JOURNAL OF BACTERIOLOGY, July 1985, p. 248–255
0021-9193/85/070248-08$02.00/0

Vol. 163, No. 1

TOL Plasmid pWW15 Contains Two Nonhomologous, Independently Regulated Catechol 2,3-Oxygenase Genes

HEINRICH KEIL, MICHAEL R. LEBENS, AND PETER A. WILLIAMS*

Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Gwynedd LL57 2UW, United Kingdom

Received 22 January 1985/Accepted 3 April 1985

Pseudomonas putida **MT15 contains a 250-kilobase-pair (kbp) TOL plasmid pWW15, encoding toluene and xylene catabolism, which undergoes large spontaneous deletions to give two classes of mutants with altered catabolic phenotypes (H. Keil and P. A. Williams, J. Gen. Microbiol, 131:1023–1033, 1985). Two structural genes for catechol 2,3-oxygenase (C23O) were cloned from pWW15. The gene for C23OI was located on the 2.1-kbp *XhoI* fragment Xh, whereas that for C23OII was found on the 11.5-kbp *BamHI* fragment BJ. The two restriction fragments and the subcloned regions of them showed no similarity in the pattern of restriction digestion, nor did they hybridize with each other. The substrate specificities of the two enzymes were also substantially different. The two structural genes were separated on pWW15 by about 100 kbp. In plasmid pWW15-510 of a B5 mutant, the 90-kbp deletion in the plasmid removed most of the intervening DNA, but it also deleted 80% of the gene for C23OI from its 3′ end. Thus, only C23OII was expressed in the host MT15-510. Conversely, in RP4::pWW15 cointegrate plasmid pWW15-1003, only the C23OI gene was present. The expression of C23O activity from these two derivative plasmids and from the wild-type pWW15 showed that only C23OI was induced by growth in the presence of *m*-toluate, whereas both activities were induced in the presence of *m*-xylene. These findings cast doubt on the earlier hypothesis that the deletions in B3 and B5 mutants remove a regulatory gene by which *m*-toluate induces the enzymes necessary for its own catabolism.**

TOL plasmids carry genes for the regulated catabolism of toluene and some substituted toluenes to central metabolites and thus support the growth of host *Pseudomonas* strains on the hydrocarbons and their metabolites (14, 25). A property of *Pseudomonas* hosts carrying members of this class of large and varied plasmids is that growth on benzoate, a metabolite of toluene on the plasmid-coded pathway, exerts a strong selection against utilization of the plasmid pathway and in favor of the alternative chromosomal β-ketoadipate pathway (23, 26). Segregants resulting from benzoate selection either have lost the plasmid or the plasmid has undergone a deletion of structural or regulatory genes which causes a change in expression of the pathway enzymes and an irreversible change in the catabolic phenotype (3, 13, 15, 26).

Benzoate acts as a particularly strong selector against the wild type of three strains, *Pseudomonas* sp. MT14 and *Pseudomonas putida* MT15 and MT20 (24), which contain large, related TOL plasmids (13). Three phenotypically distinct classes of segregants are formed. In addition to those segregants which have been cured of their plasmids and no longer grow on toluene, *m*-xylene, or its metabolite *m*-toluate (Tln^- Mxy^- $Mtol^-$), two unique classes of mutants have been found, the B3 mutants (Tln^+ Mxy^+ $Mtol^-$) and the B5 mutants (Tln^+ Mxy^- $Mtol^-$). In both B3 and B5 mutants a large, essentially common, region of about 90 to 100 kilobase pairs (kbp) is deleted from the wild-type plasmid, but the exact termini of the deletion are different in the various segregants. Results of biochemical analysis of these mutants have led to the proposal that the deletion has resulted in the loss of a regulatory gene that disables the induction by *m*-toluate of the enzymes necessary for its own catabolism but not affecting the ability of *m*-xylene or toluene to induce the complete catabolic pathway (13, 19, 26). It has also been shown that in pseudorevertants of both B3 and B5 strains which have regained the ability to grow on *m*-toluate, there is a tandem gene amplification of about 23 to 28 kbp of the plasmid DNA spanning the deletion termini. This results in a low constitutive expression of the enzymes for *m*-toluate catabolism (13).

In this paper we describe the discovery on plasmid pWW15 of *P. putida* MT15 of two nonhomologous genes for catechol 2,3-oxygenase (C23O), a key enzyme on the toluene-xylene catabolic pathway (Fig. 1). The results cast doubts on the hypothesis that the B3 and B5 mutants result from the deletion of an essential regulatory gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Media and culture conditions. *Escherichia coli* strains were grown in Luria broth supplemented with the appropriate antibiotic at the following concentrations (in micrograms per milliliter): streptomycin, 15; kanamycin, 15; ampicillin, 25; and tetracycline, 7.5. The minimal medium used for the selection of recombinant plasmids in *E. coli* JM103 after they were cloned into plasmid vectors pUC12 and pUC13 was as described previously (17). *P. putida* strains were grown as described previously (25).

Conjugations. Nonquantitative matings were performed on plates or filters. The RP4::pWW15 cointegrate pWW15-1003 was constructed by transferring RP4 from *P. putida* AC34(RP4) into *P. putida* MT15, selecting for growth on *m*-toluate in the presence of 10 μg of kanamycin per ml. An $RP4^+$ transconjugant was then used as a donor in a mating with PRSB10 used as the recipient by selecting for growth on *m*-toluate with added streptomycin (1 mg/ml) and kanamycin (10 μg/ml). PRSB1003 was one transconjugant that was retained.

