene as an inducer and is able to induce *xy*/DLEGF under conditions where *m*-xylene cannot. The work presented here does not demonstrate whether this induction involves the participation of only the *xy*/S gene product, with *m*-methylbenzyl alcohol acting as an analogue of *m*-toluate, or whether *xy*/R gene product is also required. However, the results of Franklin *et al.* (1983) suggest that functional *xy*/R and *xy*/S genes are required for *m*-methylbenzyl alcohol induction of *xy*/DLEGF.

These results are in conflict with those of Inouye *et al.* (1983) which showed that cloned *xylR* gene could initiate transcription at the cloned operator–promoter region (OP2) of the lower operon. However, their experiments were done using *E. coli* with the genes cloned in high copy number vectors, and induced levels of expression were very low. Our experiments have involved *P. putida*, the natural host for expression of the catabolic functions, and induced levels of expression were of the same orders of magnitude as in wild-type cells.

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