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Cloning of the Genes for and Characterization of the Early Stages of Toluene and *o*-Xylene Catabolism in *Pseudomonas stutzeri* OX1

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In order to study the toluene and *o*-xylene catabolic genes of *Pseudomonas stutzeri* OX1, a genomic library was constructed. A 28-kb *Eco*RI restriction endonuclease DNA fragment, cloned into the vector plasmid pLAFR1 and designated pFB3401, permitted *Pseudomonas putida* PaW340 to convert toluene and *o*-xylene into the corresponding *meta*-ring fission products. Physical and functional endonuclease restriction maps have been derived from the cloned DNA fragment. Further subcloning into and deletion analysis in the *Escherichia coli* vector pGEM-3Z allowed the genes for the conversion of toluene or *o*-xylene into the corresponding catechols to be mapped within a 6-kb region of the pFB3401 insert and their direction of transcription to be determined. Following exposure to toluene, *E. coli* cells carrying this 6-kb region produce a mixture of *o*-cresol, *m*-cresol, and *p*-cresol, which are further converted to 3-methylcatechol and 4-methylcatechol. Similarly, a mixture of 2,3-dimethylphenol and 3,4-dimethylphenol, further converted into dimethylcatechols, was detected after exposure to *o*-xylene. The enzyme involved in the first step of toluene and *o*-xylene degradation exhibited a broad substrate specificity, being able to oxidize also benzene, ethylbenzene, *m*-xylene, *p*-xylene, styrene, and naphthalene. Deletions of the 6-kb region which affect the ability to convert toluene or *o*-xylene into the corresponding methylphenols compromise also their further oxidation to methylcatechols. This suggests that a single enzyme system could be involved in both steps of the early stages of toluene and *o*-xylene catabolism.

Several soil bacteria can grow on toluene as the sole carbon and energy source. Characterization of the catabolic properties of several isolates showed that different biochemical routes for the mineralization of toluene had evolved among pseudomonads. Differences regard both upper and lower portions of the catabolic pathways. In the TOL plasmid-encoded pathway of *Pseudomonas putida* PaW1 (35), the formation of catechol from toluene is preceded by a progressive oxidation of the methyl group to produce benzoate. In contrast, in other pathways, the toluene ring is cleaved without the processing of the methyl group. For example, in *P. putida* F1, toluene is converted to 3-methylcatechol via a dioxygenation of the aromatic ring followed by dehydrogenation (14, 16). In *Burkholderia* (*Pseudomonas*) *cepacia* G4 and *Burkholderia* (*Pseudomonas*) *pickettii* PKO1, the formation of the same central intermediate, 3-methylcatechol, is accomplished by two subsequent monooxygenation reactions via phenolic intermediates, *o*-cresol and *m*-cresol, respectively (26, 30). In all these bacteria, the (methyl)catechol is further metabolized via extradiol cleavage. An upper pathway for toluene degradation combining both the methyl group oxidation and aromatic ring hydroxylation was described for *Pseudomonas mendocina* KR1 (33). In this strain, toluene is initially converted to *p*-cresol through a monooxygenation reaction followed by a progressive oxidation of the methyl group. The resulting protocatechuate is subsequently catabolized through an *ortho*-cleavage pathway.

If toluene can enter many different pathways, the catabolism of dimethylated compounds such as xylenes appears much more restricted. Several bacteria isolated so far can degrade *m*-xylene and *p*-xylene through the progressive oxidation of a methyl group (1). *o*-Xylene cannot enter this pathway, and in the few *o*-xylene-degrading bacteria described so far, this isomer appears to be oxidized via pathways involving either mono- or dioxygenation reactions of the aromatic ring without any processing of the methyl groups (2, 15, 17, 29).

Pseudomonas stutzeri OX1 can grow on *o*-xylene, 2,3- and 3,4-dimethylphenol, toluene, and cresols as the only carbon and energy sources. The pathway for the dissimilation of these compounds in *P. stutzeri* OX1 is chromosomally encoded, and several pieces of physiological evidence had previously suggested that their catabolism proceeds through the same catabolic route and involves oxygenation reactions of the aromatic ring to form mono- and dimethylcatechols which are subsequently catabolized by a *meta*-cleavage pathway (2, 3).