Plasmid purifications. pWW15 and its large derivatives

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Notes (references)
P. putida (with plasmids)	
MT15(pWW15)	Wild type; Mxy$^+$ Tln$^+$ Mtol$^+$ (13)
MT15-300(pWW15-300)	B3 mutant of MT15; Mxy$^+$ Tln$^+$ Mtol$^-$ (13)
MT15-450(pWW15-450)	Mtol$^+$ revertant of MT15-300 (13)
MT15-510(pWW15-510)	B5 mutant of MT15; Mxy$^-$ Tln$^+$ Mtol$^-$ (13)
MT15-760(pWW15-760)	Mtol$^+$ revertant of MT15-510(13)
AC34(RP4)	Ade$^-$ Ap^r Km^r Tc^r (8)
PRSB10	Spontaneous Str^r derivative of *P. putida* A.3.12 (12)
PRSB1003(pWW15-1003)	Contains RP4::pWW15 cointegrate plasmid; Mxy$^+$ Tln$^-$ Mtol$^+$ Str^r Km^r Ap^r Tc^r in PRSB10 (this study)
E. coli	
PM191	Thr$^-$ Leu$^-$ Thi$^-$ *recA* derivative of strain C600 (1)
JM103	Thi$^-$ (17)
Recombinant plasmids	
pKT230	(2)
pBR322	(4)
pUC12	(16)
pUC13	(16)
pWW15-3151	Fragment Xh in pKT230 (this study)
pWW15-3152	Fragment Xh in pKT230, reverse orientation to pWW15-3151 (this study)
pWW15-31513	Removal of *Xba*I-*Kpn*I internal fragment from pWW15-3151 (this study)
pWW15-31517	*Bal* 31 treatment of *Sma*I-cut pWW15-3151 in pKT230 (this study)
pWW15-31524	*Bal* 31 treatment of *Sma*I-cut pWW15-3151 in pKT230 (this study)
pWW15-3161	Fragment BJ of pWW15 in pBR322 (this study)
pWW15-3163	*Xho*I-*Bam*HI fragment of pWW15-3161 in pKT230 (this study)
pWW15-3165	*Sma*I-*Bam*HI fragment of pWW15-3161 in pKT230 (this study)
pWW15-3167	*Sma*I-*Hin*dIII fragment of pWW15-3165 in pKT230 (this study)
pWW15-3171	*Xba*I-*Hin*dIII fragment of pWW15-3167 in pUC12 (this study)
pWW15-3172	*Xba*I-*Hin*dIII fragment of pWW15-3167 in pUC13 (this study)
pWW15-3173	*Pst*I-*Hin*dIII fragment of pWW15-3171 in pUC12 (this study)
pWW15-3175	*Sst*I fragment of pWW15-3171 in pKT230 (this study)
pWW15-3181	*Bal* 31 treatment of *Xba*I-cut pWW15-3167 in pUC12 (this study)
pWW15-3183	*Bal* 31 treatment of *Hin*dIII-cut pWW15-3167 in pUC12 (this study)
pWW15-3191	*Sst*I fragment of pWW15-3181 in pKT230 (this study)
pWW15-3193	*Eco*RI-*Sst*I fragment of pWW15-3183 in pKT230 (this study)
pWW15-31500	Novel *Xho*I fragment of pWW15-510 in pKT230 (this study)
pWW15-31501	Novel *Xho*I fragment of pWW15-510 in pKT230 in opposite orientation to pWW15-31500 (this study)

were prepared by the sucrose gradient method described by Wheatcroft and Williams (22). Vector and recombinant plasmid DNA was extracted from *E. coli* by a cleared lysate procedure (10) followed by cesium chloride-ethidium bromide density gradient centrifugation. For screening, small-scale plasmid isolations from *E. coli* were carried out by the method of Holmes and Quigley (11).

Restriction enzyme digestion and agarose gel electrophoresis. All digestions and subsequent electrophoresis were carried out as described previously (22). Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Bethesda, Md.), or NBL Enzymes Ltd. (Northumberland, U.K.)

Cloning and transformations. Reaction conditions for restriction and ligation with T4 DNA ligase (Bethesda Research Laboratories) were carried out according to instructions provided by the manufacturer. *E. coli* PM191 and JM103 were transformed by standard procedures (5), and transformants were selected on antibiotic media appropriate for the vector. Colonies carrying recombinant plasmids expressing C23O activity were detected by the catechol spray test (6).

Digestion with *Bal* 31 exonuclease. Plasmid DNA (20 μg) was digested with an appropriate restriction endonuclease and subsequently digested with *Bal* 31 (Bethesda Research Laboratories) in a reaction volume of 100 μl according to the instructions provided by the manufacturer. The rate of digestion was controlled by altering the enzyme concentration. In a typical experiment, conditions were adjusted so that about 10 base pairs (bp) of DNA per end per minute were digested. Samples (10 μl) were removed at 6-min intervals, and the reaction was stopped by the addition of EGTA to a final concentration of 20 mM. Samples were repaired with the Klenow fragment of DNA polymerase I to give flush ends. The DNA was then cut with a second appropriate restriction enzyme to remove the remaining insert DNA from the partially digested vector. The insert DNA was ligated into a fresh, appropriately digested vector

which had been added to the reaction mixture. When the new vector was different from that in which the *Bal* 31 digestion had been carried out (for pWW15-3181 and pWW15-3183), a different antibiotic was used to select against religation into the original *Bal* 31-treated vector DNA. For pWW15-31517 and pWW15-31524, in which the

