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# Characterization of a Novel TOL-Like Plasmid from *Pseudomonas putida* Involved in 1,2,4-Trimethylbenzene Degradation

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**A strain of *Pseudomonas putida* (TMB) was found to resemble *P. putida* mt-2 (PaW1) in its ability to degrade 1,2,4-trimethylbenzene, toluene, *m*-xylene, and *p*-xylene via oxidation of a methyl substituent and reaction of the *meta* fission pathway, but a different regulatory model is suggested. The ability of *P. putida* TMB to degrade these substrates was encoded by plasmid pGB (85 kilobase pairs), which showed considerable differences in size, restriction patterns, and DNA sequence from those of plasmid pWWO of strain PaW1.**

*Pseudomonas* strains have been found to be the most versatile bacteria in metabolizing methyl-substituted aromatic compounds, such as toluene and xylenes. The genes responsible for the degradation of toluene, *m*-xylene, and *p*-xylene in *Pseudomonas putida* mt-2 (PaW1) have been shown to reside on a transmissible plasmid, referred to as the TOL plasmid (pWWO) (20). Kunz and Chapman (10) found that *P. putida* mt-2 could also metabolize 1,2,4-trimethylbenzene through the formation of 3,4-dimethylbenzyl alcohol, 3,4-dimethylbenzoate, and 3,4-dimethylcatechol, which was then cleaved in an extra-diol position to yield a semialdehyde-related compound, and provided evidence that the ability to degrade this compound was also encoded by the TOL plasmid (pWWO). A similar metabolic pathway was also proposed for *P. putida* HS1 (11).

In this work, we describe a new strain of *P. putida* (TMB) which was directly isolated from an enrichment culture supplied with 1,2,4-trimethylbenzene as the only carbon and energy source (2). We also provide evidence that the genes coding for the catabolism of this compound are encoded on a plasmid which we compared at both the genetic and regulatory levels with the TOL plasmid (pWWO).

The bacteria used are listed in Table 1. PaW340, TMB-1, and *Escherichia coli* strains were maintained on LD agar plates (4). All the other strains were maintained on minimal medium (10) with 1,2,4-trimethylbenzene or *p*-toluate as the only C source. A volatile hydrocarbon as a growth substrate was supplied in a narrow glass tube placed in the lid of a minimal agar plate. Water-soluble compounds were incorporated into the minimal medium at 5 to 10 mM. Cultivation was carried out at 30°C.

Plasmid DNA was isolated by the procedure of Hansen and Olsen (7) and then purified with an ethidium bromide-CsCl density gradient (5). Endonuclease digestion and analysis of DNA by gel electrophoresis were performed by standard procedures (4). DNA restriction fragments separated by agarose gel electrophoresis were transferred to nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) by the method of Southern (16). Plasmid DNA (ca. 1 μg) was $^{32}P$ labeled by nick translation essentially as described by Rigby et al. (15) with [$^{32}P$]dATP as the labeled precursor. Benzoate selection was carried out as described by Williams and Worsey (18). After incubation, samples showing growth were appropriately diluted and spread on LD agar plates. Individual colonies were picked out onto selective minimal agar plates and onto LD agar plates. DNA transformation was performed with PaW340 as the recipient. Cells grown in LD broth to the exponential phase (about $2 \times 10^8$ cells per ml) were harvested, washed twice by centrifugation in 0.5 volumes of cold buffer 1 (100 mM $MgCl_2$, 5 mM Tris hydrochloride [pH 7.4]) and cold buffer 2 (100 mM $CaCl_2$, 5 mM Tris hydrochloride [pH 7.4]), suspended in 0.1 volume of cold buffer 2, and held for 90 min at 0°C. The cell suspension (0.2 ml) was incubated with purified plasmid DNA (0.5 to 1 μg) at 0°C for 30 min, diluted by the addition of 3 ml of LD broth, and incubated at 30°C for 3 h with shaking prior to dilution and plating on 1,2,4-trimethylbenzene agar plates supplied with tryptophan and streptomycin for the selection of transformed strains.

