Cloning and Characterization of a Pseudomonas mendocina KR1 Gene Cluster Encoding Toluene-4-Monoxygenase

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Pseudomonas mendocina KR1 utilizes toluene as a sole carbon and energy source. Toluene is oxidized in this bacterial strain to protocatechuate, which is further oxidized through ortho ring cleavage to form substrates of tricarboxylic acid cycle (37). The initial step of this pathway is distinctively different from those of other known aromatic pathways. Earlier studies have shown that in Pseudomonas putida F1, toluene is initially oxidized to form (±)-cis-1,5,2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (cis-toluene dihydrodiol) via a dioxygenation reaction (Fig. 1) (14, 19, 45). Subsequently, it was found that in P. putida mt-2, toluene is initially attacked at the methyl group by a monooxygenase enzyme system to form benzyl alcohol (Fig. 1) (39). More recent studies have demonstrated that the initial monooxygenation reaction can also occur on the aromatic ring of toluene. In the toluene-degrading bacterium G4, this reaction hydroxylates toluene to form o-cresol (Fig. 1) (33). However, in P. mendocina KR1, toluene is initially hydroxylated by a toluene-4-monoxygenase (T4MO) system to form p-cresol (Fig. 1) (36). The T4MO enzyme system not only catalyzes a previously unknown reaction but also acts on a variety of substrates. These properties of the T4MO system provide a new opportunity for biodegradation of toxic compounds and biocarbons. We recently demonstrated the complete degradation of trichloroethylene by the T4MO system (38). In addition, many phenyl compounds, such as anilide, chlorobenzene, 2-phenylethanol, and ethylbenzene, may serve as substrates and be converted to phenolic compounds by the T4MO system (13, 42).

In this report, we describe the cloning and characterization of a gene cluster from strain KR1 that determines T4MO activity. We have cloned 20.4 kb of KR1 DNA into Escherichia coli and identified a 3.6-kb region required for T4MO activity. DNA sequencing and N-terminal amino acid determination identified five genes within this region. Expression of this gene cluster carrying mutations in the individual genes demonstrated that each of the five genes is essential for T4MO activity. Other evidence presented indicated that none of the tmo genes was involved in the regulation of the tmo gene cluster. In control of substrate transport for the T4MO system, or in major processing of the products of the tmo genes. It was tentatively concluded that the tmoABCDE genes encode structural polypeptides of the T4MO enzyme system. One of the tmo genes was tentatively identified as a ferredoxin gene.

Pseudomonas mendocina KR1 metabolizes toluene as a carbon source by a previously unknown pathway. The initial step of the pathway is hydroxylation of toluene to form p-cresol by a multicomponent toluene-4-monoxygenase (T4MO) system. The T4MO enzyme system has broad substrate specificity and provides a new opportunity for biodegradation of toxic compounds and biocarbons. Its known activities include conversion of a variety of phenyl compounds into the phenolic derivatives and the complete degradation of trichloroethylene. We have cloned and characterized a gene cluster from KR1 that determines the T4MO activity. To clone the T4MO genes, KR1 DNA libraries were constructed in Escherichia coli HB101 by using a broad-host-range vector and transferred to a KR1 mutant able to grow on p-cresol but not on toluene. An insert consisting of two SacI fragments of identical size (10.2 kb) was shown to complement the mutant for growth on toluene. One of the SacI fragments, when cloned into the E. coli vector pUC19, was found to direct the synthesis of indigo dye. The indigo-forming property was correlated with the presence of T4MO activity. The T4MO genes were mapped to a 3.6-kb region, and the direction of transcription was determined. DNA sequencing and N-terminal amino acid determination identified a five-gene cluster, tmoABCDE, within this region. Expression of this cluster carrying a single mutation in each gene demonstrated that each of the five genes is essential for T4MO activity. Other evidence presented indicated that none of the tmo genes was involved in the regulation of the tmo gene cluster. In control of substrate transport for the T4MO system, or in major processing of the products of the tmo genes. It was tentatively concluded that the tmoABCDE genes encode structural polypeptides of the T4MO enzyme system. One of the tmo genes was tentatively identified as a ferredoxin gene.

