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Naturally Occurring TOL Plasmids in *Pseudomonas* Strains Carry Either Two Homologous or Two Nonhomologous Catechol 2,3-Oxygenase Genes

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Structural genes for catechol 2,3-oxygenase (C23O) were cloned from the TOL plasmids pWW5, pWW14, pWW74, pWW84, and pWW88 isolated from *Pseudomonas* strains of diverse geographical origins. Each pKT230-based C23O$^+$ recombinant plasmid carried a 2.05-kilobase *Xho*I insert which showed strong homology in Southern hybridizations with the *xylE* gene from the archetype TOL plasmid pWW0. Fragments were mapped for restriction endonuclease sites and were classified into two closely related groups on the basis of restriction maps. C23O structural genes were located on cloned fragments by a combination of subcloning and site-specific mutagenesis. All five TOL plasmids examined yielded clones whose maps differed from that of *xylE* of pWW0 by only a single *Xba*I site, but in addition plasmids pWW5, pWW74, and pWW88 carried a second, homologous C23O gene with seven further restriction site differences. The remaining plasmids, pWW14 and pWW84, carried a second nonhomologous C23O gene related to the second C23O gene (C23OII) of TOL plasmid pWW15 described previously (H. Keil, M. R. Lebens, and P. A. Williams, J. Bacteriol. 163:248–255, 1985). Thus, each naturally occurring TOL plasmid in this study appears to carry genes for two *meta* cleavage dioxygenases.

The degradation of toluene and some substituted toluenes via the *meta* pathway by *Pseudomonas* spp. is often plasmid mediated (7, 15, 19, 26, 34, 35, 39). TOL plasmids from different strains are diverse with regard to molecular sizes, fragmentation patterns by restriction endonucleases, self-transmissibility, rate of dissimilation of *p*-methyl-substituted substrates, and formation of different classes of deletion mutants after growth on benzoate (7, 17, 27, 35, 37, 38).

Although the archetype TOL plasmid pWW0 (34) has a single *xylE* gene coding for the ring cleavage dioxygenase catechol 2,3-oxygenase (C23O), it was recently demonstrated in this laboratory that the 250-kilobase (kb) plasmid pWW15 carries two nonhomologous genes which specify the apparently unrelated enzymes C23OI and C23OII (16). To investigate the evolutionary relationship between the catabolic genes on different TOL plasmids, we cloned *xylE* genes from five TOL plasmids found in *Pseudomonas* strains isolated from soil samples of diverse geographical origins. Like pWW15, two of the plasmids reported here also carried two nonhomologous C23O genes. Unexpectedly, the other plasmids in this study each possessed two closely related C23O genes which exhibited strong homology with each other and with the *xylE* gene of pWW0.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas* strains and plasmids used in this study are described in Table 1. Strains MT74, MT84, and MT88 were isolated by selective enrichment on *m*-toluate minimal medium during this study; MT5 and MT14-26 have been described previously (7, 26, 35). *Escherichia coli* PM191, a *thr leu thi recA* derivative of the K-12 strain C600 (1), was used as host for all recombinant plasmids listed in Table 2.

Media and culture conditions. All plasmid-containing *Pseudomonas* strains were maintained on 5 mM *m*-toluate minimal media as described by Worsey and Williams (37). Other aromatic carbon sources were either added to the media to 5 mM (10 mM for benzoate) concentration or were present in the vapor phase. All *Pseudomonas* strains were cultured at 30°C. *E. coli* strains were routinely grown at 37°C in Luria broth supplemented with the appropriate antibiotic at the following concentrations (in micrograms per milliliter): streptomycin, 15; kanamycin, 20; ampicillin, 25; and tetracycline, 7.5.

Bacterial matings. Conjugation experiments were carried out with broth cultures containing donor strains mixed with the streptomycin-resistant recipient PaW340 in a 1:10 ratio. Cultures were incubated at 30°C overnight without shaking. Transfer frequencies were calculated as the number of transconjugants formed relative to the input number of donor cells. Selection for transconjugants of PaW340 was made for growth on *m*-toluate plates containing streptomycin (1 mg/ml).

Isolation of DNA. TOL plasmids were isolated by using the analytical and sucrose gradient procedures of Wheatcroft and Williams (32). Small-scale recombinant plasmid preparations were made by the method of Holmes and Quigley (12) to permit rapid screening of plasmids, and stocks of recombinant plasmid DNA for subcloning, mutagenesis, and restriction mapping were prepared by a cleared lysate procedure (10) followed by CsCl-ethidium bromide density gradient centrifugation.

