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Nucleotide Sequence Analysis of Genes Encoding a Toluene/Benzene-2-Monooxygenase from *Pseudomonas* sp. Strain JS150

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It was previously shown by others that *Pseudomonas* sp. strain JS150 metabolizes benzene and alkyl- and chloro-substituted benzenes by using dioxygenase-initiated pathways coupled with multiple downstream metabolic pathways to accommodate catechol metabolism. By cloning genes encoding benzene-degradative enzymes, we found that strain JS150 also carries genes for a toluene/benzene-2-monooxygenase. The gene cluster encoding a 2-monooxygenase and its cognate regulator was cloned from a plasmid carried by strain JS150. Oxygen ($^{18}O_2$) incorporation experiments using *Pseudomonas aeruginosa* strains that carried the cloned genes confirmed that toluene hydroxylation was catalyzed through an authentic monooxygenase reaction to yield *ortho*-cresol. Regions encoding the toluene-2-monooxygenase and regulatory gene product were localized in two regions of the cloned fragment. The nucleotide sequence of the toluene/benzene-2-monooxygenase locus was determined. Analysis of this sequence revealed six open reading frames that were then designated *tbmA*, *tbmB*, *tbmC*, *tbmD*, *tbmE*, and *tbmF*. The deduced amino acid sequences for these genes showed the presence of motifs similar to well-conserved functional domains of multicomponent oxygenases. This analysis allowed the tentative identification of two terminal oxygenase subunits (TbmB and TbmD) and an electron transport protein (TbmF) for the monooxygenase enzyme. In addition to these gene products, all the *tbm* polypeptides shared significant homology with protein components from other bacterial multicomponent monooxygenases. Overall, the *tbm* gene products shared greater similarity with polypeptides from the phenol hydroxylases of *Pseudomonas putida* CF600, P35X, and BH than with those from the toluene monooxygenases of *Pseudomonas mendocina* KR1 and *Burkholderia* (*Pseudomonas*) *pickettii* PKO1. The relationship found between the phenol hydroxylases and a toluene-2-monooxygenase, characterized in this study for the first time at the nucleotide sequence level, suggested that DNA probes used for surveys of environmental populations should be carefully selected to reflect DNA sequences corresponding to the metabolic pathway of interest.

Pseudomonas sp. strain JS150, a nonencapsulated mutant of strain JS1, metabolizes a broad range of aromatic compounds for growth (13). Bacterial utilization of substituted benzenes requires that the organism synthesize both enzymes for initial ring activation and the appropriate downstream pathways for dissimilation of the resulting intermediates. Metabolic and biochemical studies have demonstrated that strain JS150 synthesizes *ortho*-, modified *ortho*-, and *meta*-ring fission pathways in response to growth on various aromatic compounds (13). Haigler et al. report that the presence of these multiple pathways for the degradation of substituted catechols in combination with a broad-substrate toluene dioxygenase allows for the extended aromatic substrate range of strain JS150. Moreover, those authors suggested that JS150 synthesizes additional dioxygenases for the initial attack of aromatic substrates, e.g., naphthalene and benzene. In our characterization of strain JS150, we have cloned genes encoding enzymes for 2-, 3-, and 4-monooxygenase attacks on benzene and chloro- or alkyl-substituted benzenes (19, 20). Our current studies suggest that toluene monooxygenases may also contribute to the large growth substrate range reported for strain JS150.

Previous studies have described toluene-degradative pathways in which the initial ring activation is a regiospecific monooxygenation (12, 36, 45, 55). One of these pathways, characterized for *Burkholderia* (*Pseudomonas*) *cepacia* G4, uses 2-monooxygenation for initial ring oxidation (45). The degradative capabilities of strain G4 are well documented and have been demonstrated in a number of potential bioremediation applications (8, 22). However, the genetic organization and regulation of the genes encoding the 2-monooxygenase for G4 have not been reported.

Recent reports by Fries et al. (10, 11) have suggested that bacteria with an enzyme to catalyze the monooxygenation of benzene and its substituted analogs may be widely distributed in nature. For this work, a probe was derived from a region internal to our 2-monooxygenase clone and used to screen bacterial isolates for the presence of homologous sequences. This study shows that a majority of the toluene- or phenol-degrading isolates that were screened provided a positive hybridization signal in Southern blot experiments. Currently, there is great interest in determining the presence and activity of bacteria associated with the degradation of petroleum wastes in situ (25, 26, 43). Assessments of bacterial populations and ongoing degradative processes by using gene probes may suggest specific applications to accelerate bioremediation of such sites. However, effective detection of these strains is influenced by the specificity of such probes (44). This specificity can be resolved only by comparison of the DNA sequences for related enzymes. Accordingly, we determined the nucleotide sequence and organization of the genes encoding the toluene/benzene-2-monooxygenase for strain JS150.

To gain an understanding of this environmentally relevant and potentially useful degradative enzyme, we have character-

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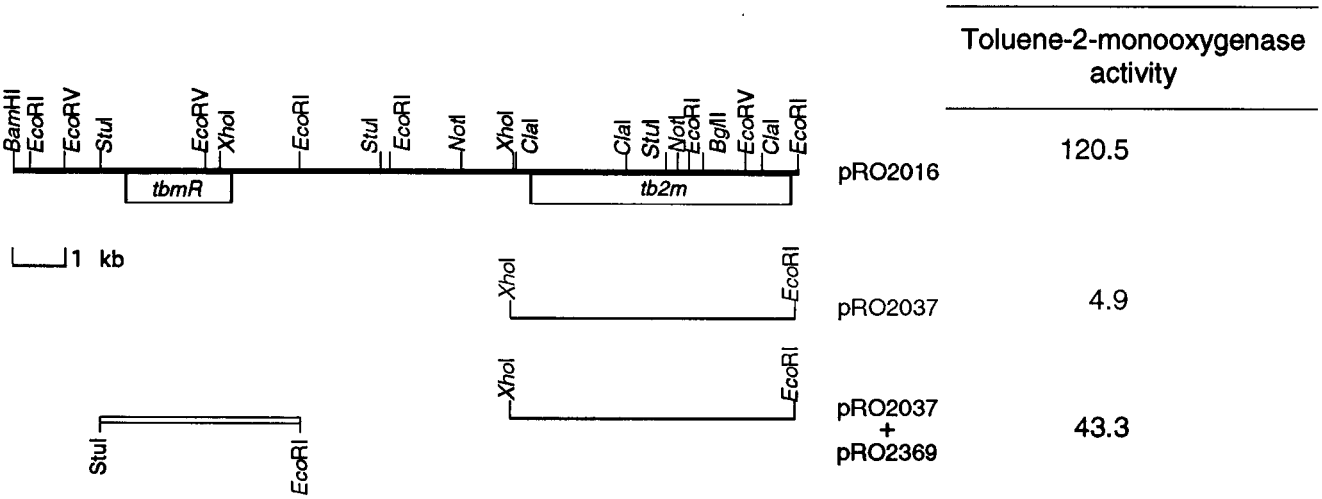

FIG. 1. Restriction maps of plasmid pRO2016 and subclones derived from it, with corresponding toluene monooxygenase activity from PAO4032 carrying the noted plasmids. Activity represents the amount of *o*-cresol (micromoles, 10^2) produced from toluene per 4 h per A_{425} unit of toluene-induced cells carrying each of the noted plasmids. Glucose-grown PAO4032(pRO2016) cells provided 6 μmol of *o*-cresol · 100/4 h/A_{425} unit (data not shown). Reported values indicate means for four or more trials. Rectangles below the pRO2016 physical map represent the loci identified in functional mapping experiments.