Here, we describe the cloning of a gene cluster encoding the early stages of catabolism of *o*-xylene and toluene in *P. stutzeri* OX1. Expression of this gene cluster in *P. putida* and *Escherichia coli* allowed reinvestigation of the first steps of this catabolic route and definition of the substrate range of the first enzymatic activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The plasmids were constructed by standard procedures (28). The gene library of *P. stutzeri* OX1 was constructed as previously described (28) by partial *Eco*RI digestion of genomic DNA and cloning of the resulting fragments in *E. coli* S17-1 with the cosmid vector pLAFR1 (11). Plasmid pNM72 is a pKT231 derivative carrying a noninducible pWW0 *meta* operon in which a mutation in the promoter region causes high-level constitutive expression in *P. putida*. Plasmid pNM185::*xylE* contains the pWW0 *xylE* gene, which encodes catechol 2,3-dioxygenase, under the control of pWW0 Pm and the *xylS* gene. A high level of catechol 2,3-dioxygenase activity can be obtained in response to pWW0 *meta*-operon effectors both in *E. coli* and in *P. putida* strains carrying pNM185::*xylE*. Plasmids were isolated from *P. putida* as described by Hansen and Olsen (18). *E. coli* plasmids were isolated by standard procedures (20) or by use of purification kits purchased from Machery-Nagel-Düren. Plas-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers or derivation[a]	Reference or source
Strains		
E. coli		
JM109	*recA1 hsdR17 thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)	36
S17-1	Mob$^+$	31
P. stutzeri OX1	Tol$^+$ *o*-Xyl$^+$ Dmp$^+$ Cre$^+$	3
P. putida		
PaW340	Tol$^-$ *o*-Xyl$^-$ Dmp$^-$ Cre$^-$ Trp$^-$; Sm^r	10
KT2442	Tol$^-$ *o*-Xyl$^-$ Dmp$^-$ Cre$^-$; Rf^r	9
F39/D	Mutant of *P. putida* F1 in the toluene *cis*-dihydrodiol dehydrogenase gene *todD*	14
Plasmids		
pLAFR1	Tc^r cloning vector	11
pNM72	Sm^r *xylXYZLTEGFJQKIH*	25
pNM185::*xylE*	Km^r *xylS xylE*	A. Polissi
pFB3401	Tc^r; 28-kb *Eco*RI partial DNA fragment from *P. stutzeri* OX1 cloned in pLAFR1	This work
pFB3405	Tc^r; 24.5-kb *Eco*RI partial DNA fragment from *P. stutzeri* OX1 cloned in pLAFR1	This work
pFB3411	Tc^r; 22.4-kb *Eco*RI partial DNA fragment from *P. stutzeri* OX1 cloned in pLAFR1	This work
pFB1229	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pFB1021	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pFB1036	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pFB1112	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pFB1230	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pFB1198	Tc^r; partial *Eco*RI deletion of pFB3401	This work
pGEM-3Z	Ap^r cloning vector	Promega Corp., Madison, Wis.
pBZ1010	Ap^r; 10-kb *Bgl*II-*Bgl*II fragment of pFB3401 cloned into *Bam*HI site of pGEM-3Z	This work
pBZ8075	Ap^r; *Kpn*I-*Not*I and *Cla*I-*Xba*I deletion of pBZ1010	This work
pBZ4085	Ap^r; 8.5-kb *Pst*I-*Pst*I fragment of pBZ1010 cloned into *Pst*I site of pGEM-3Z	This work
pBZ5065	Ap^r; 6.5-kb *Kpn*I-*Kpn*I fragment of pBZ4085 cloned into the *Kpn*I site of pGEM-3Z	This work
pBZ7045	Ap^r; 4.5-kb *Dra*I-*Dra*I fragment of pBZ1010 cloned into the *Sma*I site of pGEM-3Z	This work
pBZ7046	Ap^r; 4.5-kb *Dra*I-*Dra*I fragment of pBZ1010 cloned into the *Sma*I site of pGEM-3Z; opposite orientation with respect to pBZ7045	This work
pBZ1160	Ap^r; 6-kb *Mlu*I-*Mlu*I fragment cloned into the *Sma*I site of pGEM-3Z	This work
pBZ9047	Ap^r; 4.7-kb *Sal*I-*Sal*I fragment cloned into the *Sal*I site of pGEM-3Z	This work
pBZ1035	Ap^r; *Hin*dIII deletion of pBZ1010	This work
pBZ1048	Ap^r; *Mlu*I deletion of pBZ1010	This work
pBZ2035	Ap^r; 3.5-kb *Eco*RI-*Eco*RI fragment cloned into *Eco*RI site of pGEM-3Z	This work
pBZ1260	Ap^r; 6-kb *Not*I-*Dra*I fragment of pBZ1010 cloned into the *Sma*I site of pGEM-3Z	This work
pBZ1261	Ap^r, 6-kb *Not*I-*Dra*I fragment of pBZ1010 cloned into the *Sma*I site of pGEM-3Z; opposite orientation with respect to pBZ1260	This work

[a] Abbreviations: Ap, ampicillin; Km, kanamycin; Rf, rifampin; Sm, streptomycin; Tc, tetracycline; Tol, toluene; *o*-Xyl, *o*-xylene; Cre, *o*-cresol, *m*-cresol, and *p*-cresol; Dmp, 2,3-dimethylphenol and 3,4-dimethylphenol.

mid DNA was introduced into the bacterial host by electroporation as described previously (5).

Media and culture conditions. *P. stutzeri* OX1 was grown in M9-salts medium (22) under *o*-xylene vapors at 30°C. *P. putida* strains were grown routinely at 30°C in Luria broth (LB) (22). When tested for enzyme activity, they were grown to late exponential phase in 100 ml of M9 medium containing 20 mM malate and under toluene or *o*-xylene vapors. *E. coli* strains were grown routinely in LB at 37°C. When tested for enzyme activity, the cells were grown overnight at 30°C in 100 ml of M9 medium containing both 10 mM glucose and 20 mM malate. Induction of the *lacI*^{q}-regulated *lac* promoter of pGEM-3Z-based plasmids was performed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at 7 h after inoculation (dilution of inoculum,). Induction of the catechol 2,3-dioxygenase activity in the strains carrying pNM185::*xylE* was achieved by the addition of *m*-toluate at 2 mM. Ampicillin, kanamycin, streptomycin, and tetracycline were used in selective media at 150, 30, 50, and 25 μg/ml, respectively.

Enzyme assays. The ability to oxidize indole into blue dye indigo, previously described for several bacterial strains expressing mono- and dioxygenase activities with broad substrate ranges (6, 24, 38), was performed by plating bacteria on minimal medium with an appropriate carbon source and supplemented with 2 mM indole. After 2 days of growth, the colonies were inspected for the accumulation of blue pigment. A rapid qualitative assay of catechol 2,3-dioxygenase activity was performed by spraying colonies with a solution of 0.1 M catechol and inspecting, after a short interval (about 10 min), for the accumulation of the reaction product, 2-hydroxymuconic semialdehyde, typically yellow. The transformation of phenols or hydrocarbons into the corresponding hydroxymuconic semialdehydes was tested by growing bacteria for 2 days on LB (*E. coli*) or M9-malate (*P. putida*) plates containing the substrate at 2 mM or under vapors, in the case of *o*-xylene or toluene, and inspecting for yellow colonies. To determine the spectral properties of the ring fission products, *E. coli* or *P. putida* cells carrying the appropriate plasmid(s) were grown to the exponential phase in LB or M9-malate supplemented with the appropriated inducer(s), respectively, washed twice in 0.1 M phosphate buffer (pH 7.2), and resuspended in a half-volume of the same buffer. The soluble substrates were added to a final concentration of 2 mM, and the hydrocarbons were supplied in the vapor phase. The suspension was shaken at 30°C, and, as a yellow color appeared, the cells were removed by centrifugation and aliquots of the supernatants were acidified with concentrated HCl to pH 2.5 or basified with 10 M NaOH to pH 11. The spectra of the samples were determined with a Hewlett-Packard HP8452A spectrophotometer.