Restriction endonuclease digestion and agarose gel electrophoresis. Enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or NBL Enzymes

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TABLE 1. *Pseudomonas* strains and TOL plasmids

Strain	Phenotype[a]					TOL plasmid		Transfer frequency	C23O activity[c]	Source of isolate	Reference
	Tln	Mtol	Ptol	Mxy	Pxy	Designation	Size (kb)[b]				
PaW1	+	+	+	+	+	pWW0	117	10^{-2}–10^{-3}	+	Japan	34
MT5	+	+	+	+	+	pWW5	160	$<10^{-7}$	+	Wales	7, 35
MT5-S3	−	−	−	−	−			ND[d]	−	Cured derivative of MT5	This study
MT14-26	+	+	+	+	±	pWW14	270	$<10^{-7}$	+	Wales	27
MT14-263	−	−	−	−	−			ND	−	Cured derivative of MT14-26	This study
MT74	+	+	+	+	+	pWW74	105	$<10^{-7}$	+	Shetland Islands, Scotland	This study
MT74-1	−	−	−	−	−			ND	−	Cured derivative of MT74	This study
MT84	+	+	−	+	+	pWW84	180	$<10^{-7}$	+	Southern England	This study
MT84-1	−	−	−	−	−			ND	−	Cured derivative of MT84	This study
MT88	+	+	+	+	+	pWW88	170	10^{-1}	+	Sweden	This study
MT88-2	−	−	−	−	−			ND	−	Cured derivative of MT88	This study

[a] Abbreviations represent the ability to grow on: toluene, Tln; *m*-toluate, Mtol; *p*-toluate, Ptol; *m*-xylene, Mxy; and *p*-xylene, Pxy.
[b] Sizes of pWW74, pWW84, and pWW88 were determined by summation of sizes of restriction endonuclease-generated fragments after digestion with *Xho*I, *Pst*I, and *Pvu*II.
[c] C23O activity of strains was determined by the catechol spray test.
[d] ND, Not determined.

Ltd. (Northumberland, United Kingdom), and digestion was carried out according to the instructions of the manufacturer. Electrophoresis was as described previously (32).

Cloning experiments and transformation of *E. coli* strains. Restriction endonuclease digestion and ligation of DNA with T4 DNA ligase (Bethesda Research Laboratories) were carried out according to the instructions of the manufacturer, and *E. coli* PM191 was transformed with ligated DNA samples by the standard procedure described by Cohen et al. (5). Colonies containing recombinant plasmids were screened for C23O activity by the catechol spray test (8, 25). The vector plasmids pKT230, pBR322, and pBR328 have been described previously (2, 4, 30).

S1 mutagenesis of recombinant plasmids. Plasmid DNA samples were digested with the appropriate restriction enzyme as described in the text, and the sticky ends generated by this procedure were removed by treatment with nuclease S1 (Bethesda Research Laboratories) by using 5 U of enzyme per μg of DNA in S1 assay buffer (3) at 37°C for 30 min. After ethanol precipitation of DNA at −20°C and suspension in ligation buffer, blunt ends generated by this method were religated by using T4 DNA ligase, and plasmids were transformed into strain PM191.

DNA-DNA hybridization. DNA restriction endonuclease fragments were transferred from agarose gels to Biodyne filter paper (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) by the method of Southern (31). Cloned DNA fragments for use as probes in hybridizations were separated from the vector by restriction enzyme digestion and agarose gel electrophoresis and extracted from gels by the method of Girvitz et al. (9). Fragments were labeled in vitro with [^{32}P]dGTP and hybridized as described by Keil and Williams (17).

RESULTS

Plasmid content of strains. Strains MT5, MT14-26, MT74, MT84, and MT88 are classified as toluene-degrading *Pseudomonas* species by the API 20NE strain identification system (API Laboratory Products Ltd., Basingstoke, United Kingdom), by colony morphology on King, Ward, and Raney media (18), and by the ability of these strains to use *m*-toluate and *m*-xylene as sole carbon sources (Table 1).

TABLE 2. Recombinant plasmids

Plasmid	Source of DNA	Vector	C23O expression	Notes[a]
pWW0-3004	pWW0	pKT230	+	2.25-kb *Xho*I insert in Km^r gene
pWW5-3002	pWW5	pKT230	+	2.05-kb *Xho*I insert in Km^r gene
pWW5-3008	pWW5	pKT230	+	4.9-kb *Eco*RI insert in Sm^r gene
pWW14-3141	pWW14	pKT230	+	2.05-kb *Xho*I insert in Km^r gene
pWW14-3201	pWW14-3141	pBR328	+	1.72-kb *Sal*I partial digest in Tc^r gene
pWW14-3211	pWW14-3141	pKT230	+	Nuclease S1 digestion of *Kpn*I site
pWW14-3212	pWW14-3141	pKT230	−	Nuclease S1 digestion of *Xba*I site
pWW74-3016	pWW74	pKT230	+	2.05-kb *Xho*I insert in Km^r gene
pWW74-3209	pWW74-3016	pBR328	+	1.72-kb *Sal*I partial digest in Tc^r gene
pWW74-3211	pWW74-3016	pKT230	+	Nuclease S1 digestion of *Kpn*I site
pWW74-3212	pWW74-3016	pKT230	−	Nuclease S1 digestion of *Sst*I site
pWW74-3221	pWW74-3016	pBR322	−	1.17-kb *Sma*I-*Xho*I subclone
pWW84-3001	pWW84	pKT230	+	2.05-kb *Xho*I insert in Km^r gene
pWW88-3001	pWW88	pKT230	+	2.05-kb *Xho*I insert in Km^r gene
pWW88-3002	pWW88	pKT230	+	2.05-kb *Xho*I insert in Km^r gene

[a] Km^r, Kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

Each wild-type strain was examined for plasmid content by a rapid analytical method (32). All contained plasmid DNA, but whereas strains MT5, MT14-26, and MT74 each contained only one large plasmid, MT84 and MT88 harbored three and six distinguishable plasmids, respectively, although only one large plasmid species was observed in each strain.

