JOURNAL OF BACTERIOLOGY, Nov. 1990, p. 6355–6362
0021-9193/90/116355-08$02.00/0
Vol. 172, No. 11

Genetic Analysis of Chromosomal Operons Involved in Degradation of Aromatic Hydrocarbons in *Pseudomonas putida* TMB

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Received 15 March 1990/Accepted 22 July 1990

The catabolic pathway for the degradation of aromatic hydrocarbons encoded by *Pseudomonas putida* TMB differs from the TOL plasmid-encoded pathway as far as regulation of the upper pathway is concerned. We found, by analyzing Tn*5*-induced mutants and by Southern blot hybridization with appropriate probes derived from the TOL plasmid pWW0, that the catabolic genes of strain TMB were located on the bacterial chromosome and not on the 84-kb plasmid harbored by this strain. The catabolic genes of TMB and pWW0 had sequence homology, as shown by Southern blot hybridization, but differed significantly in their restriction patterns. The analysis of the mutants suggests that a regulatory mechanism similar to that present in pWW0 coexists in TMB with a second mode of regulation which is epistatic on the former and that the chromosomal region carrying the catabolic genes is prone to rearrangements and deletions.

Several strains of *Pseudomonas putida* have been isolated which are capable of growing on toluene and its substituted derivatives *meta*- and *para*-xylene and 1,2,4-trimethylbenzene (1,2,4-TMB) (23, 27, 39). In the best-characterized strain, *P. putida* PaW1, the catabolic genes are located in a 39-kb region of the plasmid pWW0 and organized in two operons separated by a region about 23 kb long. The upper-pathway operon *xylCMABN* is responsible for the oxidation of the hydrocarbons to their corresponding carboxylic acids, and 13 *meta*-pathway *xyl* genes are involved in conversion of the carboxylic acid into compounds which are then further metabolized to Kreb's cycle intermediates (11, 18, 20, 44). Two regulatory genes, *xylR* and *xylS*, located close to the *meta*-pathway operon, positively control these operons (12, 21); expression of both operons is induced by the upper-pathway substrates toluene, xylenes, 1,2,4-TMB, and their alcohol and aldehyde derivatives, whereas the corresponding carboxylic acids only induce the expression of the *meta*-operon (1, 35, 45). The *xyl* operons are located within the 56-kb transposon Tn*4651*, which is inserted into the 70-kb transposon Tn*4653* (40–42).

A similar operon organization and a strong DNA homology between the pWW0 *xyl* genes and plasmid or even chromosomal DNA has been found in independent *P. putida* isolates capable of catabolizing methyl-substituted aromatic hydrocarbons (24, 26, 38, 39); homology has also been found with operons involved in the catabolism of naphthalene and salicylate (19, 29). Analysis of such independent strains may therefore provide information on the evolution of these catabolic pathways and on the possibility of genetic manipulation of these strains in order to optimize their catabolic properties for practical purposes.

P. putida TMB was isolated for its ability to grow on 1,2,4-TMB as a sole carbon source (2). This strain was found to be different from PaW1 in the regulation of the catabolic genes, since in TMB the benzyl alcohol dehydrogenase (BADH), an enzyme of the upper pathway, is also induced by 3,4-dimethylbenzoic acid (3,4-DMBCOOH) and *p*-toluate, which are *meta*-pathway substrates (3). Furthermore, TMB harbors the 84-kb plasmid pGB, unrelated to pWW0 (3; unpublished data). Preliminary transformation experiments had suggested that plasmid pGB was involved in the catabolism of 1,2,4-TMB (3).

In this article we show that the *P. putida* TMB catabolic genes are located on the bacterial chromosome and have homology with the pWW0 *xyl* operons.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The *Escherichia coli* and *P. putida* strains, plasmids, and bacteriophages used are detailed in Table 1.

Media, enzymes, and chemicals. LD broth contained 1% tryptone, 0.5% yeast extract, and 0.5% NaCl; LD agar was LD broth with 1.5% agar; M9 minimal medium is described by Kunz and Chapman (27). 1,2,4-TMB, a volatile compound, was supplied in the vapor phase as described previously (3). All other compounds were added to the minimal medium at the following concentrations: 3,4-dimethylbenzylalcohol (3,4-DMBOH), 3,4-DMBCOOH, benzoate, and glutamate, 1 g/liter; kanamycin, ampicillin, and chloramphenicol, 50 μg/ml; thymine, leucine, and thiamine, 20 μg/ml; streptomycin, 500 μg/ml; tryptophan, 0.5 g/liter; and succinate, 2 g/liter. For *P. putida* strains, kanamycin was added at 30 μg/ml. Aromatic compounds and other reagents used in growth experiments were of the highest purity commercially available from Sigma Chemical Co., Aldrich Chemical Co., Merck, and J. T. Baker Chemicals.

Bacterial growth conditions. *E. coli* strains were grown at 37°C in LD broth and maintained on LD agar plates. *P. putida* strains were grown at 30°C.