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and E. coli DNA polymerase I large fragment were obtained from New England BioLabs, Inc., Beverly, Mass., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Media. L broth (21) was used as a complete medium, and phosphate ammonium salts (PAS) supplemented with a carbon source (5) was used as a minimum medium. Bacto-agar (Difco Laboratories, Detroit, Mich.) and purified agar (Oxoid USA Inc., Columbia, Md.) were used (2 g/liter) to make L agar and PAS agar, respectively.

Bacterial strains. P. mendocina KR1 was obtained from D. T. Gibson (37). Properties of P. putida KT2440 (r- m-) were described by Bagdasarian et al. (1). E. coli FM5, which contains the integrated phage lambda repressor gene cI857 (35), was described by Burnette et al. (4). E. coli JM109 [recA I hsdR17 thi D(lac-proAB)](F' traD36 proAB

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lacP*ZAM15) was described by Yanisch-Perron et al. (40). Derivation of E. coli HB101 (hsdS20 recA13 proA2 leuB6 thi-1) has also been documented elsewhere (3).

Cloning vectors. Plasmids pKMY235 and pKMY319 are broad-host-range cloning vectors in which expression of foreign genes can be regulated by the NahR protein and the inducer sodium salicylate (44). Plasmid pKMY319 was constructed to be better suitable for gene cloning and expression than is pKMY235, which was constructed and used in early experiments. The advantages of pKMY319 over pKMY235 include a lack of intervening gene sequences between the promoter and its cloning sites, the presence of a transcription terminator downstream of the cloning sites, and a smaller size (41).

Plasmid pKMY235 was constructed in several steps. In the first step, two adjoining HindIII fragments (1.1 and 3.2 kb) of pKY217 (44) that contain the nahR and nahG genes were cloned into the HindIII site of plasmid pKT240 (2). This plasmid was designated pKMY219. In the second step, an ~7-kb BamHI-SacI fragment of pKMY219 that contains the nahR and nahG genes was cloned into the BamHI and SacI sites of plasmid pKT231 (1). The resulting plasmid was designated pKMY223. In the next step, an ~5.3-kb PstI fragment from pKMY223 containing the nahR gene, approximately 200 bp of the nahG gene, and the pKT231 gene conferring kanamycin resistance was cloned into the PstI site of plasmid pUC19 (40). This plasmid was designated pKMY256. In pKMY256, the multicloning site of pUC19 from the SalI to the EcoRI site is located immediately downstream of the PstI site in the nahG gene. In the final step, a 5.4-kb BstEII-EcoRI fragment from pKMY256 containing the gene conferring kanamycin resistance, the nahR gene, ~200 bp of the nahG gene, and a multiple cloning site was end filled and used to replace the ~1-kb Smal fragment in plasmid pRK290 (9) to complete the construction of pKMY235. Plasmid pT7-5 is a ColE1-based plasmid containing the β-lactamase gene and a multiple cloning site downstream from a T7 RNA polymerase-specific promoter. It was obtained from S. Tabor. Plasmid pCFM1146 is an E. coli cloning vector similar to pCFM4722. It carries the lambda phage pt promoter, a multiple cloning site, and a gene conferring kanamycin resistance. This plasmid was constructed by Charles Morris from pCFM836 (26) by substituting the small DNA sequence between the unique ClaI and Xbal restriction sites with the oligonucleotide 5'-CGAT TGATT-3'/3'-TAAAATAGATC-5' and by destroying the two endogenous NdeI restriction sites.