ized the genes encoding the toluene/benzene-2-monooxygenase from strain JS150. We report here the cloning, characterization of activity, nucleotide sequence, and organization of the 2-monooxygenase locus from strain JS150. Specific comparisons were made to define the relationship of toluene/benzene-2-monooxygenase to monooxygenases for a similar range of substrates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Pseudomonas aeruginosa* PAO1c and *P. aeruginosa* PAO4032 (23) were used for isolation and maintenance of cloning vectors pRO1727 (5) and pRO2321 (58) containing the cloned DNA fragments from strain JS150. These clones were isolated as described previously (34). *Escherichia coli* DH5α (14) was used for isolation and maintenance of recombinant derivatives of pBluescript II KS$^+$ (Stratagene Cloning Systems, La Jolla, Calif.). *P. aeruginosa* strains containing pRO1727 and its derivatives were grown on plate count medium (TNA) (35) with carbenicillin or ticarcillin (500 or 250 μg/ml, respectively). *P. aeruginosa* strains carrying recombinant derivatives of pRO2321 were grown on mineral salts medium (VB) (54) with trimethoprim (600 μg/ml). *E. coli* cells containing derivatives of pBluescript were grown in Luria-Bertani medium (41) with ampicillin (100 μg/ml). A minimal basal salts medium (BM) (29) was used when strains were grown with benzene or phenol as the sole carbon source. Glucose (0.5%) and Casamino Acids (0.3%) (Difco Laboratories, Detroit, Mich.) were added to VB and BM media as growth substrates when required.

Toluene monooxygenase assay. Cells were grown overnight at 37°C to late log phase with shaking in 100 ml of BM medium with 0.5% glucose, 0.3% Casamino Acids, and appropriate antibiotic selection to maintain plasmids. Toluene was added (1 mM final concentration) to cultures immediately after inoculation in order to induce monooxygenase expression. Cells were harvested by centrifugation and washed twice in 25 ml of 40 mM sodium and potassium phosphate (pH 7.5). Washed cells were added to 10 ml of the same buffer containing toluene (2.8 mM) to produce an A_{425} of 1.0. These 10-ml suspensions were incubated in 125-ml bottles sealed with rubber stoppers. These assay bottles were shaken at 25°C and 0.5-ml samples were taken at 4 and 20 h. The samples were mixed with 0.5 ml of cold methanol in 1.5-ml microcentrifuge tubes and then centrifuged to remove cells. The resulting supernatants were then analyzed by reverse-phase high-performance liquid chromatography (HPLC) with a Shimadzu SCL-6B solvent delivery system and a CR501 Chromatopac computing integrator. Reverse-phase chromatography was carried out with a PhaseSep H4726 column (4.6 by 250 mm) filled with Spherisorb ODS2 (particle diameter, 5 μm) preceded by a Whatman CSKI guard column (6.5 by 65 mm). The solvent used was methanol-water (50:50) at a flow rate of 1.5 ml/min. The *o*-cresol concentration was detected by monitoring at A_{280} and calculated by comparison with a standard curve as described previously (36).

^{18}O incorporation. *P. aeruginosa* PAO4032(pRO2016) was grown overnight in BM-glucose medium with toluene. Following incubation, cells were harvested and washed as described for toluene monooxygenase assays. Washed cells were transferred to 150 ml of phosphate buffer (final A_{425} of 1.0) in a 250-ml, three-neck distillation flask, which was then sealed by using stopcocks with Teflon-lined septa and screw-on hole caps. Throughout the following steps, the cell suspension was mixed with a magnetic stirrer. The air in the headspace of the flask was removed under a vacuum and replaced with nitrogen three times. The headspace was once again evacuated and brought to 80% of ambient pressure with nitrogen; following that, the flask was filled with pure oxygen enriched with $^{18}O_2$ (95 atom% ^{18}O). An identical experiment was done with compressed dry air in place of the nitrogen-$^{18}O_2$ mixture. Following the final gas exchange, the cell suspension was stirred for 15 min to equilibrate the reaction medium. After equilibration, toluene was added by injection through the septum in the center neck of the flask (final concentration, 2.8 mM) and the suspension was incubated for 3 h with stirring. Following incubation, the suspension was sparged with nitrogen for 5 min to remove the remaining toluene. Cells were removed by centrifugation, and a portion of the supernatant (50 ml) was extracted three times with diethyl ether (25 ml each time). The organic phases from these extractions were pooled and dried by passage over anhydrous sodium sulfate. The solvent was then removed under a stream of nitrogen, and the residue was dissolved in methanol for analysis by HPLC and gas chromatography-mass spectrometry as described previously (36).

Nucleotide sequence. Plasmid pBluescript II KS$^+$ (Stratagene Cloning Systems) was used to construct the subclones necessary for DNA sequencing. Subclones and deletion derivatives of the subclones were made by using restriction endonucleases according to standard protocols (34, 41). Plasmid DNA was routinely isolated by the method of Birnboim (1). Plasmid templates for DNA sequencing were isolated and purified with Plasmid Quik (Stratagene Cloning Systems) or Qiagen T-100 columns (QIAGEN Inc., Chatsworth, Calif.) by the methods recommended by the manufacturers. Nucleotide sequences were determined directly from plasmids by the dideoxy chain termination technique as described by Sanger et al. (42) with T3, T7, or specific synthetic primers; modified T7 polymerase (Sequenase version 1.0; United States Biochemical Corp., Cleveland, Ohio); and [α-^{32}P]dATP. Duplicate reactions were performed for each template by using dITP in place of dGTP to limit band compressions in GC-rich regions. Synthetic primers were obtained from the University of Michigan Biomedical Research Core facility (Ann Arbor, Mich.).

Computer analysis. Nucleotide sequences were entered and assembled by using AssemblyLIGN sequence assembly software (Eastman Kodak, Rochester, N.Y.). Nucleotide and deduced amino acid sequences were then analyzed by using MacVector sequence analysis software version 4.0 (Eastman Kodak), the University of Wisconsin Genetics Computer Group software package version 7.3 (7), and the LaserGene software package version 1.4 (DNASTAR, Madison, Wis.).

Southern blot analysis. Chromosome and plasmid DNAs were isolated from strain JS150 by previously described methods (15, 34) and then purified by using cesium chloride-ethidium bromide density gradients. DNA digested with restriction endonucleases was transferred to nitrocellulose membranes as described previously (4). Following transfer, the DNA was fixed to membranes by baking them under a vacuum at 80°C. Membranes were prehybridized for 3 h at 42°C in a solution containing 6× SSC (1× SSC is 150 mM sodium chloride plus 15 mM sodium citrate), 5× Denhardt's solution (41), 0.5% sodium dodecyl sulfate (SDS), 100 μg of salmon sperm DNA per ml, and 50% formamide (vol/vol).