Cell extracts and enzyme assays. *P. putida* strains were grown overnight in M9 medium with glutamate supplied with either 1,2,4-TMB, 3,4-DMBOH, or 3,4-DMBCOOH as inducers or in M9-benzoate. Glutamate was used as a carbon source suitable for growing both wild-type and Tmb^- mutant strains. Cell extracts were prepared as described by Bestetti et al. (4). BADH activity was measured by following the rate of NAD^+ reduction at 340 nm (46). Catechol 2,3-oxygenase (C2,3O) activity was assayed by measuring the rate of

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Characteristics	Reference
P. putida		
TMB	Tmb$^+$(pGB)	3
PaW1	Xyl$^+$ (Tmb$^+$)(pWW0)	44
PaW340	Xyl$^-$ (Tmb$^-$) Nal^r Str^r Trp$^-$; from PaW1 by nitrosoguanidine mutagenesis	1, 12
PPM-1	*tmb-1* (class IV)(pGB103); from PPM-30 by Tn*5* mutagenesis with pGS9	This work
PPM-2	*tmb-2* (class I)(pGB); from TMB by Tn*5* mutagenesis with pGS9	This work
PPM-3	*tmb-3* (class II)(pGB); from TMB by Tn*5* mutagenesis with pGS9	This work
PPM-4	*tmb-4* (class II)(pGB); from TMB by Tn*5* mutagenesis with pGS9	This work
PPM-6	*tmb-6* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-7	*tmb-7* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-8	*tmb-8* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-9	*tmb-9* (class IV)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-10	*tmb-10* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-11	*tmb-11* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-12	*tmb-12* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-13	*tmb-13* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-14	*tmb-14* (class I)(pGB); from TMB by Tn*5* mutagenesis with P4::Tn*5* AP-1	This work
PPM-24	*tmb-24* (class III)(pGB24); from TMB by mitomycin mutagenesis	This work
PPM-25	*tmb-25* (class III)(pGB); from TMB by mitomycin mutagenesis	This work
PPM-26	*tmb-26* (class III)(pGB26); from TMB by mitomycin mutagenesis	This work
PPM-27	*tmb-27* (class I)(pGB); spontaneous mutant of TMB	This work
PPM-28	*tmb-28* (class I), spontaneous mutant of TMB; part of plasmid pGB, from 24.5 to 74.5 kb, integrated in the chromosome	This work
PPM-29	Tmb$^+$ Nal^r Str^r Trp$^-$; from PaW340 by transformation with TMB DNA	3
PPM-30	Tmb$^+$(pGB103); spontaneous derivative of TMB	This work
E. coli HB101(pGS9)	Pro$^-$ Leu$^-$ Thi$^-$ Thy$^-$ Str^r Kan^r Cam^r	37
Plasmids		
pBR322	Amp^r Tet^r	6
pED3306	Amp^r; pBR322 derivative containing the 10-kb *Hin*dIII D fragment of pWW0 which carries the upper-pathway promoter, *xylC*, *xylM*, and *xylA* genes	33
pGB	84-kb plasmid harbored by TMB	3; this work
pGB24	Rearranged pGB plasmid carried by *tmb-24* mutant	This work
pGB26	Rearranged pGB plasmid carried by *tmb-26* mutant	This work
pGB103	Spontaneous derivative of pGB, probably an insertion in the *Hin*dIII I fragment	This work
pGS9	Cam^r Kan^r(Tn*5*)	37
pGSH2960	Amp^r; pUC18 derivative containing the 2.25-kb *Xho*II fragment of pWW0 which carries *xylE*	Obtained from S. Harayama
pKT570	Str^r; pKT231 derivative containing the 6.8-kb *Xho*I D fragment of pWW0 which carries *xylR* and *xylS*	33
pWW0	Tol$^+$	44
Bacteriophages		
P4::Tn*5* AP-1	P4 *gop*24 *vir*1 Ω(*Pst*I$_{1188}$::*cat*) Ω(1.7–2.1 kb::Tn*5*) Ω(11.2–11.6 kb::*ins1*); confers Kan^r and Cam^r; P4 derivative constructed by in vivo Tn*5* transposition on P4 *cat gop*24 *vir*1	Polissi et al., unpublished data
P4 *cat gop*24 *vir*1		14

formation of the catechol ring fission product at 375 nm (27). Enzymatic activities were measured and compared with the activities of cells grown on glutamate without an inducer. Protein concentration was determined by the method of Layne (28) with bovine serum albumin as a standard. All spectrophotometric assays were carried out at 30°C.

Transposon mutagenesis. Transposon Tn*5* was introduced into *P. putida* TMB by either of two methods.