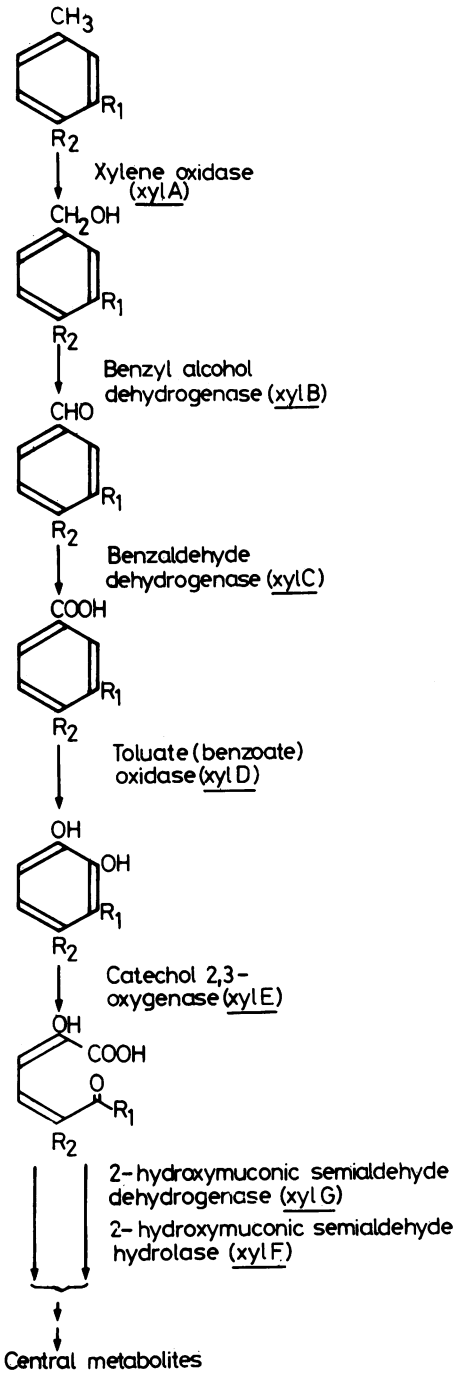

FIG. 1. Early enzymes of the TOL plasmid-encoded pathway for catabolism of toluene and the xylenes. The primary metabolites of the pathway are toluene ($R_1 = R_2 = H$), *m*-xylene ($R_1 = CH_3$; $R_2 = H$), and *p*-xylene ($R_1 = H$; $R_2 = CH_3$). *m*-Toluate is the carboxylic acid metabolite of *m*-xylene, and 2-hydroxymuconic semialdehyde is the ring cleavage product of C23O action on catechol ($R_1 = R_2 = H$).

TABLE 2. Activity and substrate specificity of C23O in *E. coli* strains carrying recombinant plasmids

Plasmid	Direction of transcription from vector promoter[a]	Sp act (mU/mg) against:		Ratio (%)[b]
		Catechol	3-Methylcatechol	
pWW15-3151	→	2,070	1,300	63
pWW15-3152	←	2	ND[c]	ND
pWW15-31513	→	<1	ND	ND
pWW15-31517	→	20	11	55
pWW15-31524	→	880	ND	ND
pWW15-31500	→	<1	ND	ND
pWW15-31501	←	<1	ND	ND
pWW15-3161	←	30	65	217
pWW15-3163	→	18	38	210
pWW15-3165	→	250	450	180
pWW15-3167	→	245	440	180
pWW15-3171	→	350	620	177
pWW15-3172	←	650	1,150	177
pWW15-3173	→	733	1,480	202
pWW15-3175	→	30	63	210
pWW15-3181	→	189	389	206
pWW15-3183	←	94	180	193
pWW15-3191	→	12	23	192
pWW15-3193	→	18	35	195

[a] Arrows indicate the direction relative to the maps of the fragments, as drawn in Fig. 2 and 3.

[b] Ratio is (activity against 3-methylcatechol/activity against catechol) × 100.

[c] ND, Not determined.

Bal 31-digested insert was religated back into pKT230, it was necessary to treat the *Bal* 31 digestion mixture with bacterial alkaline phosphatase to prevent the reduced insert from religating back into *Bal* 31-digested vector. After transformation back into *E. coli*, plates of transformants were sprayed with catechol to detect $C23O^+$ clones. A clear cutoff was seen beyond which transformants ceased to express C23O activity. The sample before this contained a mixture of expressing and nonexpressing clones and in some cases clones with different levels of expression (apparent from the intensity of the yellow color). Representatives of each group were then screened by the method of Holmes and Quigley (11).

DNA-DNA hybridization. DNA restriction fragments separated by agarose gel electrophoresis were transferred to Biodyne filter membranes (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) by the Southern blotting technique (21). Cloned fragments Xh and BJ were separated from the vector DNA by agarose gel electrophoresis and extracted from the agarose essentially as described by Girvitz et al. (9). These fragments were then labeled with [^{32}P]dGTP and hybridized as described previously (13).

Enzyme assays. Crude cell extracts of *P. putida* and *E. coli* were prepared, and C23O activity was assayed as described previously (20).

RESULTS

Molecular cloning of the C23OI gene. The structural gene for C23OI was cloned into the broad-host-range vector pKT230 from an *Xho*I digest of an RP4::pWW15 cointegrate plasmid, pWW15-1003. The 2.1-kbp cloned insert corresponded to *Xho*I fragment Xh from pWW15 (13). The restriction map of the recombinant plasmid pWW15-3151 is shown in Fig. 2. The specific activity of C23O in *E. coli* carrying pWW15-3151 (Table 2) was comparable with pub-