The rates at which *E. coli* and *P. putida* cells metabolized toluene, *o*-xylene, *m*-cresol, and 2,3-dimethylphenol were determined by monitoring changes in phenolic compound concentrations in the medium, using a colorimetric assay developed previously (8, 23). An 8-ml culture was washed twice in 0.1 M phosphate buffer (pH 7.2) and suspended in an equal volume of the same buffer. An 80-μl volume of 2 M glucose and 35 μl of 4% (vol/vol) toluene or *o*-xylene in *N*,*N*-dimethylformamide, or 20 μl of 20 mM *m*-cresol or 2,3-dimethylphenol in water, were added to the cell suspension. At 5, 10, and 15 min after incubation at 30°C with the substrate, 0.9-ml samples of cell suspension were mixed with 100 μl of 1 M NH_4OH and 25 μl of 2% 4-aminoantipyrine in a microcentrifuge tube. A 25-μl volume of 8% $K_3Fe(CN)_6$ was then mixed with the tube contents, and after brief centrifugation (14,000 × *g*), the A_{500} of the supernatant was measured.

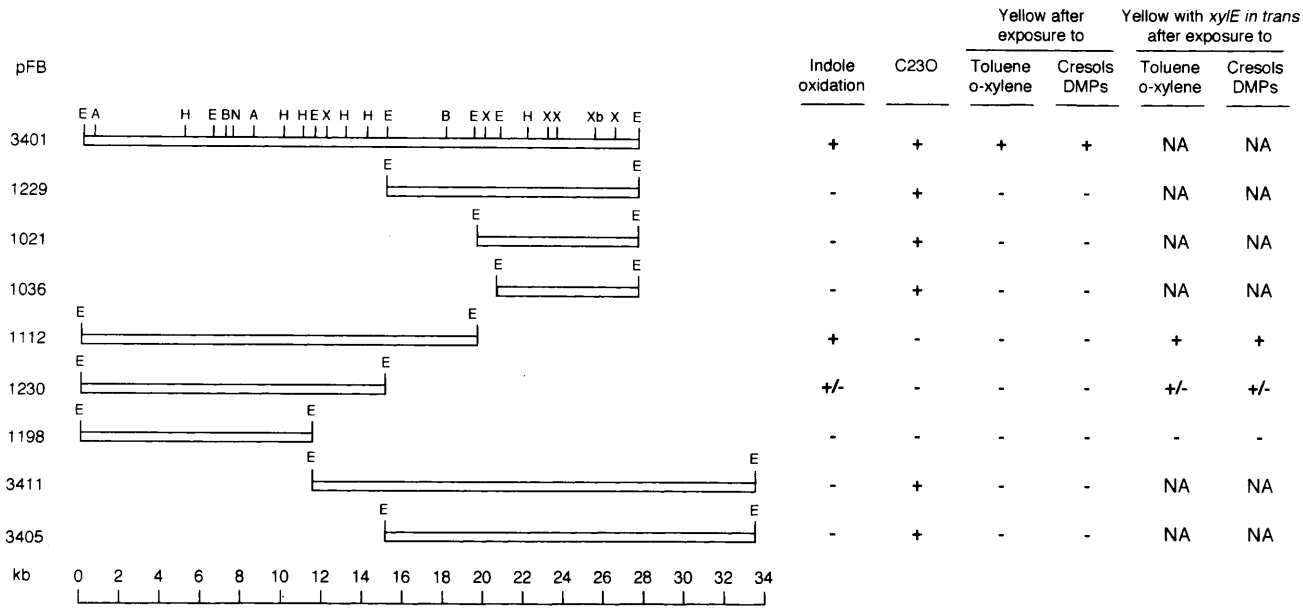

FIG. 1. Restriction endonuclease maps of the *P. stutzeri* OX1 chromosomal DNA fragment (pFB3401) which expresses indole oxidation and C23O activity in *P. putida* PaW340 and of the deletion derivatives. The physical structures of two overlapping chromosomal DNA fragments (pFB3411 and pFB3405) expressing only C23O activity are also indicated. The ability (+) or inability (−) of the plasmids to (i) allow indole oxidation, (ii) express C23O activity, or allow the production of a yellow color alone (iii) or when in *trans* with plasmid pNM185::*xylE* (iv) after exposure to toluene, *o*-xylene, *o*-cresol, *m*-cresol, and *p*-cresol (Cresols) and 2,3-dimethylphenol and 3,4-dimethylphenol (DMPs) is indicated to the right of the restriction maps. The results obtained from the exposure to toluene and *o*-xylene and to cresols and DMPs were identical for each plasmid, so they are grouped. +/−, mildly positive reaction. Abbreviations: A, *Apa*I; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; Xb, *Xba*I; X, *Xho*I; NA, not applicable.

Phenolic compound concentrations were calculated by reference to a standard curve for *m*-cresol or 2,3-dimethylphenol. Specific activities were reported as nanomoles of phenolic compound which were produced or which disappeared per minute per milligram of cell proteins. Cell protein concentrations (in milligrams per milliliter) were determined by the bicinchoninic acid method (Sigma Chemical Co.) using bovine serum albumin as a protein standard. Cells were resuspended in 0.1 M NaOH and incubated in boiling water for 20 min before protein concentration assays.

Identification of the reaction products formed from toluene, *o*-xylene, and other substrates. Cultures of IPTG-induced *E. coli* JM109(pBZ1260) (20 ml) were washed twice in 0.1 M phosphate buffer (pH 7.2) and resuspended in an equal volume of the same buffer. Substrate (25 μmol) was added to the resting cells, and the suspension was stirred at 30°C. At 30-min intervals, samples were collected, the cells were eliminated by filtration, and the medium was analyzed by reverse-phase high-pressure liquid chromatography (HPLC) or extracted. The extraction was performed with 3 volumes of dichloromethane, and the organic phase was dried with sodium sulfate and then completely evaporated under a vacuum. The residue was dissolved in 1 ml of dichloromethane and analyzed by normal-phase HPLC.