(i) Conjugation. The donor strain HB101(pGS9) and the recipient strain TMB were grown overnight in 10 ml of LD broth plus kanamycin and in LD broth, respectively. Cells were then collected by centrifugation and washed; the donor was suspended in 3 ml and the recipient was suspended in 10 ml of M9. Then, 0.1 ml of each strain were plated together on M9 minimal plates supplied with succinate, leucine, thymine, and thiamine; proline was added at a limiting concentration (1 μg/ml) to allow only a few cellular divisions of the donor strain. Cells were incubated for 3 hours at 30°C, and then kanamycin (30 μg/ml) was added. Plates were then incubated for 24 h at 30°C. Since pGS9 does not replicate in *P. putida* TMB, Kan^r Pro$^+$ clones can be obtained only by Tn*5* transposition into TMB.

(ii) P4::Tn*5* AP-1 infection. $CaCl_2$ (2.5 mM) was added to cultures of *P. putida* TMB growing exponentially; cells were then infected with P4::Tn*5* AP-1 at a multiplicity of infection of 1, and the number of nonadsorbed phage was measured 30 min after infection. The infected cells were incubated at 30°C for 2 h and then plated on M9-benzoate minimal plates supplied with kanamycin. Since P4::Tn*5* does not replicate in *P. putida* TMB (Polissi et al., unpublished data), Kan^r clones could only derive from transposition or integration events.

The Tmb phenotype of the Kan^r clones obtained by these two methods was then tested. Single colonies were picked

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TABLE 2. Enzymatic activities of *tmb* mutants in different inducing conditions

Strain	Sp act (nmol/min/mg of protein) with indicated inducer									
	Glutamate		Glutamate + 1,2,4-TMB		Glutamate + 3,4-DMBOH		Glutamate + 3,4-DMBCOOH		Benzoate	
	BADH	C2,3O	BADH	C2,3O	BADH	C2,3O	BADH	C2,3O	BADH	C2,3O
TMB	19	23	203	218	195	662	254	3,175	15	559
PaW1	3	8	121	222	134	3,078	0	1,209	5	1,158
PPM-7 (class I)	0	0	0	0	0	0	1	0	0	0
PPM-3 (class II)	2	1	3	0	3	0	0	6	2	142
PPM-25 (class III)	7	9	4	99	3	109	28	6,076	18	220
PPM-9 (class IV)	3	0	76	207	205	1,457	0	117	8	446
PaW340	4	0	0	0	0	10	0	1	0	0
PPM-29	21	18	431	172	604	2,802	286	813	12	581

with sterile toothpicks, inoculated into replica wells containing M9, and replicated on M9 agar plates supplemented with 1,2,4-TMB, 3,4-DMBOH, or 3,4-DMBCOOH. Putative Tmb$^-$ mutants were then purified by streaking on M9-benzoate-kanamycin at least twice. Twenty-five single colonies obtained from a pure clone were then tested for their ability to grow on the hydrocarbon or on the catabolic intermediates as described above.

Mutagenesis with mitomycin. Overnight cultures of TMB were diluted in LD broth to a concentration of 10^4 to 10^6 cells per ml. Mitomycin was added to the diluted cultures at a final concentration of 20 μg/ml. The cultures were then incubated at 30°C for 48 h, after which the cells were plated on LD agar, and the Tmb phenotype of single colonies was tested as described above.

Selection of spontaneous mutants. Spontaneous Tmb$^-$ mutants were also isolated from TMB cultures grown for several generations in the nonselective medium M9-benzoate. The phenotypes of single colonies were tested, and putative Tmb$^-$ mutants were purified as described above.

Preparation, analysis, and manipulation of DNA. Plasmid DNA from *E. coli* was prepared by standard methods (5). Plasmid DNA from *P. putida* strains was extracted as described by Hansen and Olsen (15) and then purified by CsCl-ethidium bromide equilibrium density gradients (9). Total bacterial DNA was prepared by the method of Ljungquist and Bukhari (31). Analysis of DNA by electrophoresis was performed on 0.6% agarose gels by standard procedures (8). Southern blot hybridization was performed as described by Maniatis et al. (32) on Hybond-N filters (Amersham). The ^{32}P-labeled DNA probes were obtained by nick translation essentially as described by Rigby et al. (36) or by random primer extension (10), with [α-^{32}P]dATP as the labeled precursor.

RESULTS

Isolation and biochemical characterization of Tmb$^-$ mutants. By Tn*5* mutagenesis, we isolated from *P. putida* TMB 11 independent mutants unable to grow on 1,2,4-TMB, as described in Materials and Methods (Table 1). The frequency of Tmb$^-$ mutants among the Kan^r clones was about 3%, following both conjugation and P4::Tn*5* infection. Other mutants were isolated following mitomycin treatment (*tmb-24*, *tmb-25*, and *tmb-26*); spontaneous mutants were also isolated after prolonged growth in nonselective medium (*tmb-27* and *tmb-28*).