To test whether the catabolic function in each strain was plasmid or host specified, cultures of MT5, MT14-26, MT74, MT84, and MT88 were grown in minimal medium containing 5 mM benzoate as the sole carbon source. This procedure (34, 35) exerts selection on TOL plasmid-containing pseudomonads against use of the plasmid *meta* cleavage pathway in favor of the alternative, chromosomally encoded β-ketoadipate pathway (34) and therefore provides a convenient method of curing strains harboring TOL plasmids. Mtol$^-$ segregants were isolated from each strain and designated MT5-S3, MT14-263, MT74-1, MT84-1, and MT88-2, respectively (Table 1). These strains were unable to grow on toluene, *m*-toluate, or *m*-xylene minimal media (Tln$^-$ Mtol$^-$ Mxy$^-$) and, unlike parental strains, did not exhibit detectable C23O activity when tested by the catechol spray method. Analysis of plasmid content by the rapid analytical and sucrose gradient methods (32) revealed that MT5-S3, MT14-263, and MT74-1 had each lost the single plasmid band observed in the parental strain. Similarly, MT84-1 differed from MT84 in the loss of the largest of its three plasmids. MT88-2 had also lost the largest of the six plasmids present in MT88, but since several of the smaller plasmids were also not discernible in MT88-2, it was not possible to conclude from these experiments alone that the TOL genes in this strain reside on a single plasmid replicon.

MT5, MT14-26, MT74, MT84, and MT88 were used as donor strains in matings with strain PaW340, a plasmid-free derivative of *Pseudomonas putida* mt-2, as recipient, with selection for transconjugants on *m*-toluate medium. Only strain MT88 was able to transfer the ability to grow on *m*-toluate, and transconjugants contained only a single plasmid band when examined by the same analytical method. Restriction endonuclease digests of plasmid DNA prepared by the sucrose gradient method from strain MT88 and its transconjugants were indistinguishable, indicating that the smaller plasmids in MT88 were not purified by this method; this agrees with our experience that the method is not suitable for preparing plasmids of less than 40 to 50 kb. The presence in transconjugants of a single TOL plasmid identical to that found in MT88 indicates that the TOL genes also reside on the single large plasmid replicon in this strain. We have therefore given the large TOL plasmid in strains MT74, MT84, and MT88 a pWW designation corresponding to the MT strain number. Previously described strains MT5 and MT14-26 have already been shown to contain the TOL plasmids pWW5 (7) and pWW14 (26), respectively.

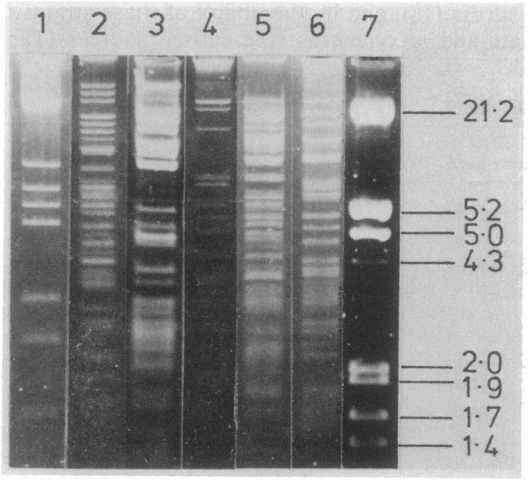

FIG. 1. Agarose gel electrophoresis of TOL plasmid DNA fragments generated by *Xho*I digestion. Lanes: 1, pWW0; 2, pWW5; 3, pWW14; 4, pWW74; 5, pWW84; 6, pWW88; 7, *Hin*dIII-*Eco*RI digest of bacteriophage λ DNA.

*Xho*I digestion of pWW5, pWW14, pWW74, pWW84, and pWW88 (Fig. 1) revealed gross differences between these plasmids, which are therefore taken to represent a diverse sample of naturally occurring TOL plasmids.