*P. putida* TMB was able to utilize 1,2,4-trimethylbenzene and additional aromatic compounds, i.e., *m*-xylene, *p*-xylene, toluene, and the corresponding alcohols and acids derived from their metabolism. Such growth characteristics are identical to the growth pattern of *P. putida* mt-2 (PaW1). The growth of TMB on benzoate for 15 to 20 generations led to the formation of the spontaneous putative cured strain TMB-1 (at a frequency of 1%), which had simultaneously lost the plasmid and the ability to grow on all the tested aromatic compounds except benzoic acid. PaW340(pGB), obtained by transforming PaW340 with plasmid pGB, was able to grow on all the substrates which were utilized by wild-type strain TMB (Table 1).

Benzyl alcohol dehydrogenase, chosen as representative of the early enzymes of the metabolic pathway, and catechol 2,3-oxygenase (EC 1.13.11.2; catechol 2,3-dioxygenase), chosen as representative of the later enzymes, were assayed in *Pseudomonas* strains after growth on various substrates. The enzymatic activities were measured and compared with those of glutamate-grown cells (Table 2). The results indicated that benzyl alcohol dehydrogenase was an inducible enzyme which showed high levels of specific activity after growth of strains TMB and PaW340(pGB) on 1,2,4-trimethylbenzene, 3,4-dimethylbenzyl alcohol, *p*-methylbenzyl alcohol, 3,4-dimethylbenzoate, or *p*-toluate. In contrast, the growth of strain PaW1 on 3,4-dimethylbenzoate and *p*-toluate did not induce the synthesis of benzyl alcohol dehydrogenase. The *meta* cleavage enzyme catechol 2,3-oxygenase was induced in strains TMB and PaW1 regardless of whether the cells were grown either on the hydrocarbon as well as the corresponding alcohol and acid or on benzoic acid; cell extracts of glutamate-grown cells contained less than 10% of the activity measured in extracts of cells grown

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TABLE 1. Bacterial strains

| Strain | Phenotype[a] or genotype | Plasmid | Parent strain | Method of production or source |
|---|---|---|---|---|
| TMB (*P. putida* wild type) | $Tmb^+$ $Tln^+$ $Mxy^+$ $Pxy^+$ $DMBA^+$ $PMBA^+$ $DMBa^+$ $Mtol^+$ $Ptol^+$ $Ben^+$ | pGB | | This work |
| TMB-1 | $Tmb^-$ $Tln^-$ $Mxy^-$ $Pxy^-$ $DMBA^-$ $PMBA^-$ $DMBa^-$ $Mtol^-$ $Ptol^-$ $Ben^+$ | | TMB | Benzoate curing (18) |
| PaW340(pGB) | $Tmb^+$ $Tln^+$ $Mxy^+$ $Pxy^+$ $DMBA^+$ $PMBA^+$ $DMBa^+$ $Mtol^+$ $Ptol^+$ $Ben^+$ $Str^r$ $Trp^-$ | pGB | PaW340 | Transformation with pGB |
| PaW1 (*P. putida* mt-2 wild type)[b] | $Tmb^+$ $Tln^+$ $Mxy^+$ $Pxy^+$ $DMBA^+$ $PMBA^+$ $DMBa^+$ $Mtol^+$ $Ptol^+$ $Ben^+$ | pWWO | | 17 |
| PaW340[b] | $Str^r$ $Trp^-$ | | PaW1 | Nitrosoguanidine mutagenesis |
| LE392 [*E. coli*(pRL3)][c] | *hsdR514 hsdM*$^+$ *metB1* $F^-$ | pRL3 | LE392 | 13 |
| LE392 [*E. coli*(pBR322-pWWO *Hin*dIII-A)][c] | *hsdR514 hsdM*$^+$ *metB1* $F^-$ | pBR322-pWWO *Hin*dIII-A | LE392 | 13 |

[a] Phenotype abbreviations: Tmb, 1,2,4-trimethylbenzene; Tln, toluene; Mxy, *m*-xylene; Pxy, *p*-xylene; DMBA, 3,4-dimethylbenzyl alcohol; PMBA, *p*-methylbenzyl alcohol; DMBa, 3,4-dimethylbenzoate; Mtol, *m*-toluate; Ptol, *p*-toluate; Ben, benzoate; $Str^r$, resistance to streptomycin; and Trp, requirement for tryptophan.