The mutants were purified and tested for their ability to grow both on 3,4-DMBOH and on 3,4-DMBCOOH, the catabolic intermediates of 1,2,4-TMB; *tmb-1*, *tmb-9*, *tmb-24*, *tmb-25*, and *tmb-26* mutants could still grow on 3,4-DMB COOH as a sole carbon source but not on 3,4-DMBOH. All the other mutants were unable to grow on either 3,4-DMBOH or 3,4-DMBCOOH. The enzymatic activities of BADH and C2,3O, chosen as representatives of the upper-pathway and the *meta*-pathway enzymes, respectively, were assayed in TMB, PaW1, and the mutants after growth in M9-glutamate in the presence of the various inducing substrates (Table 2). In TMB, both BADH and C2,3O were induced by the hydrocarbon or the corresponding alcohol or acid; in PaW1, BADH was induced by the hydrocarbon and the alcohol but not by the acid, whereas C2,3O was induced by all these compounds, as expected (3, 45, 46).

It was possible to distinguish four classes of mutants with respect to enzyme inducibility (Table 2 reports the results, one example per class). In the class I mutants (*tmb-2*, *tmb-6*, *tmb-7*, *tmb-8*, *tmb-10*, *tmb-11*, *tmb-12*, *tmb-13*, *tmb-14*, *tmb-27*, and *tmb-28*), neither enzymatic activity was detectable, regardless of the inducing substrate tested. In class II (*tmb-3* and *tmb-4*), BADH was not induced by any intermediate, whereas C2,3O was induced by benzoic acid and not by the methyl-substituted compounds. In class III (*tmb-24*, *tmb-25*, and *tmb-26*), BADH activity was low and noninducible in all conditions tested, whereas C2,3O activity was expressed, although at different levels with different inducing substrates. In class IV (*tmb-1* and *tmb-9*), the same induction pattern as in PaW1 was observed, that is, BADH activity was induced only by the hydrocarbon and the corresponding alcohol but not by the corresponding acid; C2,3O activity was detected after growth on any aromatic compound. Classes III and IV grew on 3,4-DMBCOOH, whereas classes I and II did not.

Chromosomal location of Tn*5* in Tmb$^-$ mutants. In order to determine whether, in the Tmb$^-$ mutants obtained by transposon mutagenesis, Tn*5* was transposed onto the plasmid pGB, we performed a restriction analysis of pGB DNA extracted from such Tmb$^-$ mutants with *Hin*dIII, *Eco*RI, and *Sal*I endonucleases. This analysis showed that only two mutant strains harbored a plasmid containing Tn*5* (PPM-1) or the entire P4::Tn*5* (PPM-12); all the others gave a plasmid restriction profile identical to that of the parental plasmid pGB with all three enzymes tested (data not shown).

These results suggested that in most of the transposon mutants, Tn*5* might have transposed onto the chromosome. To test this hypothesis, total DNA from wild-type and Tmb$^-$ mutants was extracted and digested with *Eco*RI, and Southern blot analysis was performed with ^{32}P-labeled P4::Tn*5* DNA as a probe. The results are reported in Fig. 1. P4::Tn*5* AP-1 digested with *Eco*RI generated four fragments of 17,

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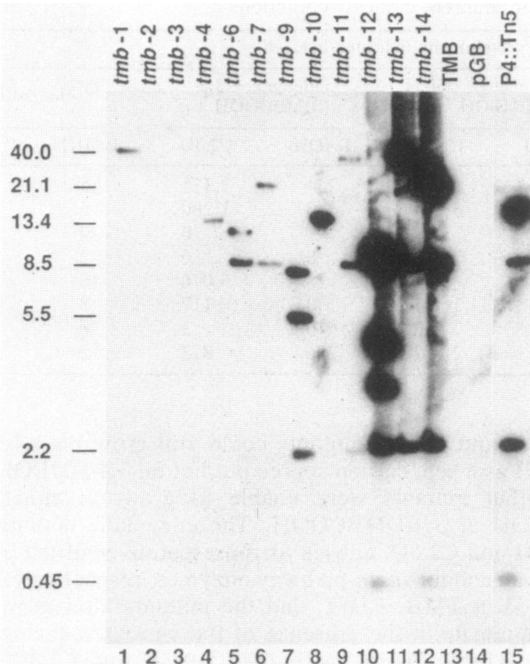

FIG. 1. Southern blot analysis of Tn*5* insertion in Tmb$^-$ mutants. Approximately 1 μg of total DNA from strain TMB and Tmb$^+$ mutants was digested with *Eco*RI and Southern blot analyzed with P4::Tn*5* AP-1 as the probe. In lanes 14 and 15 were loaded, respectively, 0.3 μg of plasmid pGB and 0.05 μg of P4::Tn*5* AP-1 DNA digested with the same enzyme. The molecular sizes (indicated on the left in kilobases) were calculated by using the P4::Tn*5* AP-1 fragments as markers.

8.5, 2.2, and 0.4 kb (the 8.5-kb fragment contained the entire Tn*5*, which does not have *Eco*RI sites [22]). TMB total DNA and pGB did not show any homology to P4::Tn*5*; the hybridization bands in the *tmb-1* and *tmb-12* mutants corresponded to the pGB fragments already identified in the above plasmid DNA analysis, as expected in the case of a single Tn*5* insertion. No hybridization signal was observed for the *tmb-2 and tmb-3* mutants, although these two mutants were still Kan^r and also exhibited streptomycin resistance (data not shown), another marker expressed by Tn*5* in some hosts (37).