Molecular cloning of C23O genes. Cloning of structural genes for C23O from each of these strains was readily achieved by using the catechol spray method to identify C23O$^+$ *E. coli* transformants. In most cases, the successful experiment was carried out by ligating *Xho*I-digested TOL plasmid DNA into the corresponding restriction site in the kanamycin resistance gene of pKT230, but for pWW5 an *Eco*RI digest gave the first C23O$^+$ transformant. Transformants from experiments with pWW5, pWW14, pWW74, pWW84, and pWW88 DNA all harbored recombinant plasmids which carried a 2.05-kb *Xho*I fragment of DNA derived from the appropriate TOL plasmid (Table 2). Even in the case of the *Eco*RI-derived recombinant plasmid pWW5-3008, the cloned 4.9-kb insert of pWW5 DNA contained two *Xho*I sites separated by 2.05 kb. Closer examination of cloned inserts revealed some internal restriction site differences (Fig. 2), and two distinct C23O$^+$ *Xho*I fragments of the same size, but which differed in their internal restriction sites, were isolated from pWW5 and pWW88. Two recombinant plasmids, one of each type derived from pWW5 and pWW88 and one each from pWW14, pWW74, and pWW84, were retained for further analysis (Table 2).

Analysis of cloned DNA fragments. All cloned DNA fragments were mapped with restriction nucleases *Ava*I, *Bam*HI, *Bst*EII, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Sst*I, *Xba*I, and *Xho*I by a combination of double and triple digestions; none contained internal *Eco*RI, *Hin*dIII, or *Sph*I sites. To aid mapping and to allow direct comparison with the *xylE* gene of pWW0, restriction enzyme digestion was also carried out on pWW0-3004, which carries the 2.25-kb *Xho*I-I fragment from the TOL plasmid pWW0. The nucleotide sequence of this gene has recently been established (21, 40), allowing accurate location of restriction sites internal to the gene. Since *xylE* is transcribed from left to right as pWW0-3004 is drawn in Fig. 2 (21, 40) and all cloned fragments in Fig. 2 are oriented in the same direction with respect to their vector promoters, it appears that their direction of transcription is also from left to right.

The maps show a remarkable conservation of restriction sites. The cloned fragments fall into two distinct, but related groups on the basis of these restriction maps. The cloned inserts carried by pWW5-3002, pWW14-3141, pWW84-3001, and pWW88-3002 are identical to each other and to the *Xho*I fragment carrying the C23OI gene of pWW15 (16). These fragments differ from the pWW0 segment of pWW0-3004 by only three sites, those for *Ava*I, *Xba*I, and *Xho*I. With the exception of a *Bam*HI site located on pWW5-3008 in a complementary position to the *Bam*HI site of pWW0-3004, the 2.05-kb *Xho*I inserts carried by pWW5-3008, pWW74-3016, and pWW88-3001 are identical to each other but differ from pWW0-3004 by 10 restriction sites.