Analytical methods. HPLC analyses of metabolites were performed with a Waters 600E delivery system equipped with a Waters 486 detector and 746 integrator. Normal-phase chromatography was carried out with a Merck LiChroCART 250-4 CN cartridge eluted with hexane-dichloromethane (85:15) at a flow rate of 1 ml/min. The eluted compounds were detected by monitoring the A_{254}. Reverse-phase chromatography was conducted with a Waters μBondapak C_{18} column eluted with acetonitrile-water (50:50) at a flow rate of 1 ml/min. The eluted compounds were detected by monitoring the A_{254} or A_{270}. In HPLC analyses, metabolites were identified by comparison of their retention times with those of commercially available standards. UV-visible spectra and single-wavelength measurements were done with a Beckman DU-40 spectrophotometer.

Chemicals, reagents, and standards. Chemicals and reagents were of the highest purity available. Reference standards of 2-vinylphenol and 3-vinylphenol were obtained by the addition of concentrated HCl to a solution of (+)1(*S*),2(*R*)-dihydroxy-3-ethenylcyclohexa-3,5-diene (*cis*-styrene dihydrodiol), nuclear magnetic resonance tested, produced from styrene by *P. putida* PpF39/D essentially as previously described (17). After neutralization of the solution, the mixture of the two isomers was extracted from the aqueous phase with dichloromethane, dried with sodium sulfate, desiccated under a vacuum, and dissolved in a suitable volume of dichloromethane, 3,4-Dimethylcatechol was a kind gift of G. Baggi (Dipartimento di Science e Tecnologie Alimentari e Microbiologiene, University of Milan).

RESULTS

Cloning and mapping of the genes for the early stages of toluene and *o*-xylene catabolism. The isolation of the genes for the early stages of toluene and *o*-xylene catabolism resulted from the screening of an *E. coli* library of *P. stutzeri* OX1 genomic DNA in which single clones of the library were transferred by conjugation into *P. putida* PaW340 and transconjugants were tested on minimal medium plates for the ability to oxidize indole or to express catechol 2,3-dioxygenase (C23O) activity. By these selection procedures the overlapping recombinant cosmids pFB3401, pFB3405, and pFB3411 were isolated (Fig. 1). None of the analyzed recombinant cosmids allowed PaW340 to grow on toluene or *o*-xylene.

When exposed to *o*-xylene or 2,3-dimethylphenol, *P. putida* PaW340 cells harboring pFB3401 accumulated in the medium a yellow compound whose spectral properties (λ_{max} = 312 nm at pH 2.5, 324 nm at pH 7.2, and 392 nm at pH 11) were identical to those of the ring fission product of 3,4-dimethylcatechol, as previously observed with *o*-xylene-exposed *P. stutzeri* OX cells (2). Yellow compounds whose spectral properties were comparable to those of the ring fission products of 3-methylcatechol (λ_{max} = 314 nm at pH 2.5, 322 and 378 nm at pH 7.2, and 388 nm at pH 11) and 4-methylcatechol (λ_{max} = 318 nm at pH 2.5, 326 and 378 nm at pH 7.2, and 382 nm at pH 11) were also observed when PaW340(pFB3401) cells were cultured under toluene vapors or in the presence of *o*-, *m*-, and *p*-cresol. Thus, we tentatively concluded that the pFB3401 insert could code for the enzymes involved in the transformation of hydrocarbons or their phenolic intermediates into the corresponding *meta* ring cleavage products. To test if the cloned genes could indeed allow growth under toluene or *o*-xylene vapors, we introduced pFB3401 into *P. putida* KT2442 carrying the plasmid pNM72, which can express constitutively the entire