We suggest that these two mutants have lost most of the Tn*5* DNA, retaining only a small region including the antibiotic resistance markers. Since the entire P4::Tn*5* AP-1 DNA of 27.8 kb was used as the probe, it is possible that in these experimental conditions we could not detect the antibiotic resistance genes. The hybridization pattern of all the other mutants was consistent with the integration of either Tn*5* (*tmb-4* and *tmb-10*) or Tn*5* plus all or part of P4. With the exception of *tmb-1* and *tmb-12*, none of the hybridization bands observed in the mutants corresponded to any pGB fragment.

Homology between TMB and the upper-pathway operon of pWW0. The above experiments strongly suggested that in strain TMB, at least a part of the information for 1,2,4-TMB catabolism is located on the chromosome. We therefore used Southern blot hybridization to see whether there was any homology between the catabolic genes of pWW0 and TMB chromosomal DNA.

By using ^{32}P-labeled pED3306 DNA (upper-pathway probe), we observed hybridization with 1 μg of total undigested DNA from strain TMB, but no hybridization signal could be detected with 0.3 μg of CsCl-purified plasmid pGB DNA (data not shown). To further investigate this homology, total DNA from *P. putida* TMB and the *tmb* mutants was digested with different restriction endonucleases, Southern blotted, and hybridized to the upper-pathway probe. An example of the results obtained is presented in Fig. 2A. It was observed that (i) hybridization occurred between TMB and pED3306 (the signals did not appear upon hybridization with the vector plasmid pBR322; data not shown); (ii) the fragments hybridizing with pED3306 did not correspond in size to the fragments expected from the digestion of pGB (this fact was confirmed by rehybridizing the same filters to ^{32}P-labeled pGB DNA; the hybridization signal obtained could not be superimposed on those given by pED3306 [data not shown]); and (iii) pGB DNA did not cross-hybridize with the pED3306 probe. These results indicate that sequences homologous to the pWW0 *Hin*dIII fragment cloned in pED3306 are present on the TMB chromosome.

Although homologous, the two regions showed a remarkably different restriction pattern. For example, the 10-kb *Hin*dIII fragment of pWW0 contains four *Eco*RI sites (33), which produced five hybridizing fragments in *Eco*RI-digested pED3306 DNA (Fig. 2A, lane 12). On the contrary, the TMB DNA hybridizing to the upper-pathway probe gave a single 6.8-kb *Hin*dIII fragment (data not shown) and only two *Eco*RI fragments of 20 and 9 kb (Fig. 2A, lane 1).

The TMB region homologous to pWW0 upper-pathway genes was either deleted or rearranged in all the *tmb* mutants we obtained. In fact, all class I and II *tmb* mutants gave no hybridization signal (data not shown), whereas class III and IV mutants gave a hybridization pattern different from that of the Tmb$^+$ strain with *Eco*RI (Fig. 2A) and two other restriction enzymes tested (*Bgl*II and *Hin*dIII; data not shown). It is interesting that even the two mutants that carried Tn*5* on the plasmid pGB also suffered the rearrangement (*tmb-1*) or the loss (*tmb-12*) of the chromosomal region homologous to pWW0.

Homology of *tmb* with the regulatory genes *xylR* and *xylS*. The same filters were also hybridized to ^{32}P-labeled pKT570 DNA, which contains *xylR* and *xylS*, the two regulatory genes of the pWW0 *xyl* operons. TMB DNA also hybridized to the *xylR-xylS* probe; with each of the three enzymes used, we obtained one hybridization band identical in size to that observed with the upper-pathway probe plus an additional band(s) (Fig. 2B and data not shown). This suggests that the regions homologous to the pWW0 upper-pathway gene probe and to the *xylR-xylS* probe are close to each other on the TMB chromosome, whereas in pWW0 they are separated by about 23 kb (29, 30). Most of the class I mutants (*tmb-6*, *tmb-10*, *tmb-11*, *tmb-12*, *tmb-13*, *tmb-14*, *tmb-27*, and *tmb-28*) did not show any homology with the *xylR-xylS* probe. All the other mutants presented more or less severe deletions or other rearrangements.