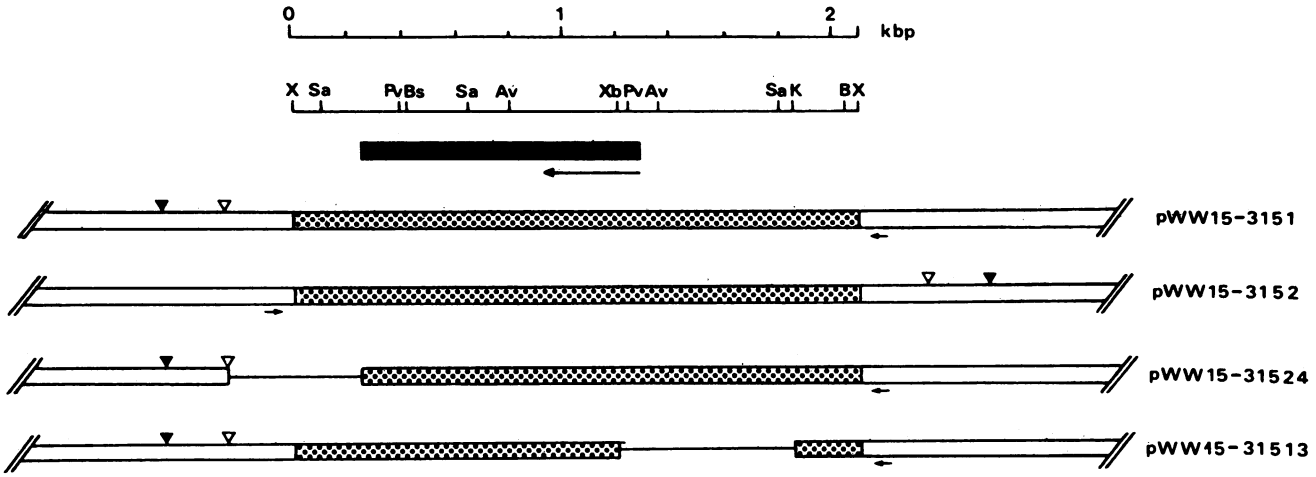

FIG. 2. Restriction endonuclease map of the *Xho*I fragment Xh of TOL plasmid pWW15 and of the recombinant plasmids constructed from it. Abbreviations: Av, *Ava*I; B, *Bam*HI; Bs, *Bst*EII; K, *Kpn*I; Pv, *Pvu*II; X, *Xho*I; Xb, *Xba*I. There are no sites for *Eco*RI, *Hin*dIII, *Pst*I, *Sst*I, and *Sma*I on Xh. The closed bar represents the position of the structural gene for C23OI, and the large arrow shows the direction of transcription. In the recombinant plasmids, the open boxes represent the vector (pKT230) DNA, and the stippled boxes represent the pWW15 DNA. The small arrows indicate the direction of transcription from the kanamycin promoter of pKT230, and restriction sites on pKT230 for *Hin*dIII (▼) and *Sma*I (▽) are shown.

lished results of the *Xho*I fragment carrying the C23O structural gene (*xylE*) from the archetypal TOL plasmid pWW0 similarly cloned into pKT230 (6). This suggests that the C23OI gene is under the control of the constitutive promoter of the vector kanamycin resistance gene. This was confirmed by reorientation of Xh to give pWW15-3152 (Fig. 2), which elicited no detectable C23O activity in *E. coli* (Table 2). This demonstrates that the direction of transcription is from right to left. A comparison of the restriction map with that of the *Xho*I fragment of pWW0 (18) suggests substantial homology because the pattern of restriction sites is similar. The only differences are the slightly smaller size of Xh and an additional *Xba*I site close to the *Pvu*II site on the right.

Localization of the structural gene for C23OI on Xh. To determine the terminus of the C23OI gene, pWW15-3151 was linearized at the unique *Sma*I site on the vector (Fig. 2) and digested with limited amounts of *Bal* 31 exonuclease. C23O activity was sharply reduced in transformants containing religated plasmid in which about 270 bp had been digested to the right of the left *Xho*I end of Xh (Fig. 2). Two such plasmids allowed for the expression of only 44% (pWW15-31524) and 1% (pWW15-31517) of the C23O activity produced by pWW15-3151 (Table 2). The remaining Xh in these differed by not more than 15 to 20 bp. This region therefore defines the position of the 5′ end of the gene and suggests that in pWW15-31517 the low activity might be the result of a modified protein which has lost some of its C-terminal residues.

The 3′ end of the gene extends to the right of the *Xba*I site because removal of the interior *Xba*I-*Kpn*I fragment and religation to give pWW15-31513 (Fig. 2) led to the total loss of C23O activity (Table 2).

Molecular cloning of the novel fragment from the deleted plasmid pWW15-510. Careful examination of the *Xho*I digests of pWW15-510 in the B5 mutant MT15-510 (13) revealed that fragment Xh, shown above to carry the C23OI gene, was absent. Repeated attempts to clone C23O activity from *Xho*I digests of the plasmid failed. Furthermore, the only restriction fragment in pWW15-510 which hybridized after Southern blotting with ^{32}P-labeled pWW15-3151 was the novel fragment generated by the 90-kbp deletion. One end of that deletion therefore must terminate within Xh, leaving the residue as part of the novel fragment. To map the deletion terminus precisely, the 3.4-kbp novel *Xho*I fragment from pWW15-510 was cloned into pKT230 in both orientations, generating pWW15-31500 and pWW15-31501. No C23O activity was found in *E. coli* carrying pWW15-31500 or pWW15-31501 (Table 2). The terminus of the deletion was located both by visual comparison of the restriction maps of the novel fragment with that of the intact Xh fragment in pWW15-3151 and by Southern hybridizations between different digests of the novel fragment and Xh. The deletion removes the entire right end of Xh up to the *Pvu*II site on the left, including most of the C23OI gene, leaving only about 130 bp at its 5′ end (Fig. 2; see also Fig. 5). The loss of C23O activity from pWW15-31500 and pWW15-31501 can be explained by the fact that neither plasmid contains more than the distal 20% of the gene.

Molecular cloning of the C23OII structural gene. B3 and B5 mutants of MT15, including MT15-510, can express C23O activity after deletion of 90 kbp from pWW15 (13). From previous results it is apparent that in MT15-510 the deletion removes about 80% of the C23OI structural gene, which indicates the possibility of a second C23O gene in MT15-510. From previous studies it has been inferred that the structural genes for the C23O and subsequent enzymes are amplified in derivatives of B3 and B5 mutants selected for the regained ability to grow on *m*-toluate (13). Therefore, we attempted to clone the putative second C23O gene from the plasmid DNA of a number of such revertants to increase our chances of obtaining the required clones. Success was achieved by cloning a *Bam*HI digest of the plasmid DNA from B3 revertant MT15-450 into pBR322. The recombinant plasmid pWW15-3161 had an 11.5-kbp insert which corresponded to fragment BJ of pWW15 (13); this fragment is amplified in MT15-450 and is also found in pWW15-510.