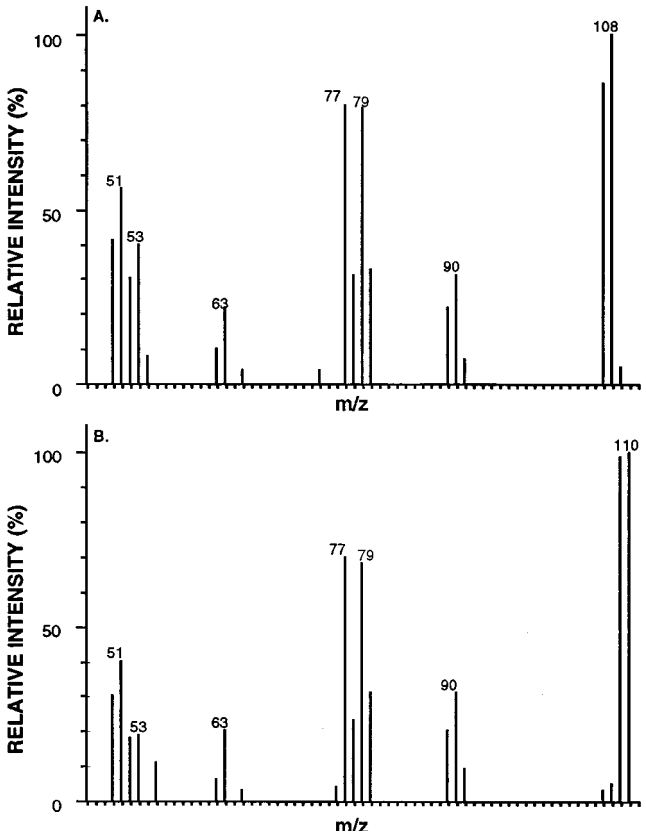

FIG. 2. Mass spectrometry analysis of *o*-cresol produced from toluene by *P. aeruginosa* PAO4032(pRO2016) in air (A) and in an $^{18}O_2$-enriched atmosphere (B).

Hybridization to radiolabeled probes (16 h at 42°C) was done by using the above-described solution with the Denhardt's solution omitted. After hybridization, membranes were given two 15-min washes in 2× SSC–0.1% SDS (25°C), one 15-min wash in 0.25× SSC–0.1% SDS (25°C), and one 30-min wash in 0.25× SSC–0.5% SDS (42°C). Washed membranes were blotted dry and used to expose imaging films (X-OMAT AR; Eastman Kodak) with intensifying screens at −70°C. Probes were prepared by randomly labeling agarose gel-purified restriction fragments with [α-^{32}P]dATP by using a nick translation kit (Boehringer Mannheim, Indianapolis, Ind.) according to methods described by the manufacturer.

Nucleotide sequence accession number. The nucleotide sequence of toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150 has been entered into the Genetic Sequence Data Bank (GenBank) under the accession number L40033.

RESULTS

Cloning and mapping of the *Pseudomonas* sp. strain JS150 toluene/benzene-2-monooxygenase structural and regulatory gene loci. A 14-kb DNA fragment cloned into vector pRO1727 and designated pRO2016 allowed *P. aeruginosa* PAO1c to grow with phenol or benzene as the sole carbon source. Subsequent testing showed that PAO1c(pRO2016) also hydroxylated substituted benzenes, i.e., toluene and chlorobenzene, to more polar compounds. When PAO1c(pRO2016) was grown in liquid minimal media containing 0.5% glucose as a growth substrate plus toluene or chlorobenzene, intermediates of aromatic metabolism accumulated in the medium. Metabolic products resulting from the oxidation of substituted benzenes were identified by comparing the product retention time with those of authentic *o*-, *m*-, and *p*-cresol (8.0, 7.5, and 7.6 min, respectively) and 2-, 3-, and 4-chlorophenol (8.3, 11.0, and 10.4 min, respectively). Toluene oxidation by *P. aeruginosa* strains carrying pRO2016 provided a product with a retention time (8.0 min) coinciding with that of *o*-cresol. Similarly, a product found following incubation with chlorobenzene (8.2 min) corresponded to 2-chlorophenol. The detection of these products suggested that pRO2016 contained genes encoding a 2-monooxygenase, i.e., an enzyme specific for the oxidation of chloro- or alkyl-substituted benzenes at the carbon adjacent to the ring substitution.

Experiments were done to determine if expression of the genes contained on pRO2016 was inducible. For this, PAO4032(pRO2016) was grown in minimal medium with or without toluene (1 mM) to late log phase and then assayed for toluene (or chlorobenzene) oxygenase activity as described in Materials and Methods. The assays showed that toluene-grown cells provided 20-fold greater toluene oxygenase activity than did cells grown with glucose alone (Fig. 1). This result suggested that the DNA insert contained on pRO2016 included loci encoding both structural and regulatory gene products.

Deletions of pRO2016 were constructed to identify regions

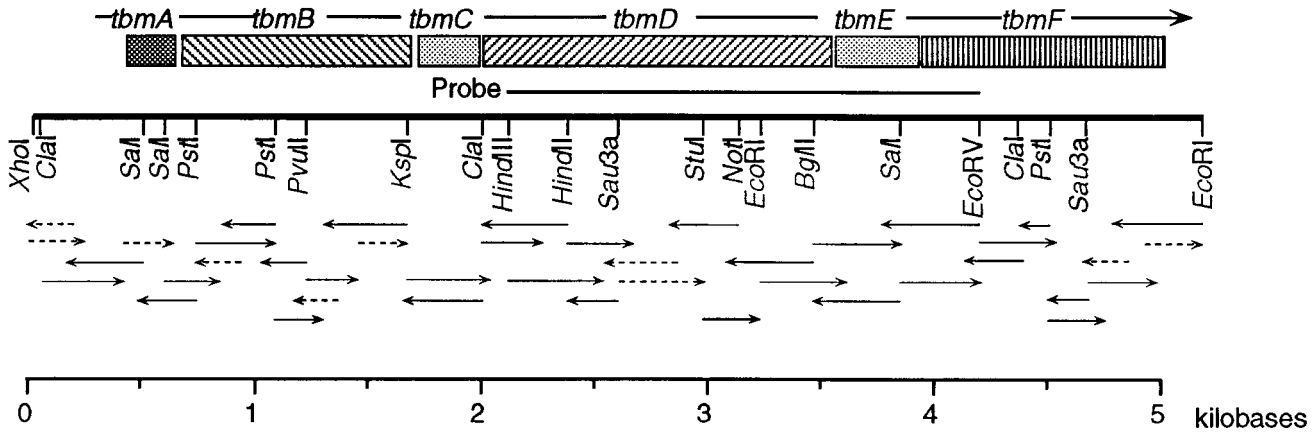

FIG. 3. Restriction map of the 5.1-kb DNA fragment in plasmid pRO2016 containing the Tb2m coding region. Solid arrows below the physical map indicate the extent of sequence obtained from subclones with the corresponding end, by using the vector T3 or T7 priming sites. Broken arrows indicate sequence obtained by using synthetic oligonucleotide primers. The direction of transcription for the Tb2m operon is indicated by the uppermost arrow. ORFs are indicated by the shaded boxes. The *Hin*dIII-*Eco*RV restriction fragment used as a probe in Southern blot experiments is shown below the ORF map.