Homology of *tmb* with the *meta*-pathway genes. To complete the analysis of homology between TMB and the catabolic region of pWW0, we performed Southern blot analysis with the pWW0 *Xho*I I fragment, which contains the complete *xylE* gene, encoding C2,3O (34), as the probe. Some results are presented in Fig. 2C. Homology between pWW0 *xylE* DNA and two TMB *Eco*RI fragments was observed. The class I mutants, which were unable to grow on 3,4-DMBCOOH and to produce C2,3O, completely lost any homology with the *xylE* probe. Among the class II mutants, which were capable of producing C2,3O in the presence of benzoate but not of 3,4-DMBCOOH and the

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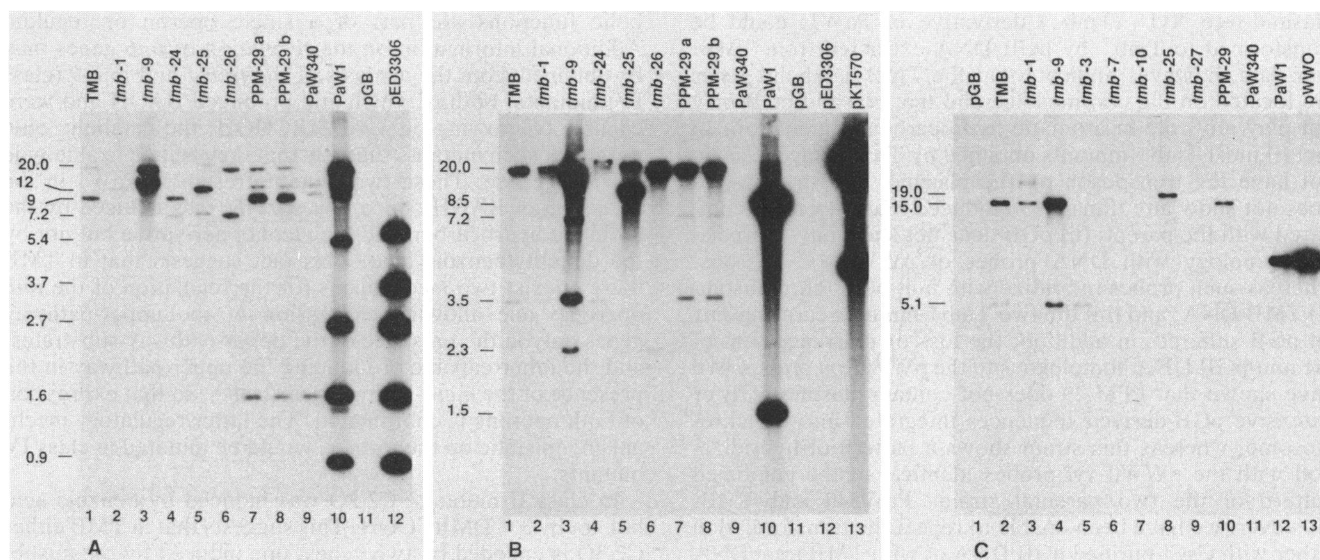

FIG. 2. Southern blot hybridization of *P. putida* TMB and derivative mutants with pWW0 *xyl* probes. Approximately 1 μg of total DNA extracted from the strains indicated above each lane was digested and Southern blot analyzed with pED3306 (upper pathway; panel A), pKT570 (*xylR-xylS*; panel B), and the *Xho*I I fragment of pWW0 (*xylE*; panel C) as probes. The *Xho*I I fragment was purified by low-melting-point agarose gel electrophoresis of *Bam*HI-digested pGSH2960 plasmid DNA and phenol recovered from the gel slice as described before (32). *Eco*RI-digested pGB (0.2 μg) was loaded in lane 11, panels A and B, and lane 1, panel C; 0.05 μg of *Eco*RI-digested pED3306 DNA was loaded in lane 12, panels A and B; 0.05 μg of *Eco*RI-digested pKT570 DNA was loaded in lane 13, panel B; and 0.2 μg of *Eco*RI-digested pWW0 DNA was loaded in lane 13, panel C. The molecular sizes (indicated on left in kilobases) were calculated by using as standards the pED3306 and pWW0 fragments, and the lambda *Hin*dIII-digested fragments were visualized by ethidium bromide staining before being transferred to the filters.

other methyl-substituted aromatics, one of the two *Eco*RI bands was missing. In the class III mutants, which grew on 3,4-DMBCOOH and regulated the expression of C2,3O like the wild type, only a single new band was observed. The hybridization pattern of class IV mutants was identical to that of TMB. These and other hybridization patterns obtained with other restriction enzymes (data not shown) suggest that in *P. putida* TMB, the gene coding for C2,3O is duplicated; one copy, still present in class II mutants, might be regulated by benzoate, the other by methyl-substituted aromatics.

Analysis of PaW340 Tmb$^+$ transformants. In previous work (3), transformation of the Xyl$^-$ (Tmb$^-$) mutant PaW340 to Tmb$^+$ was obtained with partially purified pGB DNA; these clones were able to grow on all the substrates utilized by the wild-type strain TMB and showed the same induction pattern of the catabolic enzymes as TMB. These data suggested that the catabolic activity of TMB was coded by the plasmid pGB. When we obtained evidence that chromosomal genes were involved in 1,2,4-TMB degradation, we reanalyzed the characteristics of the putative PaW340(pGB) Tmb$^+$ transformant (strain PPM-29). We confirmed that the phenotype of PPM-29 was Str^r Trp$^-$ Tmb$^+$ and that the induction profile of BADH and C2,3O expression under different inducing conditions was still similar to that of TMB (Table 2), as first published by Bestetti and Galli (3). However, PPM-29 did no carry the pGB genome, neither as a free plasmid nor integrated in the chromosomal DNA, as indicated by lack of hybridization of PPM-29 total DNA with a ^{32}P-labeled pGB DNA probe in Southern blot analysis (data not shown).