[b] *P. putida* PaW1 harboring the TOL plasmid (pWWO) and *P. putida* PaW340 were kindly supplied by P. A. Williams, University College of North Wales, Bangor, United Kingdom.

[c] *E. coli* LE392 carrying pRL3 and LE392 carrying a pBR322 hybrid recombinant plasmid containing the pWWO *Hin*dIII A fragment encoding the *meta* pathway genes were kindly provided by K. N. Timmis, University of Geneva, Geneva, Switzerland.

TABLE 2. Specific activities of benzyl alcohol dehydrogenase and catechol 2,3-oxygenase in extracts of *P. putida* strains[a]

| Strain | Inducer | Sp act ($nmol\ min^{-1}\ mg^{-1}$ of protein) of: | | |
|---|---|---|---|---|
| | | Benzyl alcohol dehydrogenase | | Catechol 2,3-oxygenase (catechol) |
| | | 3,4-Dimethylbenzyl alcohol | *p*-Methylbenzyl alcohol | |
| TMB | 1,2,4-Trimethylbenzene | 390 | 360 | 1,520 |
| | 3,4-Dimethylbenzyl alcohol | 460 | 406 | 2,000 |
| | 3,4-Dimethylbenzoate | 203 | 177 | 1,890 |
| | *p*-Methylbenzyl alcohol | 786 | 752 | 890 |
| | *p*-Toluate | 304 | 414 | 936 |
| | Benzoate | 0 | 0 | 1,090 |
| | Glutamate | 0 | 0 | 305 |
| PaW340(pGB) | 1,2,4-Trimethylbenzene | 305 | 267 | 1,006 |
| | 3,4-Dimethylbenzyl alcohol | 330 | 380 | 1,745 |
| | 3,4-Dimethylbenzoate | 203 | 152 | 1,581 |
| | *p*-Methylbenzyl alcohol | 257 | 208 | 740 |
| | *p*-Toluate | 213 | 198 | 849 |
| | Benzoate | 0 | 0 | 835 |
| | Glutamate | 0 | 0 | 13 |
| PaW340 | Benzoate | 0 | 0 | 0 |
| | Glutamate | 0 | 0 | 0 |
| PaW1 | 1,2,4-Trimethylbenzene | 857 | 733 | 2,812 |
| | 3,4-Dimethylbenzyl alcohol | 318 | 224 | 3,272 |
| | 3,4-Dimethylbenzoate | 0 | 0 | 3,257 |
| | *p*-Methylbenzyl alcohol | 355 | 482 | 2,000 |
| | *p*-Toluate | 14 | 7 | 1,657 |
| | Benzoate | 3 | 5 | 4,727 |
| | Glutamate | 0 | 0 | 155 |

[a] All spectrophotometric assays were carried out at room temperature in a Perkin-Elmer model 550 S spectrophotometer. Specific activity is expressed as the amount of activity required to convert 1 nmol of substrate per min per mg of protein. Protein determinations were carried out by the method of Layne (12), with bovine serum albumin (Sigma Chemical Co.) as a standard. Benzyl alcohol dehydrogenase was measured by following the rate of $NAD^+$ reduction at 340 nm within 8 h of preparing the extract, which was stored on ice. The activity was assayed with 200 μmol of Tris hydrochloride buffer (pH 8.7)–7.5 μmol of $NAD^+$–8 μmol of substrate–cell extract in a volume of 3 ml (20). Catechol 2,3-oxygenase activity was assayed by measuring the rate of formation of the ring fission product of catechol (375 nm; $\varepsilon = 33{,}000\ mM^{-1}\ cM^{-1}$) in a 3-ml reaction mixture which contained 0.3 mM catechol, 50 mM phosphate buffer (pH 7.5), and cell extract (10). Cell extracts were prepared as previously described (3).