TABLE 1. Comparisons of proteins with similarities to products of *tbm* ORFs

tbm ORF	Similar gene	Organism[a]	Enzyme	% Identity[b]	% Similarity[b]
tbmA	*dmpK*	*P. putida* CF600	Phenol hydroxylase	31.8	51.5
	pheK	*P. putida* BH	Phenol hydroxylase	31.8	51.5
	phhK	*P. putida* P35X	Phenol hydroxylase	30.3	51.5
tbmB	*dmpL*	*P. putida* CF600	Phenol hydroxylase	44.0	61.8
	pheL	*P. putida* BH	Phenol hydroxylase	44.0	61.8
	phhL	*P. putida* P35X	Phenol hydroxylase	44.0	61.8
	tbuA2	*B. pickettii* PKO1	Toluene-3-monooxygenase	24.4	46.8
	mmoX	*M. trichosporium* OB3b	Methane monooxygenase	22.1	46.7
	tmoE	*P. mendocina* KR1	Toluene-4-monooxygenase	18.7	43.7
	mmoX	*M. capsulatus* Bath	Methane monooxygenase	21.8	42.3
tbmC	*dmpM*	*P. putida* CF600	Phenol hydroxylase	48.9	75.6
	pheM	*P. putida* BH	Phenol hydroxylase	48.9	75.6
	phhM	*P. putida* P35X	Phenol hydroxylase	48.9	75.6
	tmoD	*P. mendocina* KR1	Toluene-4-monooxygenase	35.6	58.9
	tbuV	*B. pickettii* PKO1	Toluene-3-monooxygenase	35.6	53.3
	mmoB	*M. trichosporium* OB3b	Methane monooxygenase	27.8	46.7
tbmD	*dmpN*	*P. putida* CF600	Phenol hydroxylase	64.5	78.2
	pheN	*P. putida* BH	Phenol hydroxylase	64.5	78.2
	phhN	*P. putida* P35X	Phenol hydroxylase	64.4	77.1
	tbuA1	*B. pickettii* PKO1	Toluene-3-monooxygenase	25.6	50.2
	mmoX	*M. capsulatus* Bath	Methane monooxygenase	23.0	50.1
	tmoE	*P. mendocina* KR1	Toluene-4-monooxygenase	24.6	46.9
	mmoX	*M. trichosporium* OB3b	Methane monooxygenase	18.3	42.4
tbmE	*dmpO*	*P. putida* CF600	Phenol hydroxylase	35.3	57.8
	pheO	*P. putida* BH	Phenol hydroxylase	35.3	57.8
	phhO	*P. putida* P35X	Phenol hydroxylase	35.3	57.8
tbmF	*dmpP*	*P. putida* CF600	Phenol hydroxylase	56.5	72.7
	pheP	*P. putida* BH	Phenol hydroxylase	56.2	72.7
	phhP	*P. putida* P35X	Phenol hydroxylase	54.3	72.7
	tbuC	*B. pickettii* PKO1	Toluene-3-monooxygenase	31.7	56.2
	tmoF	*P. mendocina* KR1	Toluene-4-monooxygenase	32.6	55.8
	ascD	*Y. pseudotuberculosis*	Glucoseen reductase	33.4	54.6
	xylA	*P. putida* mt-2(pWWO)	Xylene monooxygenase	31.8	53.5
	nahAa	*P. putida* G7	Naphthalene dioxygenase	31.7	53.4
	pahAa	*P. putida* OUS82	PAH[c] dioxygenase	32.3	53.0
	nahAa	*P. putida* NCIB 9816-4	Naphthalene dioxygenase	30.7	52.6
	mmoC	*M. capsulatus* Bath	Methane monooxygenase	28.1	51.1
	benC	*A. calcoaceticus* BD413	Benzoate dioxygenase	30.2	50.2
	rfbI	*Y. pseudotuberculosis*	Glycosyl transferase	30.2	50.1
	xylZ	*P. putida* mt-2(pWWO)	Toluate dioxygenase	31.3	50.0
	mmoC	*M. trichosporium* OB3b	Methane monooxygenase	25.8	49.0

[a] Citations for genes not specifically noted in the text are as follows: *ascD*, reference 24; *xylA*, reference 49; *nahAa* (strains G7 and NCIB 9816-4), reference 47; *pahAa*, reference 51; *benC*, reference 31; *rfbI*, reference 21; *xylZ*, reference 16.

[b] Percentage of amino acids that are identical (or similar) when sequences are aligned with sequences in the GenBank database by using the algorithm of Needleman and Wunsch via the GAP program of the University of Wisconsin Genetics Computer Group software package.

[c] PAH, polycyclic aromatic hydrocarbon.

of DNA associated with the 2-monooxygenase structural genes. When the region corresponding to map coordinates kb 8.9 to 14.0 was deleted from pRO2016 and the resulting plasmid was transferred to PAO1c, neither phenol nor benzene supported growth. This suggested that essential genes for benzene metabolism were present on the deleted region. PAO1c carrying another derivative plasmid, pRO2037, which retained this region, could grow with benzene as the sole carbon source. In addition, PAO4032(pRO2037) transformed toluene to yield *o*-cresol when tested in assays as described above (Fig. 1). Metabolic pathways for which toluene metabolism is initiated by hydroxylation to yield a cresol isomer have been previously reported (12, 36, 45). These oxidations are catalyzed by multicomponent monooxygenase enzymes. On the basis of the similarities with previously described enzymes and the activity demonstrated by bacteria containing pRO2037, we tentatively designated the region contained on pRO2037 the toluene/benzene-2-monooxygenase (Tb2m) locus.

Preliminary analysis showed that PAO4032(pRO2037), when grown under inducing conditions, expressed only low levels of toluene-2-monooxygenase in comparison with pRO2016 grown under these conditions (Fig. 1). This observation suggested that a positive regulatory gene was lost with the region deleted from pRO2016 to form pRO2037. To further localize the region encoding a putative regulatory component, subclones of pRO2016 were constructed with pRO2321,