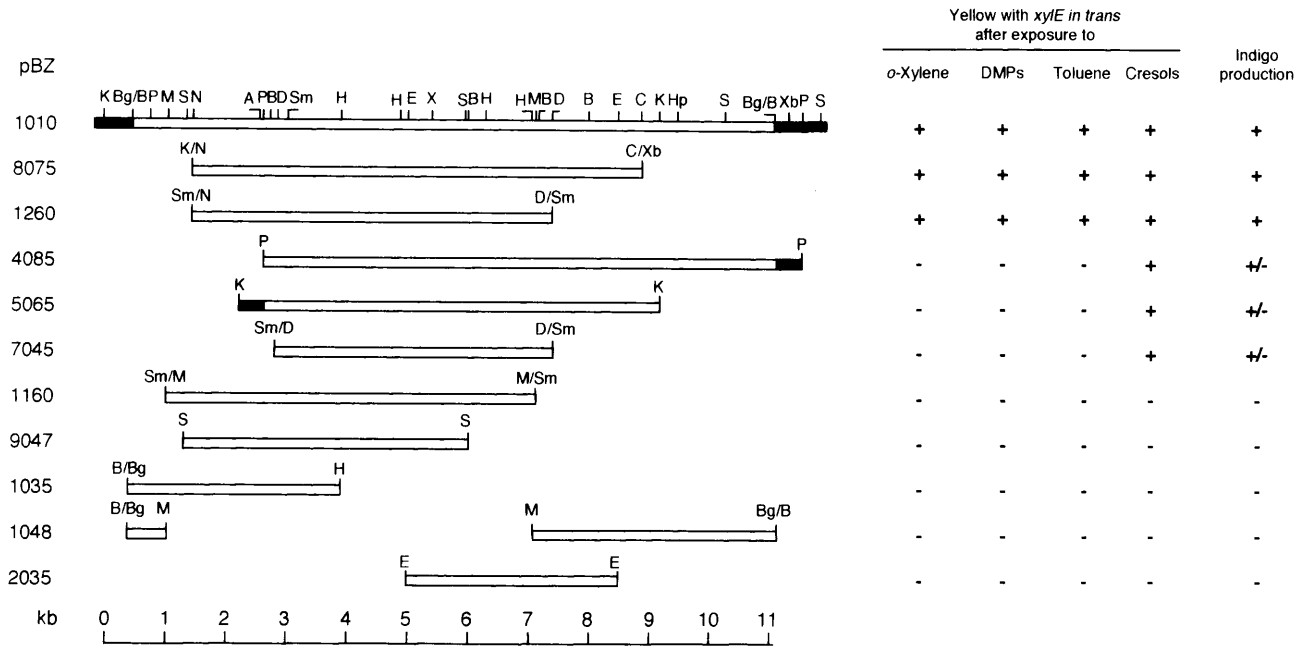

FIG. 2. Restriction maps of a pFB3401 subfragment expressing activities related to the early stages of toluene and *o*-xylene catabolism in *E. coli* JM109 and of various deletion constructs derived from it. The ability (+) or inability (−) of the plasmids to (i) allow the production of a yellow color when in *trans* with pNM185::*xylE* after exposure to *o*-xylene, 2,3-dimethylphenol and 3,4-dimethylphenol (DMPs), toluene, *o*-cresol, *m*-cresol, and *p*-cresol (Cresols) or (ii) allow the formation of a blue pigment (indigo production) on LB plates is indicated to the right of the restriction maps. +/−, mildly positive reaction. The positions of relevant restriction sites from the cloning vector are shown on solid boxes flanking the fragments. A P_{lac} promoter is located near the right end of each fragment on the cloning vector. Abbreviations: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Xb, *Xba*I; X, *Xho*I.

meta-cleavage operon isolated from the TOL plasmid pWW0. The strain harboring the two plasmids was able to grow on *o*-xylene and on toluene as the only carbon and energy sources.

For preliminary mapping of the cloned catabolic genes, we generated a series of derivatives with progressive *Eco*RI deletions into the pFB3401 insert (Fig. 1), which were tested for oxidation of the same substrates used with pFB3401. The deletion derivatives which did not express C23O activity were tested for their ability to direct the oxidation of the cited hydrocarbons and (di)methylphenols into catechols by providing *xylE* in *trans* and inspecting for the production of yellow color. The smallest fragment retaining the ability to convert the tested substrates into catechols was the one cloned in pFB1230. From these data, the genes involved in the early stages of *o*-xylene and toluene degradation could be mapped in a region to the left of the *Eco*RI site at coordinate 15 kb (Fig. 1), whereas the C23O gene was mapped within the 7.2-kb *Eco*RI fragment at the right end of the pFB3401 insert (pFB1036).

To further localize the genes of the upper portion of the pathway, the 10-kb *Bgl*II-*Bgl*II region of the pFB3401 insert was subcloned in *E. coli* JM109 into the IPTG-inducible multicopy vector pGEM-3Z to give plasmid pBZ1010. Cultures of *E. coli* cells harboring pBZ1010 accumulated in LB medium an insoluble blue pigment which could be extracted with chloroform and which presented an adsorption spectrum similar to that of indigo. The same strain appeared dark blue when plated on minimal medium supplemented with indole.

The insert of pBZ1010 was further subcloned in several overlapping fragments which were examined for indigo production in LB medium and, in *trans* with *xylE*, for their ability to convert the tested substrates into the corresponding catechols (Fig. 2). The spectral properties of the yellow compounds accumulated after exposure to *o*-xylene, toluene, dimethylphenols, and cresols by *E. coli* cells in this assay were identical to those previously reported for the semialdehydes produced by PaW340(pFB3401) exposed to the same substrates.

Remarkably, in this assay, the 4.5-kb *Dra*I-*Dra*I fragment cloned in pBZ7045 maintained the ability to convert cresols into the corresponding catechols, whereas the ability to oxidize both the hydrocarbons and the dimethylphenols was undetectable. However, the assays of hydrocarbon and phenol hydroxylation activities upon induction with IPTG (Table 2) showed that in cells carrying pBZ7045, the hydroxylation activities were not completely lost but only greatly reduced in comparison with those observed with pBZ8075 and pBZ1260, which instead showed levels of hydroxylation activities similar to each other both under induced and uninduced conditions. Since the insert in pBZ7045 has an identical end in front of the P_{lac} promoter with respect to pBZ1260, it seems that the region deleted in pBZ7045 is not essential for the expression of hydroxylation activities in *E. coli* cells but can enhance the activity levels. pBZ1261 and pBZ7046, which differ in the orientation of the inserted DNA with respect to pBZ1260 and pBZ7045, respectively, showed only basal activities upon addition of IPTG. The other subclones tested showed no detectable hydroxylation activities (Table 2). These results suggested that the genes involved in the early stage of *o*-xylene and toluene degradation are located in the 6-kb *Not*I-*Dra*I region cloned in pBZ1260 and that they are transcribed in the same direction, from right to left with respect to the map shown in Fig. 2.

Identification of the first intermediates in toluene and *o*-xylene catabolism. Supernatants of JM109(pBZ1260) cultures reacted with toluene or *o*-xylene for 30 min were extracted with dichloromethane as described in Materials and Methods, and the extracts were then analyzed by normal-phase HPLC. As shown in Fig. 3A, three reaction products from toluene could

TABLE 2. Expression in *E. coli* JM109 of recombinant plasmids carrying different *P. stutzeri* OX1 DNA fragments

Plasmid	IPTG induction	Activity (nmol min^{-1} mg of $protein^{-1}$)			
		Step I[a]		Step II[b]	
		Toluene	*o*-Xylene	*m*-Cresol	2,3-Dimethylphenol
pBZ8075	−	0.29	0.09	0.20	0.11
	+	6.30	1.35	3.10	2.33
pBZ1260	−	0.37	0.12	0.32	0.25
	+	6.92	1.81	3.68	2.62
pBZ1261	−	0.70	0.10	0.58	0.56
	+	0.67	0.14	0.86	0.61
pBZ7045	−	0.07	0.04	0.07	0.05
	+	0.86	0.48	0.81	0.59
pBZ7046	−	0.13	0.04	0.12	0.02
	+	0.14	0.04	0.13	0.07
pBZ9047	−	0.00	0.00	0.00	0.00
	+	0.00	0.00	0.00	0.00
pBZ1160	−	0.00	0.00	0.00	0.00
	+	0.00	0.00	0.00	0.00
pBZ2035	−	0.00	0.00	0.00	0.00
	+	0.00	0.00	0.00	0.00
pBZ1035	−	0.00	0.00	0.00	0.00
	+	0.00	0.00	0.00	0.00