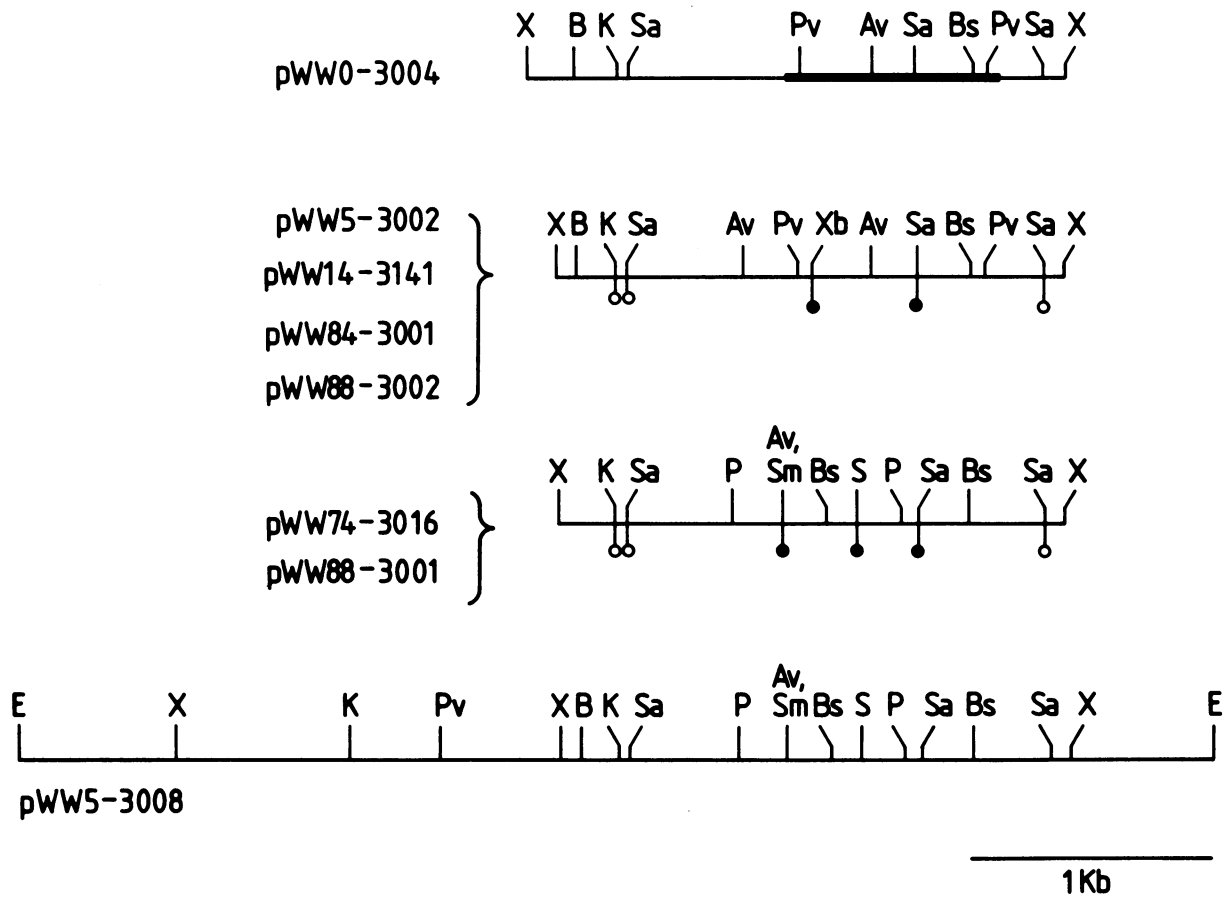

FIG. 2. Restriction endonuclease maps of cloned fragments of TOL plasmid DNA carrying genes for C23O. Recombinant plasmids bearing these fragments are listed in Table 2. Abbreviations: Av, *Ava*I; B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sst*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. The position of *xylE* on pWW0-3004 is represented by the thick line, and transcription from the kanamycin resistance promoter of pKT230 in each case is deduced to be from left to right. Circles indicate sites on the cloned DNA fragments from recombinant plasmids pWW14-3141 and pWW74-3016 that are deduced to lie outside (○) or within (●) the genes for C23O (see text).

Location of structural genes on cloned fragments. To confirm that the conservation of restriction sites reflects a corresponding conservation of the location of the structural gene on each cloned fragment, recombinant plasmids pWW14-3141 and pWW74-3016 were taken as representative of each of the two groups of related DNA inserts. Both were partially digested with *Sal*I and recloned into the *Sal*I site of pBR328. The only transformants which were C23O$^+$ contained both internal *Sal*I fragments (1.18 and 0.54 kb) from each parental plasmid aligned continuously and in the same orientation (pWW14-3201 and pWW74-3209; Table 2). Clones carrying single *Sal*I fragments were C23O$^-$. The structural gene must therefore span the central *Sal*I (Fig. 2) site. Similarly, the *Sma*I-*Xho*I internal fragment of pWW74-3016 was subcloned by ligation into pBR322 in the correct orientation for transcription to form pWW74-3221 (Table 2). No C23O activity was detected, and therefore the *Sma*I site is deduced to lie within the gene.

S1 mutagenesis of both pWW14-3141 and pWW74-3016 was carried out to produce blunt ends at the unique *Kpn*I and *Xba*I sites on these plasmids and at the cloned *Sst*I site on the latter by using a partial *Sst*I digest to circumvent the presence of the *Sst*I site on the vector (Table 2). The blunt ends were then religated. S1 mutagenesis of the *Kpn*I sites in each case (to give the plasmids pWW14-3211 and pWW74-3211; Table 2) had no effect on C23O expression, whereas similar treatment of the *Xba*I site of pWW14-3141 (plasmid pWW14-3212; Table 2) and the *Sst*I site of pWW74-3016 (plasmid pWW74-3212; Table 2) resulted in the loss of C23O activity. Therefore, these results confirm that the C23O structural gene is in an analogous position on the cloned TOL plasmid fragments as on pWW0-3004.

DNA-DNA hybridization with the pWW0 *xylE* probe. The relatedness between C23O genes indicated by the overall similarity of restriction maps was borne out by results of Southern hybridization experiments. Since the *xylE* gene of pWW0 is bound internally by two *Pvu*II sites (Fig. 2), pWW0-3004 was digested with *Pvu*II, and the internal DNA fragment was isolated from agarose gels to yield a highly specific probe for *xylE* DNA sequences. This fragment was nick translated by using [^{32}P]dGTP and hybridized against Southern blots of *Xho*I-digested TOL plasmid DNA prepared by the sucrose gradient method (32) from strains MT5, MT14-26, MT74, and MT84 and the transconjugant strain PaW340(pWW88). Each TOL plasmid carries at least one *Xho*I fragment homologous with *xylE* and of the size indicated by cloning and restriction data (Fig. 3).

Total cellular DNA was extracted from the cured *Pseudomonas* strains MT5-S3, MT14-263, MT74-1, MT84-1, and MT88-2 by the method of Roussel and Chabbert (29), and *Xho*I digests of this DNA were used in similar hybridization experiments with the same *xylE*-specific [^{32}P]dGTP-labeled

DNA fragment from pWW0-3004. No hybridization of this probe was observed against any of these DNA samples, showing that sequences sharing homology with *xylE* are not present on either the chromosome of these strains or on the residual plasmids in MT84-1 or MT88-2.