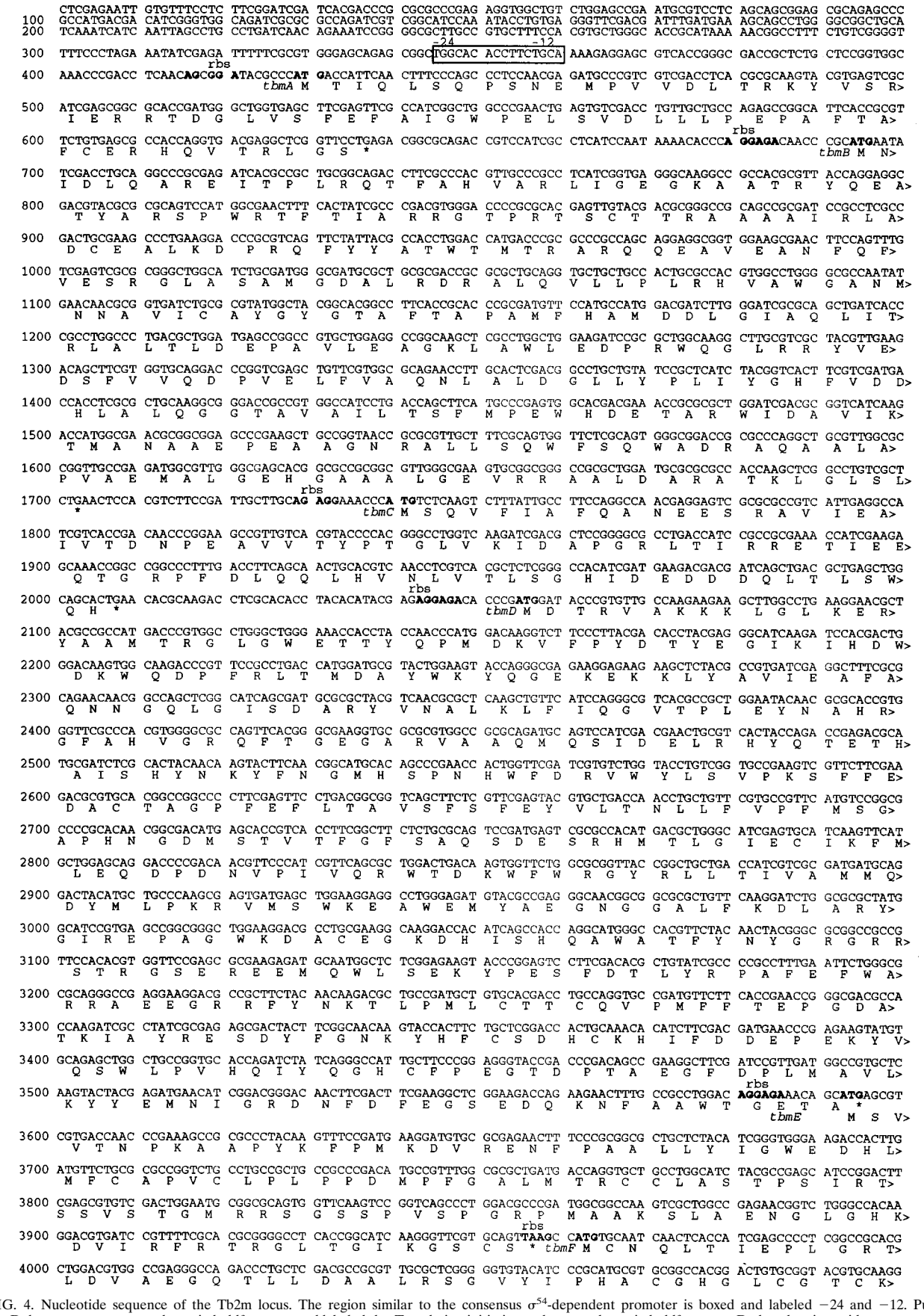

FIG. 4. Nucleotide sequence of the Tb2m locus. The region similar to the consensus σ^{54}-dependent promoter is boxed and labeled −24 and −12. Putative Shine-Dalgarno sequences are shown in boldface type and labeled rbs. Translation initiation codons are shown in boldface type. Deduced amino acid sequences of the individual polypeptides are displayed in single-letter code, centered below the respective codons. Translational stop codons are indicated by asterisks. The nucleotide sequence is numbered starting from the *Xho*I restriction site (Fig. 1 and 3).

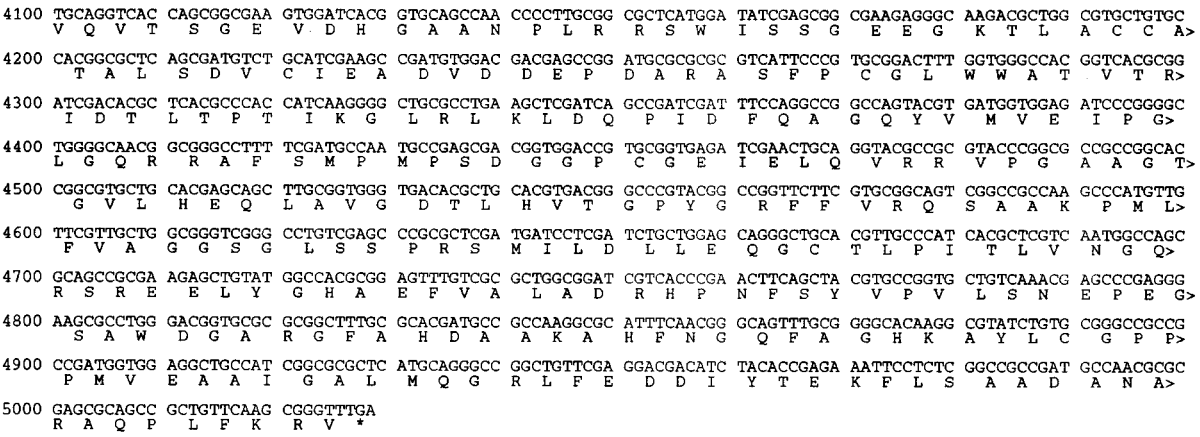

FIG. 4—*Continued.*

a cloning vector compatible with pRO2037. Such clones were transferred to strain PAO4032(pRO2037), and the toluene-2-monooxygenase activity from the resulting strains was determined. These experiments showed that the 2-monooxygenase activity was inducible in strains containing pRO2369 in *trans* with pRO2037 (Fig. 1). No toluene monooxygenase activity was detected in assays using PAO4032(pRO2369). These results, taken together with the basal-level activity from PAO4032(pRO2037), suggested that pRO2369 contained a gene encoding a regulatory protein that activated Tb2m expression. More deleted subclones were constructed and tested as before, which allowed a better delineation of the regulatory locus (*tbmR*), as indicated in Fig. 1.

PAO4032(pRO2037, pRO2369) was tested for its ability to catalyze the oxidation of *o*-cresol. This was shown by incubating toluene-induced cells with *o*-cresol and then analyzing the products from this incubation by reverse-phase HPLC. This experiment showed that *o*-cresol was transformed to a more polar compound during incubation. This product was identified as 3-methylcatechol on the basis of the corresponding retention times of authentic standards (5.1 min) and the product (5.1 min). This result showed that the enzyme encoded on pRO2037, like the toluene-*ortho*-monooxygenase previously described for *B. cepacia* G4 by Shields et al. (45), catalyzed cresol hydroxylation in addition to the oxidation of toluene to *o*-cresol. Experiments were also done to examine whether a toluene dioxygenase may be involved in the toluene oxidation encoded by pRO2037, as done previously for the toluene-3-monooxygenase of *Burkholderia* (*Pseudomonas*) *pickettii* PKO1 (36). For this, samples were withdrawn from toluene-metabolizing resting cell suspensions and either acidified to pH 2.0 or maintained mildly alkaline. These samples were analyzed by HPLC. In the present work, no evidence was found to suggest that a *cis*-dihydrodiol intermediate was involved in the pathway encoded on pRO2016. Therefore, this analysis supported our assertion that a 2-monooxygenase was encoded on pRO2037.

^{18}O incorporation experiments. Experiments were done to demonstrate that formation of *o*-cresol from toluene was due to incorporation of a single atom of dioxygen into the aromatic nucleus via a 2-monooxygenase reaction. Toluene was incubated with whole cells of strain PAO4032(pRO2016) in air or an $^{18}O_2$-enriched atmosphere as described in Materials and Methods. Following incubation, reaction mixtures were extracted with diethyl ether and analyzed by gas chromatography-mass spectrometry. A product (retention time, 15.42 min) that produced mass spectra characteristic of *o*-cresol was detected. These spectra showed a molecular ion and reference peak of 108 atomic mass units and a fragmentation pattern corresponding well to an authentic standard (Fig. 2A). The molecular ion value of *o*-cresol obtained in an $^{18}O_2$-enriched atmosphere increased 2 atomic mass units over the value for the product obtained in air (Fig. 2B). This shift showed that the oxygen in *o*-cresol was derived from molecular oxygen and that one atom of oxygen was incorporated into toluene to form the product. Accordingly, these experiments confirmed that the oxidation of toluene to *o*-cresol was catalyzed through a 2-monooxygenase reaction (17).