The restriction map of pWW15-3151 (Fig. 2) is presented such that transcription from the tetracycline promoter of pBR322 would be from the right to the left. The expression

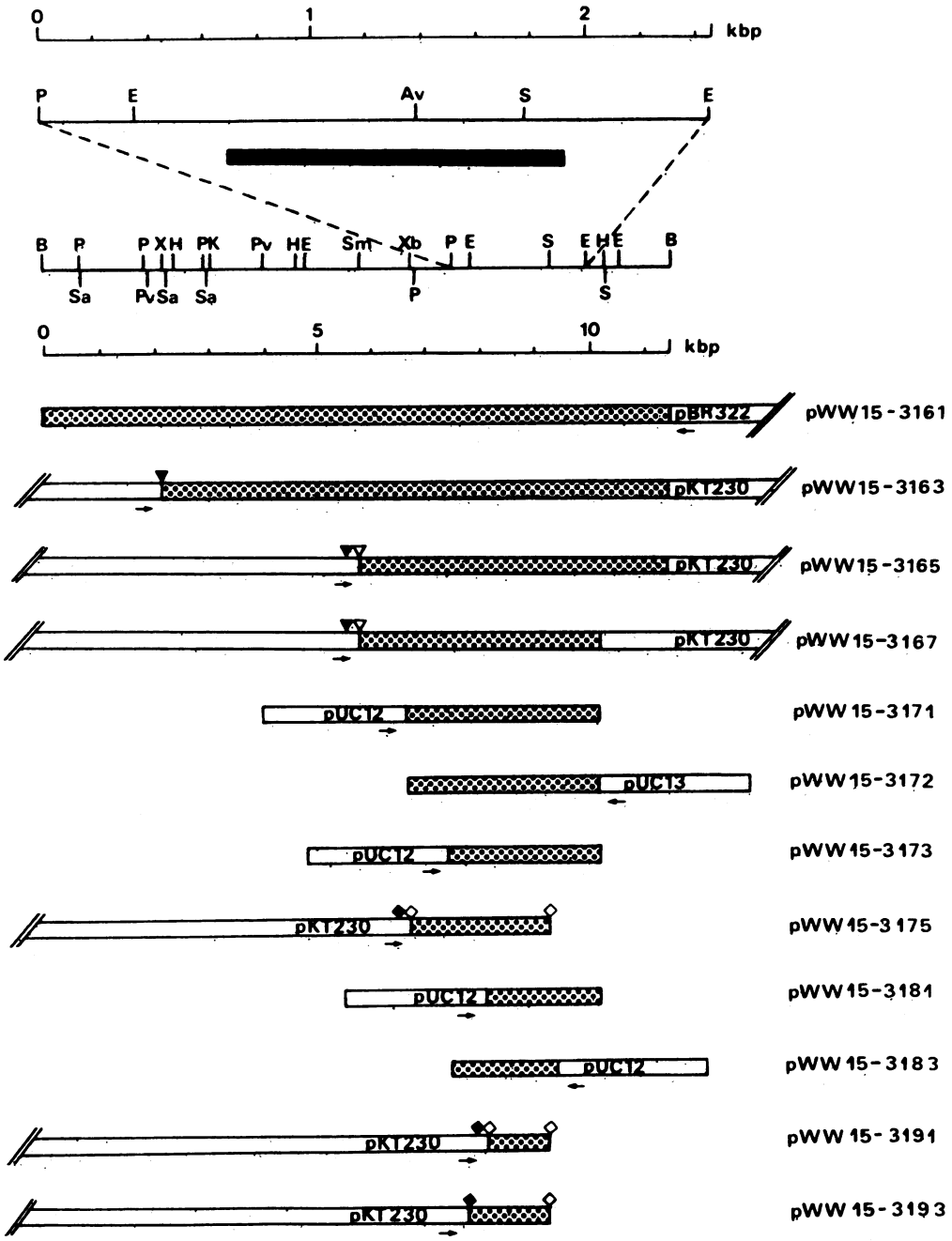

FIG. 3. Restriction endonuclease map of the *Bam*HI fragment BJ of TOL plasmid pWW15 and of the recombinant plasmids constructed from it. Abbreviations: Av, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. The *Pst*I-*Eco*RI region is expanded to show the position of the structural gene for C23OII, represented by the closed bar. In the recombinant plasmids the open boxes represent the vector DNA, and the stippled boxes represent the pWW15 DNA. The small arrows indicate the direction of transcription from the vector promoters, and restriction sites on vectors *Xho*I (▼), *Sma*I (▽), *Eco*RI (◆), and *Sst*I (◇) are shown.

of C23O in *E. coli* containing pWW15-3161 was very low (~2%) compared with *E. coli* carrying the cloned Xh fragment in pWW15-3151 (Table 2), suggesting that the tetracycline promoter might not be the element that regulates expression. This was confirmed by subcloning from pWW15-3161 into pKT230 such that the kanamycin promoter of this vector would direct transcription from the left to the right. Three recombinant plasmids were constructed, pWW15-3163 (containing *Xho*I-*Bam*HI), pWW15-3165 (containing *Sma*I-*Bam*HI), and pWW15-3167 (containing *Sma*I-*Hin*dIII) (Fig. 3). Expression from pWW15-3163 was at about the same level as that from the original pBR322 recombinant, but from pWW15-3165 and pWW15-3167 it was about 10-fold higher (Table 2).

Localization of the structural gene for C23OII on BJ. Plasmid pWW15-3167 was further subcloned by double digestion with *Xba*I and *Hin*dIII and by ligation into pUC12 and pUC13 to give pWW15-3171 and pWW15-3172, respec-