[a] Conversion of the specified hydrocarbon assay substrate into phenolic compound.
[b] Consumption of the specified phenolic compound assay substrate.

be detected. To assess whether these products could be the three isomers of cresol, samples of dichloromethane extracts were mixed with pure *o*-cresol, *m*-cresol, or *p*-cresol. As shown in Fig. 3B to D, the reaction products coeluted with the authentic cresols. From *o*-xylene, two main products were observed (Fig. 3E) which showed retention times characteristic of 2,3- and 3,4-dimethylphenol. To test whether phenolic compounds observed upon toluene oxidation could derive from spontaneous dehydration of a *cis*-dihydrodiol intermediate under the reaction conditions adopted, both IPTG-induced JM109(pBZ1260) and toluene-induced *P. putida* PpF39/D cells resuspended in phosphate buffer (pH 7.2) were supplemented with toluene and shaken for 3 h. Samples of the reacting mixtures were filtered to remove cells, and an aliquot of each sample was acidified with concentrated HCl to pH 1. Analysis of the samples by reverse-phase HPLC showed that under the mildly alkaline conditions used to react the cells, the toluene *cis*-dihydrodiol produced from toluene by *P. putida* PpF39/D cells was detectable, and only upon acidification of the medium could we detect a product which coeluted with *o*-cresol. In contrast, the products obtained from JM109(pBZ1260) did not change their retention times upon acidification of the medium.

Identification of catechols formed from toluene and *o*-xylene. To identify other more polar intermediates, such as catechols, supernatants of JM109(pBZ1260) cultures reacted with toluene or *o*-xylene for longer times were directly analyzed by reverse-phase HPLC. After exposure to toluene, two reaction products were identified as 3-methylcatechol and 4-methylcatechol (Fig. 4A). The other two peaks corresponded to *o*-cresol and *m*-cresol or *p*-cresol, which under these experimental conditions showed the same retention times. 4-Methylcatechol was also detected after exposure to *m*-cresol and *p*-cresol, and 3-methylcatechol was detected after exposure to *o*-cresol (data not shown). A more polar compound derived from *o*-xylene oxidation, different from 2,3- and 3,4-dimethylphenol, was identified as 3,4-dimethylcatechol (Fig. 4B); 3,4-dimethylcatechol was also detected after exposure to 2,3-dimethylphenol (Fig. 4C). The fourth product derived from *o*-xylene (Fig. 4B) showed the same retention time as the product formed after exposure to 3,4-dimethylphenol (Fig. 4D) and did not coelute with 3,4-dimethylcatechol. Because of the lack of a suitable standard, this compound can only be supposed to be 4,5-dimethylcatechol. Indeed, upon addition of purified C23O, the supernatants of JM109(pBZ1260) cultures reacted with 3,4-dimethylphenol turned yellow and showed λ_{max} values of 326 nm at pH 7.2, 310 nm at pH 2.5, and 394 nm at pH 11. Thus, the more polar compound arising from 3,4-dimethylphenol should, at least, be cleaved at the *meta* position.

Substrate range of the cloned enzyme activity. IPTG-induced JM109(pBZ1260) cells were reacted with several hydrocarbons (Table 3), and the metabolic products were analyzed by normal-phase HPLC upon extraction with dichloromethane as described in Materials and Methods. We found that benzene was converted to phenol. Ethylbenzene was converted mainly to 3-ethylphenol even though 2- and 4-ethylphenol could be detected with a yield of approximately 1/20 of the amount of 3-ethylphenol. *m*-Xylene and *p*-xylene were converted to 2,4-dimethylphenol and 2,5-dimethylphenol, respectively, as previously reported for *o*-xylene-induced *P. stutzeri* OX1 cells (4). Styrene was converted into a mixture of three compounds which were more polar than styrene. Two of them coeluted with the standards of 2-vinylphenol and 3-vinylphenol (see Materials and Methods). The third one showed a longer retention time, and because of the lack of a standard of 4-vinylphenol, it could only tentatively be thought to be 4-vinylphenol. However, the possibility that such a product could be a styrene epoxide could not be totally excluded. The yield of the styrene products was approximately 1:16:30. Naphthalene was converted to α-naphthol and β-naphthol in a ratio of 6:1.

DISCUSSION

To date, several different pathways for the degradation of toluene have been identified among members of the genus *Pseudomonas*, but none of them has been reported to allow them to grow on *o*-xylene as the sole carbon and energy source.

In this study, we investigated the gene organization of a novel pathway for the degradation of both toluene and *o*-xylene in *P. stutzeri* OX1. In a cloned fragment of the OX1 genome, genes involved in the early stages of the pathway have been located within a 6-kb region (at 1.5 to 7.5 kb in Fig. 2). The activity of the enzyme(s) involved in the first step of this catabolic pathway on toluene results in the formation of a mixture of *o*-cresol, *m*-cresol, and *p*-cresol, and we could exclude the formation of a toluene *cis*-dihydrodiol. Furthermore, we reconfirmed that 2,3-dimethylphenol and 3,4-dimethylphenol are formed from *o*-xylene. These data, together with those previously reported on cross-inducibility of toluene- and *o*-xylene-dependent O_2 uptake in *P. stutzeri* OX1 cells grown on either toluene or *o*-xylene (13) and similarities with previously described enzymes, strongly suggest that a monooxygenase activity is responsible for the first step of toluene and *o*-xylene degradation; we tentatively designate this activity toluene/*o*-xylene-monooxygenase. Moreover, the abilities to oxidize indole, previously reported for other bacterial mono- and dioxygenases (6, 24, 38), and to oxidize several hydrocarbons give further evidence for a broad-spectrum oxygenase activity. Remarkably, its ability to hydroxylate more than one position on the aromatic ring of toluene should be considered unique with respect to the known toluene monooxygenases, such as toluene/benzene-2-monooxygenase (Tb2m) in *Pseudomonas* sp. strain JS150 (21) and toluene-3-monooxygenase (TTM) in *B. pickettii* PKO1 and toluene-4-monooxygenase (T4MO) in *P.*