Although these results demonstrate the relatedness of C23O genes on the TOL plasmids pWW5, pWW14, pWW74, pWW84, and pWW88 between each other and the *xylE* gene of pWW0, this experiment does not differentiate between the two classes of cloned DNA fragments represented by the recombinant plasmids pWW14-3141 and pWW74-3016 (Fig. 2), since all these genes reside on *Xho*I DNA fragments of the same size. One convenient distinction between the genes exemplified by pWW14-3141 and pWW74-3016 is the presence of two *Pvu*II sites within the former and of two *Pst*I sites (one inside and one upstream of the gene) in the latter (Fig. 2). By using the *xylE Pvu*II fragment of pWW0-3004 as a probe, we again performed hybridizations against TOL plasmid DNA digested with either *Pvu*II (Fig. 4) or *Pst*I (Fig. 5). A 0.75-kb *Pvu*II fragment in each track hybridized (Fig. 4), and it follows that all TOL plasmids in this study, including pWW74, specify a C23O gene corresponding to that exemplified by pWW14-3141. Furthermore, the presence of an additional 4.3-kb *Pvu*II fragment in the tracks containing pWW5, pWW74, or pWW88 DNA indicates that these TOL plasmids carry a second, homologous C23O gene. This is confirmed by Fig. 5, which shows that this second gene corresponds to that carried by pWW74-3016 since pWW5, pWW74, and pWW88 possess a 0.6-kb *Pst*I fragment which hybridizes to the *xylE* probe. The 4.7-kb fragment hybridizing in these tracks presumably results from the region of overlap between the *Pvu*II-derived probe and the *Pst*I site within the C23O gene, while the 12.7-kb restriction fragment is deduced to carry the other structural gene (identical to that of pWW14-3141) by analogy with the tracks containing pWW14 or pWW84 DNA (Fig. 5).

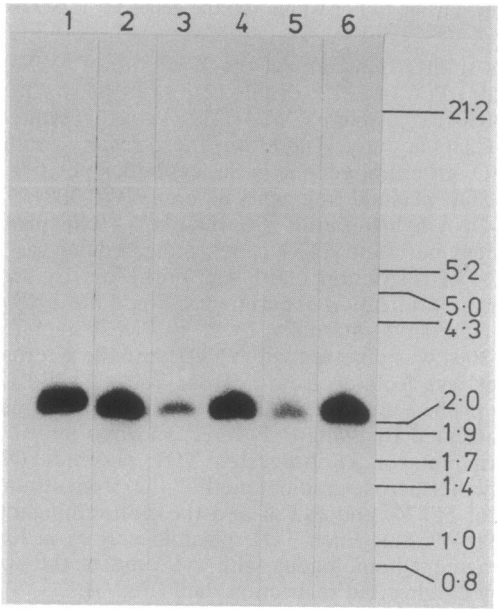

FIG. 3. Southern hybridization analysis of *Xho*I-digested TOL plasmid DNA. The probe was prepared from pWW0-3004 by nick translation of the *Pvu*II fragment internal to *xylE* after its extraction from an agarose gel. Lanes: 1, pWW0; 2, pWW5; 3, pWW14; 4, pWW74; 5, pWW84; 6, pWW88.

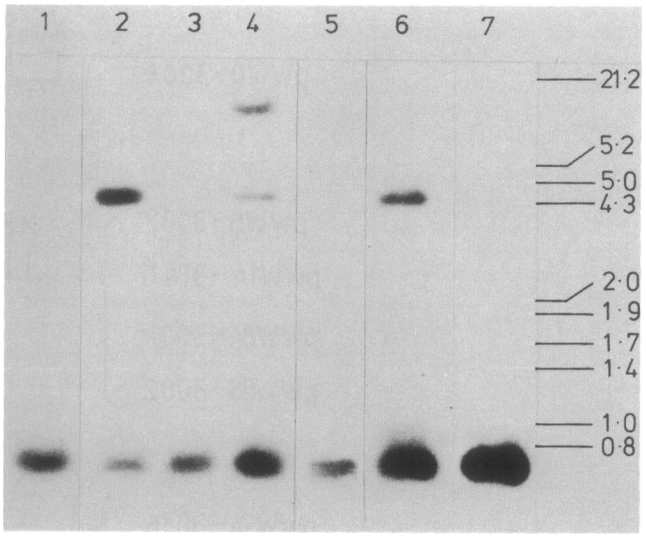

FIG. 4. Southern hybridization analysis of *Pvu*II-digested plasmid DNA. The *Pvu*II probe was as described for Fig. 3. Lanes: 1, pWW0; 2, pWW5; 3, pWW14; 4, pWW74; 5, pWW84; 6, pWW88; 7, pWW14-3141.