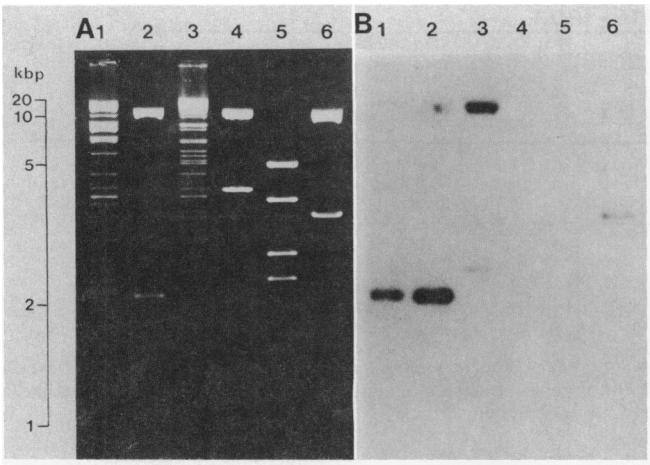

FIG. 4. (A) Hybridization analysis of TOL plasmid pWW15 and of recombinant plasmids containing pWW15 DNA. (A) Ethidium bromide-stained agarose gel; (B) autoradiogram of the same gel after hybridizing to ^{32}P-labeled fragment Xh. Lane 1, pWW15 digested with *Xho*I; lane 2, pWW15-3151 digested with *Xho*I; lane 3, pWW15 digested with *Bam*HI; lane 4, pWW15-3161 digested with *Bam*HI; lane 5, pWW15-3161 digested with *Bam*HI and *Hin*dIII; lane 6, pWW15-31500 digested with *Xho*I. The low intensity of the band in lane 6 is due to a 400-bp homology of pWW15-31500 with Xh; that of the 2.2.-kbp *Bam*HI fragment in lane 3 is the result of a common sequence of only 50 bp. No homology was detected in lanes 5 and 6 under the conditions used (50% formamide).

tively (Fig. 3), allowing transcription from the *lac* promoter in both directions. The C23O-specific activity from pWW15-3172 was about twice that from pWW15-3171 (Table 2), but in neither case was expression affected by isopropyl-β-D-thiogalactopyranoside, nor was it significantly higher than in the pKT230-derived plasmids pWW15-3165 and pWW15-3167.

Additional subcloning from pWW15-3171 gave pWW15-3173 (*Pst*I-*Hin*dIII fragment in pUC12) and pWW15-3175 (*Xba*I-*Sst*I fragment in pKT230) (Fig. 3). Both plasmids expressed C23O (Table 2), indicating that the gene is located on the common 1.8-kbp *Pst*I-*Sst*I region, although the sharp decline in activity from pWW15-3175 suggests the *Sst*I site might be close to one end of the gene. The *Sma*I-*Hin*dIII fragment on pWW15-3167 was further reduced by *Bal* 31 digestion after the unique *Xba*I site on the insert was cut. Expression of C23O was lost after digestion about 700 bp to the right of the *Pst*I site. The smallest plasmid obtained that was expressed was pWW15-3181, which retained the *Sst*I site (Fig. 3) and which resulted in 54% of the activity determined for pWW15-3167 (Table 2). A second *Bal* 31 digestion of pWW15-3167 at the other end of the insert was initiated after it was cut with *Hin*dIII, and the digested DNA was further cut with *Pst*I before religation. The activity directed by the smallest C23O$^+$ plasmid, pWW15-3183 (Fig. 3), was about 27% that of the parent plasmid pWW15-3167 (Table 2). Expression of C23OII activity therefore appeared to require no more than 1.1 kbp of DNA.

The two plasmids produced by *Bal* 31 digestion were further subcloned into pKT230 to give pWW15-3191 (*Sst*I fragment of pWW15-3181) and pWW15-3193 (*Eco*RI-*Sst*I fragment of pWW15-3183) (Fig. 3). Both plasmids directed low C23O activity (Table 2), which was comparable with the results achieved with the other plasmid (pWW15-3175) which had the *Sst*I site at its right end, supporting the presumption that this site is close to or actually in the structural gene.

Comparison of the two structural genes. The restriction maps of the two C23O genes suggest little or no homology. This was confirmed by hybridization after Southern blotting with ^{32}P-labeled Xh and BJ fragments (in pWW15-3151 and pWW15-3161, respectively) used as probes. The results for ^{32}P-labeled Xh (Fig. 4) show that it hybridizes to only Xh in *Xho*I-digested pWW15 and to only two fragments (BF and Bd) in *Bam*HI digests (there is one *Bam*HI site on Xh). There is clearly no homology with either *Bam*HI digests (Fig. 4, lane 4) or *Bam*HI-*Hin*dIII double digests (Fig. 4, lane 5) of pWW15-3161. The homology of fragment Xh with the *Xho*I novel fragment, cloned from pWW15-510 into recombinant plasmid pWW15-31500, is detectable (Fig. 4, lane 6).

Comparison of C23OI and C23OII activities. The relative rates of conversion of catechol and 3-methylcatechol by crude extracts of *E. coli* strains carrying cloned Xh-derived plasmids (C23OI) and BJ-derived plasmids (C23OII) are significantly different (Table 2). C23OI oxidized 3-methylcatechol at about 60% of the rate of catechol, whereas the corresponding figure for C23OII was between 180 and 220%.

Mapping the second deletion terminus on pWW15-510. The *Xho*I novel fragment in pWW15-510 caused by the deletion and fragment BJ share a common 2.3-kbp *Bam*HI-*Xho*I fragment located at the left end of BJ (Fig. 3). This was shown by comparison of the restriction map and confirmed by Southern hybridizations (data not shown). Because the novel fragment contains 3.4 kbp and was shown above to share 400 bp with Xh, it follows that the other end of the deletion in pWW15-510 is 700 bp to the left of the end of fragment BJ (Fig. 5).

Mapping the termini of the pWW15 DNA in the cointegrate plasmid pWW15-1003. The RP4::pWW15 cointegrate plasmid pWW15-1003 supports the growth of host *P. putida* strains on *m*-xylene and *m*-toluate but contains only about 150 kbp of the 250 kbp of DNA in pWW15, as judged by comparison of their respective restriction digests (data not shown). In the cointegration there appears to have been a loss of about 100 kbp of the TOL plasmid. About 80 to 90% of the deletion, which confers a B5 phenotype, is common to both pWW15-1003 and pWW15-510 (Fig. 5). The differences in the two deletions are crucial because pWW15-1003 contains fragment Xh (from which it was cloned), which is absent from pWW15-510. Conversely, BJ is present in pWW15-510, but is missing from pWW15-1003, although the adjacent *Bam*HI fragment BZ (data not shown) is present in the RP4 cointegrate. This suggests that the right-hand terminus of the deletion in pWW15-1003 is in BJ. This could be more accurately located by searching the digests of pWW15-1003 for the internal *Hin*dIII and *Eco*RI fragments which are part of BJ. Its position appears to be at the right-hand end of BJ beyond the structural gene for C23OII (Fig. 5). The deletion of C23OII from pWW15-1003 could be confirmed by measuring the substrate specificity of the activity induced in PRSB1003 (Table 3) which was identical to that found in *E. coli* strains carrying plasmids containing cloned Xh (Table 2).