Nucleotide sequence of the toluene/benzene-2-monooxygenase locus. To further characterize the organization and structure of the genes encoding the 2-monooxygenase, we determined the nucleotide sequence of the Tb2m locus using the strategy summarized in Fig. 3. Analysis of the nucleotide sequence revealed an operon containing six open reading frames (ORFs), which were designated *tbmABCDEF* (Fig. 3). A single region resembling the consensus σ^{54}-dependent promoters for *Pseudomonas* operons was found upstream of the *tbmA* gene (Fig. 4, nucleotides 345 to 360) (6). All the ORFs identified by computer analysis were initiated by the canonical ATG start codon and were preceded by potential ribosome binding sites (Fig. 4) (46). Translation of the six individual ORFs provided polypeptides with calculated molecular masses of 7.9, 37.0, 10.0, 60.1, 13.0, and 38.0 kDa, respectively. The G+C content of the *tbmA*, *tbmB*, *tbmC*, *tbmD*, *tbmE*, and *tbmF* genes ranged from 60.1% for *tbmD* to 67.8% for *tbmB*. This is similar to percent G+C values found for chromosomes of *Pseudomonas* species (27).

Comparison of the deduced amino acid sequences of *tbmABCDEF* products with the peptide sequences of other bacterial enzymes. Comparison of predicted *tbm* polypeptide sequences with sequences in the GenBank database revealed strong homology to the multicomponent phenol hydroxylases encoded by *dmp*, *phe*, and *phh* operons of *Pseudomonas putida* CF600 (47), BH (51), and P35X (33), respectively. The organization of genes encoding these similar polypeptides was conserved among *tbmABCDEF* and the *dmp*, *phe*, and *phh* operons. In addition to those in the phenol hydroxylase, homologous peptides were also found in other enzyme systems, most notably, the toluene-3- and toluene-4-monooxygenases from *B. pickettii* PKO1 (2) and *Pseudomonas mendocina* KR1 (56, 57), respectively, as well as the soluble methane monooxygenases from *Methylococcus capsulatus* Bath (49) and *Methylosinus tricho-*

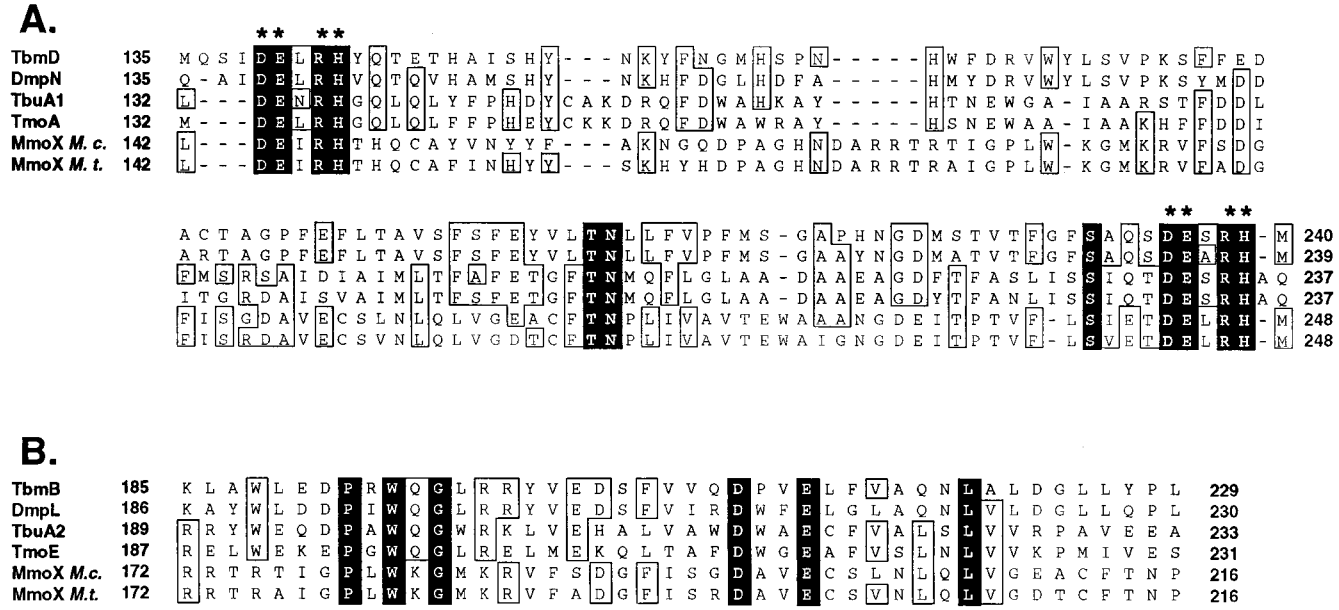

FIG. 5. Conserved regions in phenol hydroxylase, toluene monooxygenase, and methane monooxygenase oxygenase subunits. The first and final residues in each sequence are numbered to indicate their positions in the respective polypeptides. (A) Comparison of the region including the two binuclear iron centers from six oxygenase subunits. (B) Comparison of the conserved regions from TbmB, DmpL, TbuA2, TmoE, and MmoX from *M. capsulatus* and *M. trichosporium*. Sequences were aligned by the method of Jotun and Hein by using the MegAlign program included in LaserGene software package (DNASTAR, Inc.). The gap weight was 30, and the gap extension penalty was 10. Amino acid residues conserved in all proteins are indicated by a black background, and residues conserved in a majority of the proteins are shown boxed. Dashes indicate gaps introduced into the peptide sequences to maximize alignment of proteins. *M. c.*, *M. capsulatus*; *M. t.*, *M. trichosporium.*

sporium OB3b (3). A comparison of these relationships is summarized in Table 1.

TbmD, the largest polypeptide encoded by the Tb2m operon, shared homology with the large oxygenase subunit of other bacterial monooxygenase enzymes. These proteins were all similarly sized, ranging from 501 to 527 amino acids. A region resembling a dinuclear iron binding ligand was found in each of these large oxygenase subunits. This motif is found in several enzymes which catalyze reactions involving activated oxygen (9). These regions were identified by a pair of conserved domains with the amino acid sequence Asp-Glu-X-Arg-His. An alignment of the polypeptide sequences including the iron binding domains showed that the spacing between the two domains was also conserved, as the motifs were separated by 91 to 94 amino acids (regions denoted by asterisks in Fig. 5A). TbmB shares homology with the small oxygenase subunits of phenol hydroxylase and toluene monooxygenase enzymes. In addition, pairwise comparisons of TbmB with the large (MmoX) and small (MmoY) oxygenase subunits from methane monooxygenase revealed that TbmB shared greater overall homology with MmoX than with the similarly sized MmoY. Alignments with the TbmB homologs (Table 1) indicated that the conservation among TbmB, DmpK, TbuA2, and TmoD was distributed throughout the proteins. However, when the MmoX proteins from each of the methane monooxygenases were included in the multiple peptide alignments, one region was noted to contain the greatest concentration of identical residues (Fig. 5B). Database searches using this conserved region as a query sequence did not yield any additional proteins with significant homology.