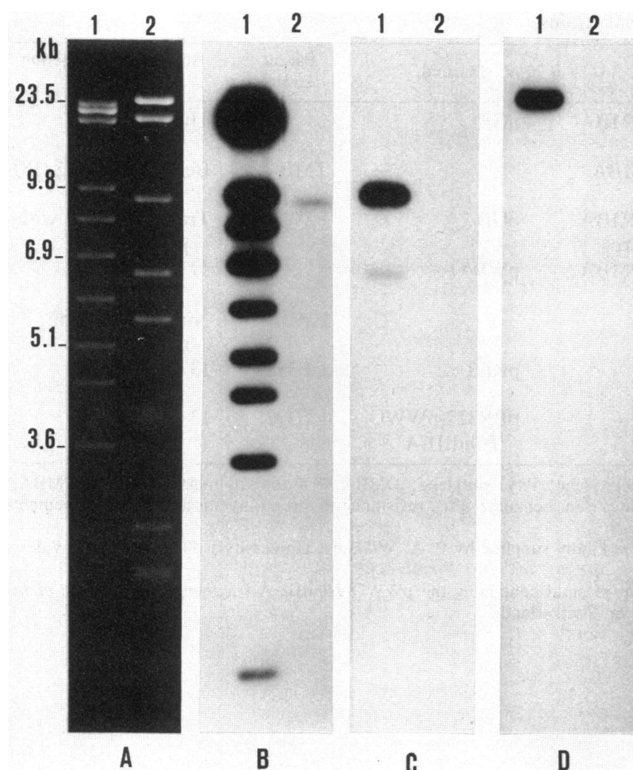


FIG 1. Hybridization of *Hin*dIII restriction digests of plasmids pWWO and pGB with nick-translated, $^{32}$P-labeled pWWO DNA (lanes 1B and 2B), $^{32}$P-labeled pRL3 DNA (lanes 1C and 2C), or $^{32}$P-labeled pBR322-pWWO *Hin*dIII-A DNA (lanes 1D and 2D) used as a probe. Electrophoretic analysis of *Hin*dIII-digested plasmids pWWO and pGB (lanes 1A and 2A) was carried out for 16 h at 40 mA in 0.6% agarose gels. Autoradiography was performed for 3 days to reveal weakly hybridizing bands. kb, Kilobases.

on an aromatic substrate. No enzymatic activity was detectable in cell extracts of strain TMB-1, which had lost the ability to grow on each of the aromatic substrates, with the exception of benzoic acid.

Strain TMB was demonstrated to carry a plasmid designated pGB, the size of which was estimated to be 85 kilobase pairs (kbp) by comparing the sizes of the fragments (determined from their electrophoretic mobilities) with the sizes of pWWO digestion fragments. A plasmid identical to pGB was detected in PaW340(pGB). No plasmid DNA was found in strain TMB-1 after benzoate curing.

On the basis of the metabolic features, a close metabolic relationship between strains TMB and PaW1 was postulated, suggesting that plasmid pGB might share a common trait with plasmid pWWO. To study the relationship between pWWO and pGB, we first compared their DNA endonuclease restriction patterns and then tested the relatedness of cleavage fragments by DNA-DNA hybridization techniques with whole plasmid pWWO or its particular restriction fragments as $^{32}$P-labeled DNA probes. The plasmids differed in both size (117 kbp for pWWO and 85 kbp for pGB) and restriction patterns (Fig. 1).

The DNA-DNA hybridization data obtained with $^{32}$P-labeled pWWO and Southern blots of *Hin*dIII fragments of pGB demonstrated a weak homology distributed over the *Hin*dIII C fragment (Fig. 1B). To investigate the homology level further, we hybridized $^{32}$P-labeled pRL3 DNA carrying the upper pathway genes of pWWO and a pBR322 hybrid recombinant plasmid containing the pWWO *Hin*dIII A fragment encoding the *meta* pathway genes to *Hin*dIII fragments of pGB transferred to nitrocellulose filters. In both experiments, no DNA sequence homology was detected with pGB (Fig. 1C and D).