Regulation of C23OI and C23OII genes. Growth of PRSB1003, which carries only C23OI gene, on *m*-toluate or *m*-xylene causes induction of C23OI. However, in MT15-510, which contains only a functional C23OII gene, only *m*-xylene induces the activity (*m*-toluate is unable to induce activity [Table 3]), which is in agreement with previously

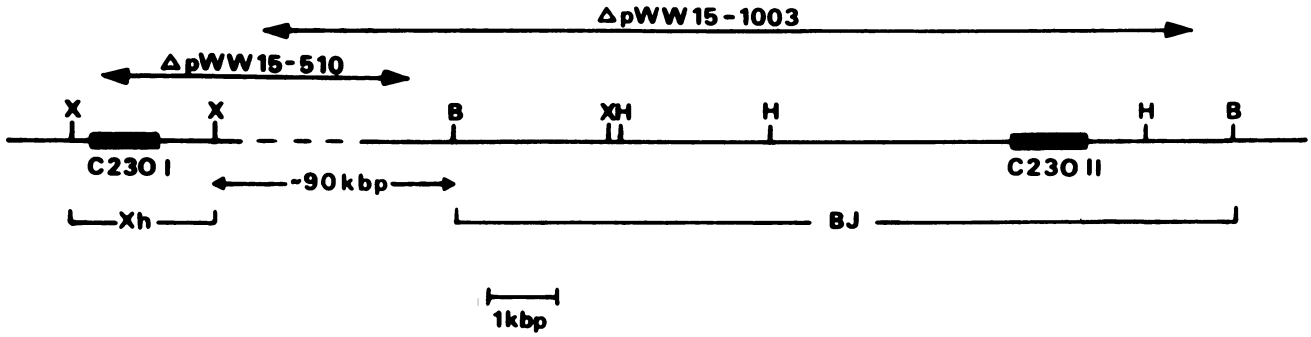

FIG. 5. Physical map of the region around the deletion sites on TOL plasmid pWW15. The deletions in pWW15-510 and pWW15-1003 are represented by the horizontal, double-headed arrows, and the unmapped 90-kbp region between Xh and BJ is indicated by the broken line. The position of restriction sites for *Xho*I (X), *Bam*HI (B), and *Hin*dIII (H) only are shown.

reported results for this strain and other B3 and B5 mutants (13). When the substrate specificity of C23O activity in wild-type MT15 was measured, the results were in agreement with the induction found in the two strains carrying deleted plasmids. Growth of MT15 in the presence of *m*-toluate resulted in the induction of C23OI almost exclusively, as judged by the low relative rate of conversion of 3-methylcatechol, whereas growth with *m*-xylene resulted in an intermediate specificity, indicating that both C23OI and C23OII genes were induced (Table 3).

DISCUSSION

The TOL plasmid pWW15, together with related plasmids pWW14 and pWW20, continues to provide novel results. These include strong selection against strains carrying the wild-type plasmid afforded by growth on benzoate minimal medium and the resulting segregation not only of the expected plasmid-free derivatives, but also of the B3 deletion mutants (24), investigation of which led to the first model of the regulation of a TOL plasmid catabolic pathway (26). More recently we have discovered the new class of phenotypically distinct B5 mutants (13) which have plasmid deletions that are very similar to those in the B3 mutants. It was a further investigation of one of these derivatives containing pWW15-510 which led us to the finding reported here that pWW15 codes for two nonhomologous C23O structural genes.

The lack of homology between the two genes, judged both by a lack of common restriction sites and a failure to hybridize, is surprising because recently we have cloned eight different C23O genes (*xylE*) from independently isolated TOL plasmids and two C23O genes from *P. putida* chromosomes (L. Chatfield and P. A. Williams, unpublished data), and all of them show a recognizable similarity in restriction sites and also hybridize with the cloned *xylE* gene of the archetypal TOL plasmid pWW0 (6). The C23OI gene of pWW15 (Fig. 2) fits into this family of related genes, but the C23OII gene is the only one we have cloned so far which appears to have a distinctly different structure. Unlike *xylE*-like gene C23OI, it is not obvious from the results presented here in which direction the C23OII gene is transcribed, as it is expressed in various pBR322, pKT230, pUC12, and pUC13 recombinants in both orientations, apparently independent of the vector promoters (Table 2). This suggests that it carries a sequence upstream which can be recognized as a weak constitutive promoter in *E. coli*, and it may also carry sequences further upstream which terminate transcription from the vector promoters. It is not clear whether this putative promoter sequence has any significance to its expression in *P. putida* because other TOL plasmid catabolic promoters function to a negligible extent in *E. coli*.

It is of particular interest that the two genes are separated on pWW15 by about 100 kbp but that both appear to be expressed during growth on *m*-xylene (Table 4). We have preliminary evidence, not presented here, that the genes for the upper enzymes of the toluene-xylene pathway (*xylA*, *xylB*, and *xylC*) are located to the right of the C23OII gene and that the genes for the lower pathway (*xylF* and *xylG*) are located to the left of the C23OI gene and possibly are part of the same operon. Plasmid pWW15 therefore would represent an extreme example of the previously reported, but much smaller, separation of the two operons of a plasmid-coded catabolic pathway in pWW0 (7) and the naphthalene plasmid nah7 (27). It is interesting that the 90-kbp deletion in the B3 and B5 mutants removes much of this intervening DNA, bringing the two operons into much closer proximity (Fig. 5).