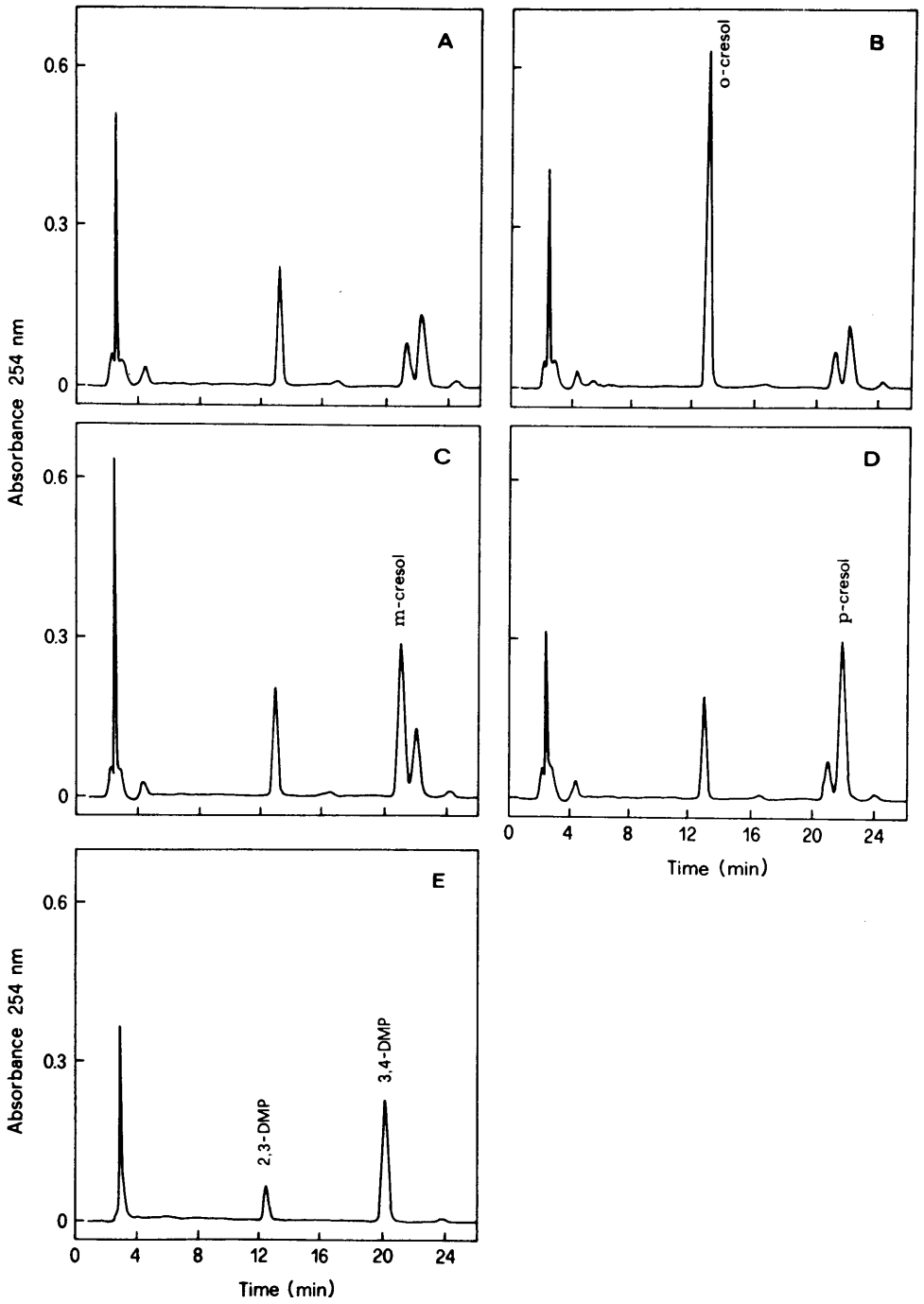

FIG. 3. Normal-phase HPLC of the first intermediates of toluene and *o*-xylene oxidation by cells of JM109(pBZ1260). (A) The products of toluene (retention times, 13.30, 21.45, and 22.37 min). (B to D) Products of the sample shown in panel A to which authentic *o*-cresol (retention time, 13.40 min), *m*-cresol (retention time, 21.60 min), and *p*-cresol (retention time, 21.56 min) were added, respectively. (E) The products formed from *o*-xylene (retention times, 12.20 and 19.89 min). Their retention times were comparable with those of authentic 2,3-dimethylphenol (2,3-DMP) (12.23 min) and 3,4-dimethylphenol (3,4-DMP) (19.78 min), respectively.

mendocina KR1 (26, 34). Little is known about the determinants of regioselectivity of toluene monooxygenase systems. In the case of methane monooxygenase from *Methylosinus trichosporium* OB3b (12), it was suggested that one component of the system, MmoB, may control the regioselectivity of the reaction altering the active site in a way that changes the orientation of the substrate molecule with respect to the reactive oxygen species. The multicomponent systems Tb2m, TTM, and T4MO show a high degree of homology (21) but display different regioselectivities. Thus, it is conceivable that not-drastic changes in a putative regulatory subunit (e.g., TmoD, TbuV, or TbmC) may alter, by restricting or expanding, the regioselectivity of a monooxygenase system.

The expression of the toluene/*o*-xylene-monooxygenase locus also allowed the conversion of phenolic intermediates into catechols. As could be predicted, *o*-cresol, *p*-cresol, and 2,3-