The TbmF peptide shared homology with a number of other bacterial iron-sulfur flavoproteins. These proteins serve as oxidoreductases for several enzyme systems, including monooxygenases (2, 3, 33, 47, 49–51, 56), aromatic dioxygenases (16, 31, 48, 52), and reductases involved in biosynthesis of deoxy sugars (21, 24). Despite the dissimilar functions of their respective systems, all the oxidoreductase proteins share a basic conservation of structure. The N-terminal portion of TbmF and its homologs resembled chloroplast-type ferredoxins with a functional domain characterized by the consensus sequence Cys-XXX-Cys-XX-Cys-29 amino acids-Cys (37). The C-terminal half of the proteins contained motifs resembling flavin adenine dinucleotide-isoalloxazine ring- and NAD(P) ribose-binding domains (16, 31). Analysis of the 15 TbmF homologs by the method of Joten and Hein (DNASTAR) separated the proteins into six phylogenetic groups as follows: group I, DmpP, PheP, and PhhP; group II, TbuC, TmoF, and XylA; group III, RfbI and AscD; group IV, MmoC (*M. capsulatus* and *M. trichosporium*); group V, BenC and XylZ; group VI, PahAa and NahAa (*P. putida* G7 and NCIB 9816-4). Not surprisingly, these groupings reflected the biochemical function of each enzyme system (Table 1). A single representative of each, together with TbmF, was used in a multiple sequence alignment to demonstrate the conservation of primary structure among the individual homologs (Fig. 6). This conservation was best demonstrated in the domains noted above, as this reflected the similar generic functions of the proteins, since all serve as oxidoreductases to transfer electrons from the reduced NAD(P) cofactors to a terminal electron acceptor via the flavin and [2Fe-2S] center (28).

Database comparisons using the deduced amino acid sequences of TbmA, TbmC, and TbmE as query sequences showed that each of the three gene products had homologous counterparts in the *P. putida* phenol hydroxylases. In addition, TbmC shared similarity with polypeptides from the bacterial monooxygenase systems described above (Table 1). Unlike the situation with the oxygenase subunits and the oxidoreductase protein encoded by the Tb2m locus, analysis of TbmA, TbmC, and TbmE sequences did not suggest the functions of the corresponding individual polypeptides in the enzyme system.

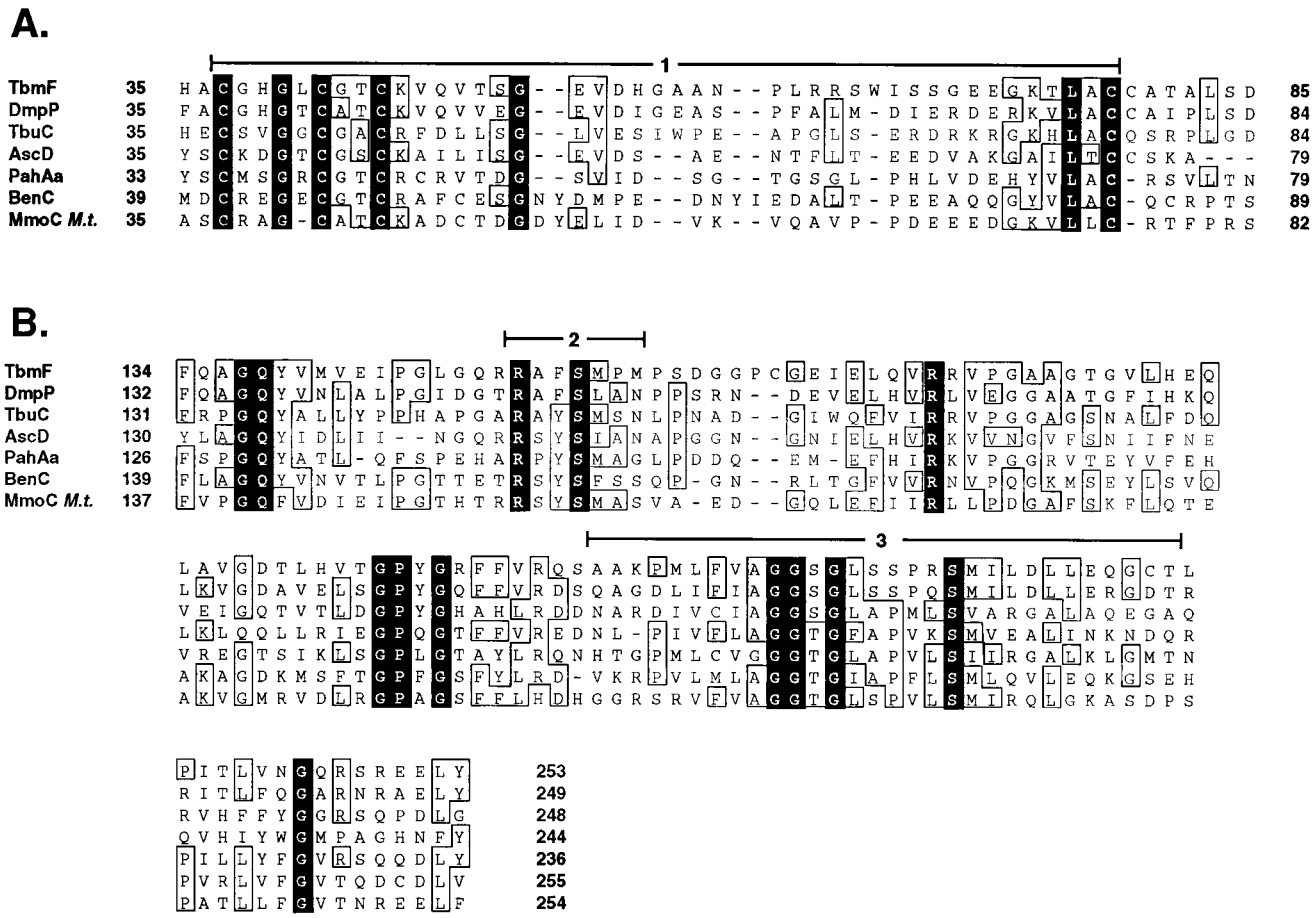

FIG. 6. Amino acid alignment of TbmF from *Pseudomonas* sp. strain JS150 with selected oxidoreductases listed in Table 1. (A) Conserved region containing the cysteine residues proposed to form the [2Fe-2S] center (bracket 1). (B) Conserved C-terminal region showing potential binding regions for the flavin adenine dinucleotide-isoalloxazine ring (bracket 2) and NAD(P)-ribose (bracket 3). Analysis parameters and alignment presentations are as described in the legend for Fig. 5.

Southern hybridization analysis. Plasmid or chromosomal DNA isolated from strain JS150 was cleaved with restriction endonucleases and hybridized on Southern blots to a probe derived from the Tb2m locus (the *Hin*dIII-*Eco*RV DNA fragment) as shown in Fig. 3. This analysis was done to determine whether the genes encoding the 2-monooxygenase originated on the chromosome or on one of the plasmids maintained by JS150. Unexpectedly, both chromosome and plasmid DNA preparations provided hybridizing fragments (Fig. 7). However, the size and relative intensity of the hybridizing 5.3-kb fragment in the plasmid lane (Fig. 7A, open arrow) compared with those of the other hybridizing fragments in chromosomal and plasmid lanes suggested that the 5.3-kb fragment represented the Tb2m locus. Moreover, we have reported previously that two other monooxygenases were also present in strain JS150 (19, 20). These monooxygenases, distinguished by unique endonuclease restriction patterns and regiospecificities, may share some homology with the Tb2m pathway. An additional complementary fragment was also detected in the plasmid DNA lane (3.8 kb) (Fig. 7A). Since the size of this fragment did not correspond to an *Xho*I restriction fragment from pRO2016, it likely represented a second plasmid-borne locus that shared homology with the probe sequence. A chromosomal origin for the Tb2m locus was precluded by the hybridization pattern from Southern blots of chromosomal DNA (Fig. 7A, lane 1, and Fig. 7B). In this case, the complementary restriction fragments detected in the *Eco*RI-cleaved (3.2- and 0.7-kb) and *Cla*I-cleaved (5.5-kb) DNAs (Fig. 7B, lanes 1 and 2, respectively) did not correspond to the pRO2016 physical map. Therefore, the complementary fragments from the chromosomal DNA also represented a cross-reacting sequence, not the Tb2m locus.