The regulation of the two genes is also different. Only C23OI appears to be inducible by *m*-toluate, whereas growth in the presence of *m*-xylene results in both C23OI and C23OII being expressed. It cannot be deduced from these results whether the induction of both genes is due directly to *m*-xylene or to some metabolite of *m*-xylene. However, the inability of *m*-toluate to induce C23OII is clear both in MT15 and in MT15-510, whereas *m*-xylene (or a metabolite of it) is able to induce C23OII. This also explains why, in pseudorevertants of MT15-510, which have regained the capability to degrade *m*-toluate, sufficient amounts of C23O cannot be obtained by an induction process but require

TABLE 3. Activity and substrate specificity of C23O in *P. putida* strains

Strain	Inducer	Sp act (mU/mg) against:		Ratio (%)[a]
		Catechol	3-Methylcatechol	
MT15	None	120	ND[b]	ND
	m-Toluate	1,900	1,500	79
	m-Xylene	3,000	3,800	126
MT15-510	*m*-Toluate	4	ND	ND
	m-Xylene	1,150	2,110	180
PRSB1003	*m*-Toluate	250	200	57
	m-Xylene	1,960	1,300	66

[a] Ratio is (activity against 3-methylcatechol/activity against catechol) × 100.

[b] ND, Not determined.

amplification of genes, including the structural gene of C23OII (13). The evidence presented here casts into serious doubt our previous suggestions that the inability of B3 and B5 mutants to grow on *m*-toluate is due to the deletion of a regulatory gene which enables *m*-toluate to induce the enzymes necessary for its own dissimilation (13, 19, 26). The fact that it has been demonstrated unequivocally that in the B5 mutant MT15-510 the deletion removes most of the C23OI structural gene, which is the one induced in MT15 by growth on *m*-toluate, makes it unnecessary to hypothesize that a regulatory gene is also lost in the deletion event. Furthermore, if, as preliminary evidence indicates, the genes for the later enzymes of the pathway *xylF* and *xylG* are downstream of the C23OI gene and part of the same operon, the low expression of the *xylF* and *xylG* gene products in B5 mutants (13) might be explained by a polar effect of the deletion terminating in the C23OI gene.

We are currently attempting to answer some of the questions posed by these results by precisely mapping the relative locations of the other catabolic genes and by sequencing the two structural genes.

ACKNOWLEDGMENTS

We acknowledge the award of a European Science Exchange Programme Fellowship to H.K. by the Deutsche Forschungsgemeinschaft and the Royal Society, and the financial support of Shell Research (U.K.).

LITERATURE CITED

1. **Bachman, B. J.** 1972. Pedigrees of some mutant strains of *E. coli* K12. Bacteriol. Rev. **30**:525–557.
2. **Bagdasarian, N., F. C. H. Franklin, R. Lurz, B. Ruckert, N. N. Bagdasarian, and K. N. Timmis.** 1981. Specific purpose vectors. II. Broad host range high copy number RSF1010-derived vectors, and a host:vector system for gene cloning. Gene **16**:237–247.
3. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154**:203–204.
4. **Bolivar, F., R. L. Rodgriguez, P. J. Green, H. C. Belach, H. L. Heynecker, H. W. Boyer, J. J. Crosa, and S. Falkow.** 1977. Construction and characterisation of new cloning vehicles. II. A multi-purpose cloning system. Gene **2**:95–113.
5. **Cohen, S. N., A. C. Y. Chang, and C. L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
6. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. U.S.A. **78**:7458–7462.
7. **Franklin, F. C. H., M. Bagdasarian, and K. N. Timmis.** 1981. Manipulation of degradative genes in soil bacteria, p. 111–132. *In* R. Hutter and T. Leisinger (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. Academic Press, Inc., London.
8. **Franklin, F. C. H., and P. A. Williams.** 1980. Construction of a partial diploid for the degradation pathway encoded by the TOL plasmid (pWW0) from *Pseudomonas putida* mt-2: evidence for the positive nature of the regulation by the *xylR* gene. Mol. Gen. Genet. **177**:321–328.
9. **Girvitz, S. C., S. Bacchetti, A. J. Rainbow, and F. W. Graham.** 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. Anal. Biochem. **106**:492–496.
10. **Guerry, P., D. J. Leblanc, and S. Falkow.** 1973. General method for isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
11. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
12. **Jeenes, D. J., and P. A. Williams.** 1982. Excision and integration of degradative pathway genes from TOL plasmid pWW0. J. Bacteriol. **150**:188–194.
13. **Keil, H., and P. A. Williams.** 1985. A new class of TOL plasmid deletion mutants in *Pseudomonas putida* MT15 and their reversion by tandem gene amplification. J. Gen. Microbiol. **131**:1023–1033.
14. **Kunz, D. A., and P. J. Chapman.** 1981. Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida* (*arvilla*) mt-2: evidence for new functions of the TOL (pWW0) plasmid. J. Bacteriol. **146**:179–191.
15. **Kunz, D. A., and P. J. Chapman.** 1981. Isolation and characterization of spontaneously occurring TOL plasmid mutants of *Pseudomonas putida* HS1. J. Bacteriol. **146**:952–964.
16. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
17. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
18. **Nakai, C., H. Kagamiyana, M. Nozaki, T. Nakazawa, S. Inouye, Y. Ebina, and A. Nakazawa.** 1983. Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. J. Biol. Chem. **258**:2923–2928.
19. **Pickup, R. W., and P. A. Williams.** 1982. Spontaneous deletions in the TOL plasmid pWW20 which give rise to the B3 regulatory mutants of *Pseudomonas putida* MT20. J. Gen. Microbiol. **128**:1385–1390.
20. **Sala-Trepat, J. M., and W. C. Evans.** 1971. The meta cleavage of catechol by *Azotobacter* species. 4-Oxalocrotonate pathway. Eur. J. Biochem. **20**:400–413.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:505–518.
22. **Wheatcroft, R., and P. A. Williams.** 1981. Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. J. Gen. Microbiol. **124**:433–437.
23. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
24. **Williams, P. A., and M. J. Worsey.** 1976. Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bacteriol. **125**:818–828.
25. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.
26. **Worsey, M. J., and P. A. Williams.** 1977. Characterization of a spontaneously occurring mutant of TOL20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. J. Bacteriol. **130**:1149–1158.
27. **Yen, K.-M., and I. C. Gunsalus.** 1982. Plasmid gene organization: Naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. U.S.A. **79**:874–878.