Neither of the cloned C23O gene types contains *Eco*RI sites, and these genes are shown to reside on separate *Eco*RI fragments by the hybridization of the *xylE* probe to *Eco*RI-digested TOL plasmids in Fig. 6. Two bands, of 5.2 and 4.9 kb, are detected in tracks containing pWW5, pWW74, and pWW88, and the relevant 4.9-kb fragment of pWW5 corresponds to that cloned in pWW5-3008. Only a single *Eco*RI fragment was detected in tracks containing pWW14 and pWW84, and so these TOL plasmids specify only a single C23O gene with homology to *xylE* of pWW0.

DNA-DNA hybridizations with the pWW15 C23OII gene as probe. To test for the presence of DNA sequences sharing homology with the C23OII gene of pWW15 (16), the fragment specifying the C23OII gene from pWW15 was isolated from the recombinant plasmid pWW15-3183 (16) and used as

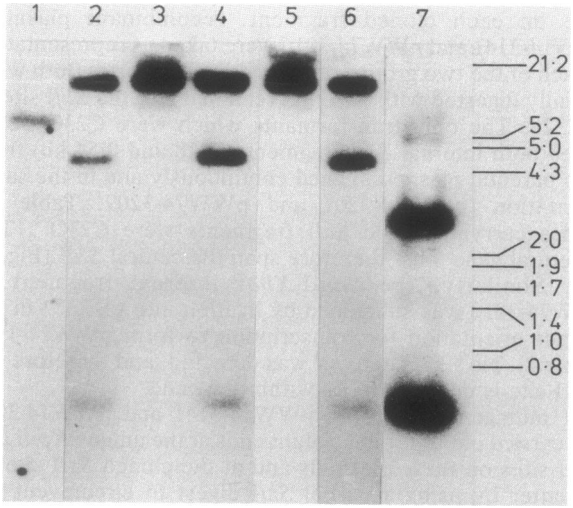

FIG. 5. Southern hybridization analysis of *Pst*I-digested plasmid DNA. The *Pvu*II probe was as described for Fig. 3. Lanes: 1, pWW0; 2, pWW5; 3, pWW14; 4, pWW74; 5, pWW84; 6, pWW88; 7, pWW74-3016.

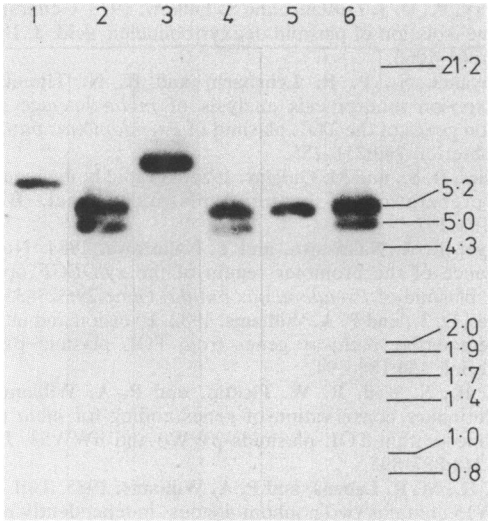

FIG. 6. Southern hybridization analysis of *Eco*RI-digested plasmid DNA. The *Pvu*II probe was as described for Fig. 3. Lanes: 1, pWW0; 2, pWW5; 3, pWW14; 4, pWW74; 5, pWW84; 6, pWW88.

a probe in Southern hybridizations against *Xho*I-digested TOL plasmid DNA from strains MT5, MT14-26, MT74, MT84, and MT88. Both pWW14 and pWW84 contain a single large *Xho*I restriction fragment which possesses homology with the C23OII structural gene and is easily distinguishable from the 2.05-kb fragments found in pWW14-3141 and pWW84-3001 (Fig. 7). Since it was shown above that these TOL plasmids generated only a single band homologous with *xylE*, both pWW14 and pWW84 are deduced to specify both C23OI and C23OII enzymes by analogy with pWW15. Furthermore, since the *Xho*I bands shown in Fig. 7 are each about or greater than 20 kb, the failure to isolate C23OII genes from these TOL plasmids in cloning experiments with *Xho*I can be accounted for by the greater efficiency with which smaller DNA fragments can be cloned.

DISCUSSION

Despite the differences in restriction endonuclease digests and sizes of the TOL plasmids used in this study, the degree of conservation of restriction sites in the structural genes for C23O is remarkable, especially when the distinct geographical origins of the isolates are considered. All the TOL plasmids examined here carry a C23O gene similar to the *xylE* gene of pWW0 and, on the basis of restriction maps, identical to the C23OI gene of pWW15. Surprisingly, each plasmid also carries a second C23O gene, either homologous to the first (plasmids pWW5, pWW74, and pWW88) or nonhomologous but related to the C23OII gene of pWW15 (plasmids pWW14 and pWW84). To clarify the nomenclature of C23O genes, it is proposed that the enzyme specified by pWW14-3141 be designated C23OIa and that the enzyme encoded by pWW74-3016 be designated C23OIb. Thus, genes coding for C23OIa and C23OIb represent two closely related and homologous loci which have arisen from a common ancestor, whereas the C23OII genes of pWW14, pWW15, and pWW84 belong to a separate gene family which either is more distantly related or is totally unrelated. To add to this list, we have recently isolated a third C23OI-type gene from the chromosome of the phenol-utilizing *P. putida* strain NCIB 10015 (6), which shares fewer restriction sites in common with C23OIa and C23OIb genes but still exhibits a marked degree of homology (unpublished results).