DISCUSSION

Previous work by others with *Pseudomonas* strain JS150 focused on the description of a toluene dioxygenase pathway for the catabolism of aromatic hydrocarbons (13, 40). We have also shown, using molecular cloning, that multiple monooxygenase pathways for the activation of benzene and alkyl- or chloro-substituted benzenes also are present in strain JS150 (19, 20). Heitkamp et al. have described a similar situation for another hydrocarbon-degrading bacterium. They characterized a polycyclic aromatic hydrocarbon-degrading *Mycobacterium* sp. that degraded pyrene by both a dioxygenase pathway and a monooxygenase pathway (18). This apparent redundancy of isofunctional enzyme systems is intriguing. It may allow for a wider range of substrates and activity under disparate environmental conditions than could be specified by a single metabolic pathway. However, the question of whether such duplication increases ecological fitness is unresolved.

Currently, many studies which employ DNA probes to de-

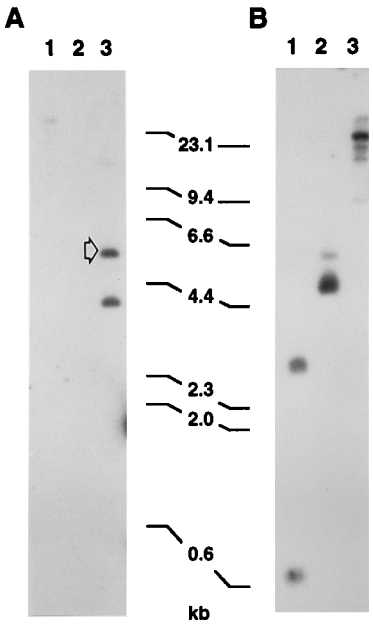

FIG. 7. Autoradiogram of Southern blot showing hybridization of 2.1-kb *Hin*dIII-*Eco*RV probe (Fig. 3) to JS150 DNA. (A) *Xho*I-cleaved chromosomal DNA (lane 1), DNA molecular size markers (lane 2), and *Xho*I-cleaved plasmid DNA (lane 3). (B) *Eco*RI-cleaved chromosomal DNA (lane 1), *Cla*I-cleaved chromosomal DNA (lane 2), and *Xho*I-cleaved chromosomal DNA (lane 3). The mobilities of the molecular size markers are indicated in the center. The band denoted by the arrow represents the *Xho*I restriction fragment, including the Tb2m-encoding region plus additional JS150 DNA (ca. 200 bp) not cloned on pRO2016.

tect and quantify the presence of bacteria which may be involved with ongoing degradation of pollutants from petroleum or other hydrocarbon contaminants occurring in the subsurface are under way. DNA probes can also be used to detect gene expression in environmental samples when combined with the extraction of mRNA (30, 38, 53). A further refinement is the use of PCR technology to enhance the sensitivity of such analyses in comparison with that of probe-based studies. This technology is also used in combination with the extraction of mRNA as a specific indicator of an ongoing degradative process. Another use of probes has been to assess the diversity of microbial populations present in a pollutant plume for bacteria specifying different metabolic pathways. The presence of relevant bacterial strains and evidence for their activity, then, may support the application of particular approaches for in situ bioremediation. However, the efficacy of these applications may be limited by the specificity of such probes and also, perhaps, by the presence of single microorganisms, such as strain JS150, which maintain more than one degradative pathway. Previous work in our laboratory described the relationship of a toluene-3-monooxygenase to other oxygenases with significant homology and similar organization (2). In the results reported here, the presence of multiple monooxygenase pathways for benzene/toluene is accompanied by weak hybridization of the Tb2m probe to DNA fragments different from the source of the probe. This is perhaps the result expected in view of the significant homology among various enzymes systems indicated here (Table 1) and in previous studies. This work suggests to us that the choice of a DNA sequence to identify a particular metabolic pathway needs to be made judiciously with knowledge at the level of the DNA sequence to promote the identification of unique regions and avoid the utilization of probes which lack specificity.

In the present work, our results expand this concern to include a 2-monooxygenase cloned and sequenced from strain JS150. The choice of this clone for further investigation was influenced by previous work reported by others. Other investigators described a similar if not identical toluene-2-monooxygenase for *B. cepacia* G4 which showed favorable characteristics for the degradation of trichloroethylene and alkyl-substituted benzenes (8, 22, 32). Our previous and current work suggested that a high degree of similarity exists between Tb2m and the toluene-*ortho*-monooxygenase described for strain G4. These similarities included the aromatic substrate range as well as the localization of genes associated with such activity on plasmids for both strains JS150 and G4 (39).

When DNA sequence comparisons are made between the 2-monooxygenase described here and other bacterial monooxygenases, both differences and similarities between them obtain. Most notable is the dissimilar organization of the *tbm* genes in comparison with their counterparts with similar functions reported for the toluene monooxygenases from *B. pickettii* PKO1 (*tbu*) (2) and *P. mendocina* KR1 (*tmo*) (56, 57). In contrast to the genes for the systems described above, the genes for the 2-monooxygenase of strain JS150 and the phenol hydroxylases from *P. putida* CF600 (*dmp*) (47), P35X (*phh*) (33), and BH (*phe*) (51) are identically juxtaposed. In spite of this conserved organization, further analysis suggested that Tb2m is distinct from the phenol hydroxylase family of multicomponent oxygenases. Comparisons among the *dmp*, *phe*, and *phh* operons indicated that the nucleotide sequences of the three operons are 95.84 to 99.98% identical. Likewise, the deduced amino acid sequence for polypeptides from the three phenol hydroxylases showed few nonconserved residues. Further analysis showed that the phenol hydroxylases were very closely related, since even the intergenic regions were conserved among these three operons. In contrast, *tbm* was 64.3, 63.2, and 62.8% identical to *dmp*, *phe*, and *phh* at the nucleotide level and the *tbm*-encoded polypeptides were 31.8 to 64.5% identical to their counterparts in the phenol hydroxylase enzyme, and no significant conservation in the intergenic regions was found. A distinction was also drawn from the functional characterization of Tb2m. To our knowledge, there are no reports showing that the previously described phenol hydroxylases catalyze oxidation of an unactivated benzene ring. However, this study unequivocally demonstrated that benzene, toluene, and chlorobenzene were hydroxylated by Tb2m.

Characterization of genes which encode degradative activities may contribute to the evaluation of microbial populations optimal for biodegradation and bioremediation in situ. Specific gene probes may be constructed for unique bacterial strains known on the basis of physiological characterizations to be suitable for in situ activities. However, we acknowledge that the presence of DNA corresponding to a particular sequence does not necessarily mean that such genes are expressed under the conditions which prevail in the subsurface. Moreover, the presence of several degradative pathways in a single bacterium may further complicate the assessment of potential microbial activity.

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