We also probed PPM-29 DNA digested with different restriction enzymes with the upper-pathway, *xylR-xylS*, and *xylE* probes. The parent strain PaW340 gave hybridization signals with the upper-pathway probe (Fig. 2A) but not with the *xylR-xylS* or *xylE* DNA (Fig. 2B and C). PPM-29 exhibited the same hybridization signals as TMB with each of the three probes tested. In addition, in the *Eco*RI digestion hybridized with the upper-pathway probe, PPM-29 also showed the bands present in PaW340 (Fig. 2A). We therefore suggest that PPM-29 is a PaW340 derivative which acquired TMB chromosomal DNA.

It should be noted that the hybridization of PaW1 total DNA with the upper-pathway probe, in addition to the fragments expected from the digestion of pWW0 DNA (9 kb for *Hin*dIII and 16, 5.1, 2.5, 1.6, and 0.9 kb for *Eco*RI [29]), gave further signals: a 6.8-kb *Hin*dIII fragment (also observed in PaW340; data not shown) and *Eco*RI fragments 13, 10, and 8.5 kb long (Fig. 2A). This indicates that at least part of the pWW0 upper pathway is duplicated on the PaW1 chromosome.

DISCUSSION

Localization of *tmb* genes in *P. putida* TMB. *P. putida* TMB was originally isolated from soil by enrichment culture in the presence of 1,2,4-TMB (2). Although the catabolic properties of TMB resembled those of PaW1, the best-characterized strain carrying the TOL plasmid pWW0, analysis of the induction pattern of BADH and C2,3O indicated an interesting difference in the regulation of catabolic gene expression in the two strains. In fact, BADH is not induced by 3,4-DMBCOOH in PaW1, whereas it is in TMB. In addition TMB harbors plasmid pGB, unrelated to pWW0 as far as DNA homology is concerned.

Preliminary data had suggested that the catabolic activity of TMB was encoded on the plasmid pGB (3). This conclusion was based mainly on the observation that PaW340, a

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plasmid-free Xyl⁻ (Tmb⁻) derivative of PaW1, could be transformed to Tmb⁺ by pGB DNA extracted from TMB. Our data, however, indicate that the TMB catabolic genes are located on the chromosome and that plasmid pGB may not play any role in aromatic hydrocarbon degradation. In fact (i) most Tmb⁻ mutants obtained by Tn*5* mutagenesis do not have the transposon on the plasmid, and the plasmid does not show any transposon-induced rearrangement compared with the parent; (ii) pGB does not show any appreciable homology with DNA probes of pWW0 *xyl* operons, whereas such probes hybridize with non-pGB (chromosomal) TMB DNA; and (iii) the two Tmb⁻ mutants carrying Tn*5* on pGB suffered, in addition, the loss or rearrangement of the non-pGB DNA homologous to the pWW0 *xyl* probes. We have shown that PPM-29 does not contain plasmid pGB or extensive pGB-derived sequences integrated into the chromosome, whereas this strain shows a pattern of hybridization with the pWW0 *xyl* probes identical to the combined pattern of the two parental strains PaW340 and TMB. However, we have been unable to repeat the transformation either with CsCl-purified pGB DNA or with TMB total DNA or to obtain transformation for other chromosomal markers even with more efficient transforming procedures such as electroporation. This might exclude the most obvious hypothesis, that transformation was due to contaminating chromosomal DNA present in the pGB DNA, which had been prepared by the method of Wheatcroft and Williams (43) and purified only once on a sucrose gradient. We should conclude that the transformant PPM-29 originated from a rare stochastic event; we suggest, for example, that the pGB DNA preparation used for that transformation experiment was rich in pGB-*tmb* recombinants, possibly generated by transposition, that allowed transfer of *tmb* genes to PaW340, followed by the loss of pGB.

Analysis of Tn*5*-induced Tmb⁻ mutants. Tn*5* mutagenesis was very efficient, and use of the P4::Tn*5* vector made the procedure simple and straightforward. However, we were surprised to observe that all the mutants had suffered chromosomal rearrangements involving the region homologous to the pWW0 *xyl* probes, which in several cases was completely lost, and we were unable to detect any case of simple Tn*5* insertion in this region, whereas in other strains of *P. putida* Tn*5* mutagenesis allowed the isolation of simple insertion mutations in single genes (16, 17). This does not seem to be due to abnormal behavior of Tn*5* in *P. putida* TMB, since both mitomycin-induced and spontaneous Tmb⁻ mutants suffered similar rearrangements, and we observed simple Tn*5* insertions in the TMB plasmid pGB.