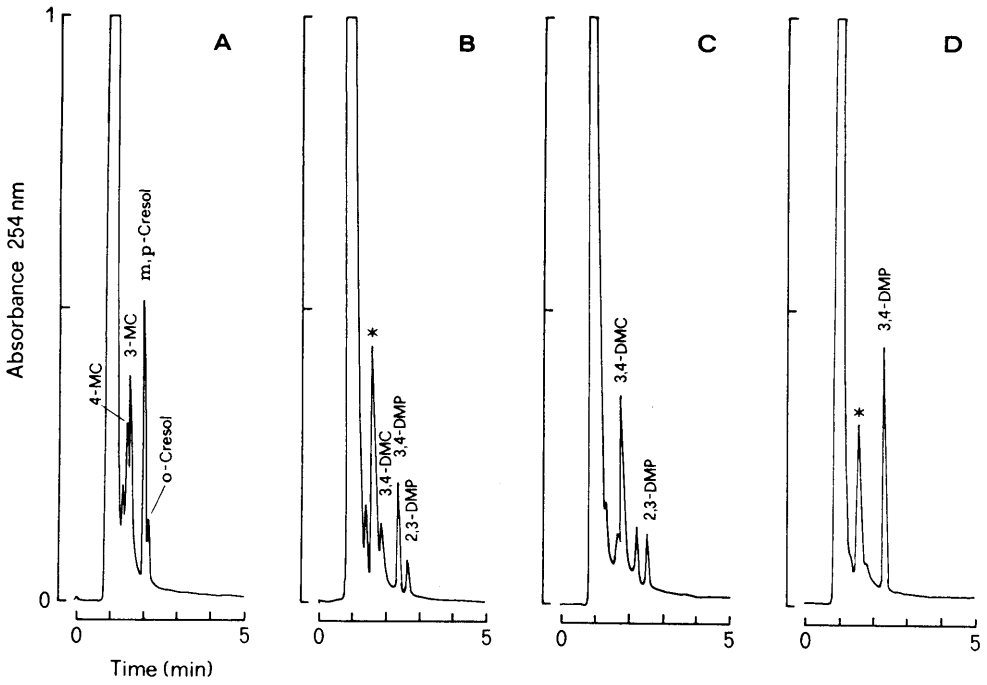

FIG. 4. Reverse-phase HPLC of products of toluene and *o*-xylene oxidation by JM109(pBZ1260). (A) The products formed from toluene were identified as 4-methylcatechol (4-MC) (retention time, 1.57 min), 3-methylcatechol (3-MC) (retention time, 1.66 min), and *o*-cresol (retention time, 2.23 min). Under the adopted experimental conditions, *m*-cresol and *p*-cresol showed the same retention time (2.10 min). (B) The products formed from *o*-xylene were identified as 2,3-dimethylphenol (2,3-DMP) (retention time, 2.74 min), 3,4-dimethylphenol (3,4-DMP) (retention time, 2.46 min), and 3,4-dimethylcatechol (3,4-DMC) (retention time, 1.93 min), while the compound eluted at 1.72 min (asterisk) has not been identified. 3,4-Dimethylcatechol (3,4-DMC) was also formed from 2,3-dimethylphenol (C), while the unidentified compound seemed to be the main product of 3,4-dimethylphenol oxidation (D).

dimethylphenol are converted into 3-methylcatechol, 4-methylcatechol, and 3,4-dimethylcatechol, respectively. *m*-Cresol and 3,4-dimethylphenol are converted mainly into 4-methylcatechol and supposedly 4,5-dimethylcatechol, respectively. Thus, according to these results, we propose that the early stages of toluene and *o*-xylene oxidation in *P. stutzeri* OX1 consist of two subsequent monooxygenations. First, toluene or *o*-xylene is converted into a mixture of all the possible methylphenols, and then the methylphenols are converted into methylcatechols. In the latter reaction, the regioselectivity of hydroxylation appears more restricted, with a preference for the position more distant from the methyl group(s).

TABLE 3. Substrates for the *P. stutzeri* toluene/*o*-xylene-monooxygenase from IPTG-induced *E. coli* JM109(pBZ1260) cells

Substrate	HPLC[a] retention time of product (min)	Product identified	HPLC retention time of standard (min)[b]
Benzene	4.63	Phenol	4.61
m-Xylene	12.06	2,4-Dimethylphenol	12.02
p-Xylene	11.14	2,5-Dimethylphenol	11.09
Ethylbenzene	11.27	2-Ethylphenol	11.05
	19.41	3-Ethylphenol	19.11
	21.30	4-Ethylphenol	20.79
Styrene	15.31	2-Vinylphenol	15.07
	26.47	3-Vinylphenol	27.05
	29.09	Unidentified	
Naphthalene	24.77	α-Naphthol	24.84
	35.75	β-Naphthol	35.49

[a] Normal-phase HPLC except for the product of benzene, which was analyzed by reverse-phase HPLC.
[b] Retention time for standard of predicted product.

In several *Pseudomonas* strains, the oxygenases acting on hydrocarbons have been shown or presumed to be multicomponent systems in which proteins interact to form an electron transport chain that transfers electrons from NAD(P)H to a terminal oxygenase. This organization is common to oxygenases found in catabolic pathways for several aromatic compounds, such as benzene, naphthalene, phenol and methylated phenols, benzoate, and toluate, and also for aliphatic compounds, such as alkanes (7, 19, 27, 32, 34). In the *P. stutzeri* system described here, a 1.5-kb deletion (pBZ7045) involving the 3′ end of the 6-kb region containing the toluene/*o*-xylene-monooxygenase locus strongly reduced in *E. coli* the specific rates of both hydrocarbon hydroxylation and phenol consumption. Further deletions at either end of the fragment cloned in pBZ7045 simultaneously abolished both activities and prevented the formation of substrates of C23O.

These observations suggest that the *P. stutzeri* toluene/*o*-xylene-monooxygenase might be a multicomponent enzyme system; this is also suggested by the detection of at least four different polypeptides (data not shown) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of different subclones overlapping the 6-kb region cloned in pBZ1260. The decrease of enzyme activity upon deletion at the 3′ end of the toluene/*o*-xylene-monooxygenase locus can be interpreted as the result of a knockout of a nonessential subunit, giving an effect similar to that described by Yen and Karl (37) with regard to the *tmoF* gene encoding NADH:ferredoxin oxidoreductase of the T4MO system in *P. mendocina* KR1, whose coexpression with the *tmoABCDE* gene cluster, although non-

essential, stimulates T4MO activity in *E. coli*. Moreover, the same enzyme system appears to be involved in methylphenol production and the further oxidation of methylphenols to methylcatechols. In this respect, the *P. stutzeri* system resembles what has been shown previously for the toluene-2-monooxygenases encoded by *Pseudomonas* sp. strain JS150 and *B. cepacia* G4 (21, 30) which catalyze cresol hydroxylation in addition to the oxidation of toluene to *o*-cresol.

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