The conservation of restriction endonuclease sites flanking the cloned C23O genes indicated by both restriction maps of the cloned fragments and Southern hybridization experiments is also of significance. Most C23OIa genes reside on *Pst*I and *Eco*RI fragments of similar sizes, and C23OIb genes also lie on apparently identical *Pvu*II and *Eco*RI segments. Southern hybridizations with the entire *Xho*I insert from pWW0-3004 rather than the *xylE*-specific *Pvu*II fragment from this plasmid as the probe have allowed identification of the flanking *Pvu*II fragments of C23OIa genes (data not shown), and both C23OIa and C23OIb genes are flanked by identically positioned *Pvu*II sites 4.3 kb apart. Furthermore, the 4.9-kb *Eco*RI insert of pWW5-3008, which carries a C23OIb-type gene, displays a high degree of site conservation when compared with the maps for the corresponding *meta* pathway regions of TOL plasmids pWW0 and pWW53 (11, 13, 15), which specify C23OIa genes. Thus, from the conservation of restriction endonuclease sites outside the structural genes for C23O, it appears that each C23O gene may be part of an entire *meta* pathway operon (*xylDLEGF*) and that the other structural genes of these operons are also conserved and closely related. This argument is strengthened by our having recently cloned two complete and homologous *meta* pathway operons from TOL plasmid pWW53; one of them encodes a C23OIa gene, and the other encodes a C23OIb gene (D. J. Osborne, H. Keil, and P. A. Williams, unpublished results).

Presumably, the conservation of C23O genes and their flanking DNA regions results from the inheritance of catabolic genes as a block by sequences of recombination or transposition events. We have demonstrated that a region of 56 kb from pWW0, carrying the entire catabolic genes of this plasmid, can integrate into the host chromosome, from which it can be rescued as part of other plasmids by appropriate selection (14, 20), and there are a number of reports of R plasmid-TOL cointegrate formation in which a part of the TOL plasmid carrying the catabolic genes has

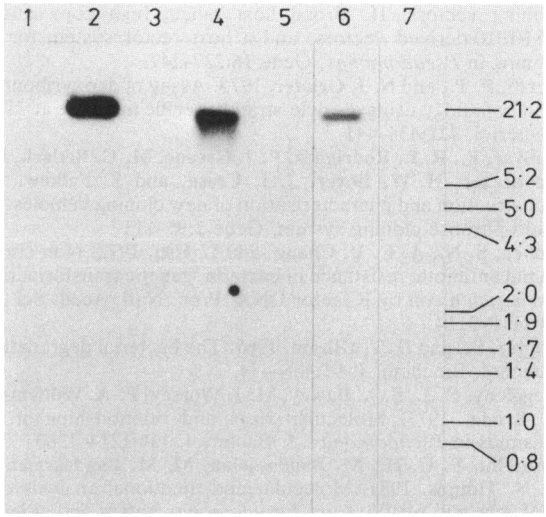

FIG. 7. Southern hybridization analysis of *Xho*I-digested plasmid DNA. The probe was prepared from pWW15-3183 (16) by nick translation of fragment BJ carrying the C23OII gene of pWW15 after its extraction from an agarose gel. Lanes: 1, pWW0; 2, pWW15; 3, pWW5; 4, pWW14; 5, pWW74; 6, pWW84; 7, pWW88.

become integrated into an R plasmid without any detectable chromosome-linked intermediate (22, 33).

The role of conjugative plasmids in the dispersal of catabolic genes among bacterial populations has long been recognized (28, 36), and the absence of any correlation between classes of C23O gene and geographical origin of isolates in this study is taken as evidence that this mechanism is highly efficient in nature. This situation appears to be analogous to the dispersal of antibiotic resistance genes, but whereas it is easy to imagine the epidemiological spread of drug resistance plasmids and transposons by human travel, the routes taken by catabolic genes between soil-living bacteria isolated from four different land masses are less evident.

The finding of two related C23O genes on a number of TOL plasmids is perhaps surprising in light of studies on pWW0, which specifies only a single gene, but these data would suggest that this is a common feature of many naturally occurring TOL plasmids. The reasons for this are obscure, although there may prove to be a further analogy with the clustering of antibiotic resistance genes on large R plasmids. Okada and co-workers (23, 24) have identified two catabolic genes (*nylB* and *nylB'*) with 88% homology on the nylon oligomer-degradative plasmid pOAD2, but this situation has been taken to reflect a recent gene duplication and mutation in the evolution of a novel enzyme active on unnatural synthetic compounds. Although cloned C23O genes are active under the influence of vector promoters, it is not yet known whether or how these genes and their respective *meta* pathways are expressed and regulated in *Pseudomonas* spp. or even whether each pathway is involved with the degradation of the same primary substrate.

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