It is possible that deletions or other chromosomal mutations involving the *tmb* genes of *P. putida* TMB occur at a much higher frequency than point or simple insertion mutations. These rearrangements might be favored if the *tmb* operons were part of a transposable element, as in the case of the pWW0 *xyl* operons (40, 41). Alternatively, it may be that the catabolic functions of TMB are duplicated and therefore chromosomal mutations involving both sets of functions would be the most frequent event producing Tmb⁻ mutants. Our data give some evidence of duplication of at least one gene of the *meta*-pathway operon.

Regulation of *tmb* catabolic genes. In pWW0, the catabolic functions are encoded in two independently regulated operons, controlling the upper pathway and the *meta*-cleavage pathway (35, 45). Bestetti and Galli (3) have shown that in strain TMB, the enzymes of both the upper and the *meta*-pathway are coordinately induced by the same set of catabolic intermediates. This suggested that in TMB the catabolic functions are part of a single operon or regulon. Additional information on the regulation of *tmb* genes may be obtained from the analysis of the *tmb-1* and *tmb-9* (class IV) mutants, both of which still produced BADH and were capable of growing on 3,4-DMBCOOH; the catabolic phenotype of such mutants suggests that they lack benzaldehyde dehydrogenase. These two mutants resemble PaW1 in the induction of BADH, since this enzyme was induced by the aromatic hydrocarbon and the alcohol derivative but not by the dimethylbenzoic acid. This fact suggests that in TMB there coexist two mechanisms for the regulation of the *tmb* operons: one allowing expression of the upper-pathway genes only in the presence of the upper-pathway substrates, and the other capable of inducing the upper pathway in the presence of the *meta*-pathway substrates, so that expression of both operons is coordinated. The latter regulatory mechanism, epistatic on the former, would be mutated in class IV mutants.

In class II mutants, C2,3O was induced by benzoic acid but not by 3,4-DMBCOOH. This suggests that in TMB either C2,3O is encoded by two genes, one induced by the unsubstituted aromatics and the other by the methyl-substituted aromatic compounds, or the same gene enjoys two independent induction mechanisms. Alternatively, in these two mutants a common positive regulator may be mutated so as to respond to one inducer and not to the other. Our hybridization data support the hypothesis of a duplication of the gene encoding C2,3O; this situation is similar to that observed in other TOL plasmids, in which either two homologous or two nonhomologous C2,3O-coding genes have been found on the same plasmids (7, 25).

Evolutionary relationship among TOL systems. Chromosomal rearrangements involving *P. putida* catabolic operons have been commonly observed and attributed to the localization of such functions within transposable elements. For example, in pWW0 the *xyl* operons are part of two transposons, one inside the other, which may be lost by the plasmid and transposed to other genetic elements. This may be relevant for the evolution of such catabolic systems, which can easily be transferred by transposition to conjugative plasmids or bacteriophages, thus favoring the horizontal diffusion of the catabolic properties, or create duplications within an organism, which may allow the evolution of one set of genes toward new related catabolic pathways.

Chromosomal localization of aromatic catabolic genes seems to occur more frequently than previously thought. Our data show that even in PaW1 there is significant homology between the upper-pathway *xyl* genes of pWW0 and the PaW1 chromosome. These regions do not seem to be sufficient to perform all the catabolic functions, as shown by the fact that PaW340 is Tmb⁻; however, they could complement defective catabolic operons that might be introduced into such hosts by plasmids or bacteriophages or provide alternative catabolic routes to otherwise nondefective *xyl* operons.

The *xyl* operons harbored by several TOL plasmids so far isolated present a very strict homology with each other (24, 26, 38). In *P. putida* MW1000, a naturally occurring strain isolated by Sinclair et al. (39), the TOL genes are located within a 56-kb segment of the bacterial chromosome exhibiting very strong similarity to Tn*4651*, the 56-kb transposon carrying the TOL genes of pWW0 (40, 42). A greater divergence is observed between the *tmb* genes of *P. putida* TMB and the *xyl* operons of pWW0. In fact, we have shown that the restriction patterns of the TOL regions of pWW0 and TMB differ significantly and that in TMB the regions

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homologous to the *xyl* upper-pathway and regulatory genes are possibly close to each other, whereas in pWW0 they are separated by about 23 kb of DNA. Moreover, the two differ in the pattern of induction of the catabolic genes, although the analysis of *tmb* mutants suggests that in TMB there may still be a regulatory mechanism similar to the one present in PaW1. This suggests that the *tmb* genes are more distantly related to pWW0 *xyl* operons than all the other systems for the catabolism of methyl-substituted aromatic hydrocarbons mentioned above. The cloning of the *tmb* region, now in progress, may allow a more detailed comparison of the catabolic systems and shed more light on their evolutionary relationships.

ACKNOWLEDGMENTS

We thank Beatrice Kiesswetter and Chiara Bulli for technical assistance and Shigeaki Harayama for providing pED3306, pKT570, and pGSH2960.

This work was supported by grant 890016070 of the Target Project on Biotechnology and Bioinstrumentation, Consiglio Nazionale delle Ricerche, Rome, and by grants from the Ministero della Pubblica Istruzione, Rome.

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