In summary, the findings reported here provide evidence that *P. putida* TMB, the first known isolate from a 1,2,4-trimethylbenzene enrichment culture, carries a plasmid of 85 kbp involved in the degradation of this compound. The induction pattern of the benzyl alcohol dehydrogenase in strain TMB seems to be different from that in strain PaW1, in which two regulons are associated, respectively, with the oxidation of the methyl group (*xylABC*) and the further oxidation of the carboxylic acids (*xylDEGF*) (6, 8, 9, 19). In strain TMB, it could be assumed that only one regulatory gene, induced either by a hydrocarbon or by alcohol and carboxylic acid, is involved. A similar induction pattern has been reported for *P. putida* PpG7, which harbors plasmid NAH encoding the naphthalene catabolic pathway enzymes (1). The NAH genes appear to comprise at least two operons, namely, the genes specifying naphthalene conversion to salicylate and the genes specifying the *meta* cleavage pathway. Salicylate is the inducer of all the enzymes involved in this catabolism, suggesting that they share a regulatory gene.

Despite a functional homology, strain TMB harbors an 85-kbp plasmid with a molecular size and restriction patterns different from those of plasmid pWWO. No DNA sequence homology was observed between plasmid pGB and pWWO catabolic genes. Nevertheless, the extent of similarity between pGB and pWWO at a functional level suggests that pGB could be considered a new TOL plasmid, a class of large *Pseudomonas* plasmids which carry the genes for the catabolism of toluene, *m*-xylene, and *p*-xylene and thus support the growth of host strains on these compounds as sole sources of carbon and energy.

This assumption agrees with previous reports demonstrating that strains of *Pseudomonas* capable of using *m*-xylene and *p*-xylene are widespread in the soil microbial population and bear TOL plasmids which, however, vary in size, structure, and properties (14, 21). Very few studies have been published on other TOL plasmids at a comparable molecular biological level, mainly because of their large sizes (200 kbp). An investigation of the relationship among DNAs encoding the isofunctional catabolic pathway genes should yield information which would cast light on the evolution of complex inducible pathways and catabolic plasmids.

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## LITERATURE CITED

1. **Austen, R. A., and N. W. Dunn.** 1980. Regulation of the plasmid-specified naphthalene catabolic pathway of *Pseudomonas putida*. J. Gen. Microbiol. **177**:521–528.
2. **Baggi, G., D. Catelani, C. Sorlini, and V. Treccani.** 1982. Microbial degradation of methylbenzenes: metabolism of 1,2,4-trimethylbenzene by *Pseudomonas putida*. Ann. Microbiol. **32**:37–45.
3. **Bestetti, G., and E. Galli.** 1981. Plasmid-coded degradation of ethylbenzene and 1-phenylethanol in *Pseudomonas fluorescens*. FEMS Microbiol. Lett. **21**:165–168.
4. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 148–155. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and**

P. Broda. 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. **130**:1274–1280.

6. **Franklin, F. C. H., and P. A. Williams.** 1980. Construction of a partial diploid for the degradative pathway encoded by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2: evidence for the positive nature of the regulation by the *xylR* gene. Mol. Gen. Genet. **177**:321–328.
7. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
8. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1981. Molecular cloning of gene *xylS* of the TOL plasmid: evidence for positive regulation of the *xylDEGF* operon by *xylS*. J. Bacteriol. **148**: 413–418.
9. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1983. Molecular cloning of regulatory gene *xylR* and operator-promoter regions of the *xylABC* and *xylDEGF* operons of the TOL plasmid. J. Bacteriol. **155**:1192–1199.
10. **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 (pWWO) plasmid. J. Bacteriol. **146**:179–191.
11. **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.
12. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring protein—biuret method. Methods Enzymol. **3**:447–454.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **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.
15. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–245.
16. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
17. **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.
18. **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.
19. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
20. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas* ([sic] *putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.
21. **Worsey, M. J., and P. A. Williams.** 1977. Characterization of a spontaneously occurring mutant of the TOL20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. J. Bacteriol. **130**:1149–1158.