Construction of plasmids carrying the T4MO gene(s). The various inserts contained in these plasmids are shown in Fig. 2. Plasmid pKMY277 was constructed by cloning a 10.2-kb Sall fragment shown in Fig. 2 into the Sall site of pUC19. In pKMY277, a major portion of the multiple cloning site derived from pUC19 is located downstream from the T4MO genes (Fig. 2). Deletion of a 4.3-kb XmaI fragment of pKMY277 (Fig. 2) generated plasmid pKMY282. Plasmid pKMY287 was constructed by cloning a 4.7-kb XhoI fragment of pKMY282 containing the tmoABCDE genes into pCFM1146 (Fig. 2). Insertion of the same XhoI fragment into the Sall site of pUC19 generated pMY401. Cloning of a 2.6-kb HindIII fragment of pMY401 containing the tmoABCDE genes (Fig. 2) into pUC19 produced pMY419. Substitution of the XbaI-Asp 718 fragment of pKMY287 extending from coordinates 0.4 to 3.1 (Fig. 2) with a corresponding XbaI-Asp 718 fragment of pMY419 extending from coordinates 1.2 to 3.1 generated pMY421 (Fig. 2).

Several intermediate plasmids were involved in the construction of pMY437 (Fig. 2). Deletion of a 0.9-kb SspI fragment downstream from the tmoABCDE genes in pMY401 produced pMY424. Insertion of an XhoI linker into the SspI site of pMY424 generated pMY436. Substitution of an Asp 718-XhoI fragment of pMY424 extending from coordinates 3.1 to 5.1 with the corresponding Asp 718-XhoI fragment of pMY436 extending from coordinates 3.1 to 4.8 generated pMY437 (Fig. 2).

Construction of pMY448 (Fig. 2) involved using plasmids pMY476 and pKMY336. Insertion of the 0.8-kb HindIII fragment within the tmoE gene (Fig. 2) into the SmaI site of pUC19 produced pMY476. Substitution of the Asp 718-BamHI fragment of pMY421 extending from coordinates 3.1 to 5.1 with the longer Asp 718-BamHI fragment of pKMY282 extending from coordinates 3.1 to 5.9 produced pKMY336 (Fig. 2). Substitution of the EspI-BamHI fragment of pKMY336 extending from coordinates 3.9 to 5.9 with the EspI-BamHI fragment of pMY476 extending from coordinates 3.9 to 4.6 produced pMY448 (Fig. 2). Deletion of a 1.2-kb NcoI fragment from pMY436 generated a derivative of the tmoA gene in pKMY287 produced pMY449 (Fig. 2).

E. coli plasmids carrying a single mutation in a given T4MO gene were constructed by cloning the tmoABCDE genes into the plasmid pT7-5 and then introducing a DNA sequence change into the individual genes. Plasmid pKMY341, which carries the tmoABCDE genes, was constructed by cloning the entire insert in pKMY336 in an XbaI-BamHI fragment into the XbaI and BamHI sites of pT7-5. Plasmids pMY459, pMY458, pMY482, pMY484, and pMY472 carry mutations in the tmoA, tmoB, tmoC, tmoD, and tmoE genes, respectively. Cleavage of the NcoI site in the tmoA gene (Fig. 2) of pKMY341 followed by end filling and ligation generated pMY458. The same treatment at the NcoI site in the tmoB gene, at the Asp 718 site in the tmoC gene, and at the ClaI site in the tmoD gene generated the mutations in plasmids pMY458, pMY482, and pMY484, respectively (Fig. 2). Cleavage of the BamHI site in the tmoE gene (Fig. 2) followed by removal of the overhangs and ligation generated the mutation in plasmid pMY472.

Plasmids pMY438, pMY447, pMY474, pMY479, and pMY327 are derivatives of pKMY319 that carry the tmoA, tmoB, tmoC, tmoD, and tmoE genes, respectively. For the construction of pMY438, pMY430 was initially constructed.
by cloning a 1.6-kb Dral fragment extending from coordinates 1.2 to 2.8 and containing the tmoA gene (Fig. 2) into the Smal site of pUC19 in an orientation that placed the XbaI site of pUC19 at the 5' end of the tmoA gene. Plasmid pMY438 was constructed by cloning the 1.6-kb XbaI-SacI fragment of pMY430 carrying the tmoA gene into the XbaI and SacI sites of pKMY319.

Plasmids pKMY332 and pMY446 were used in the construction of plasmid pMY447. Plasmid pKMY332 was obtained by deleting a 2-kb Hpal-Xmal fragment of pKMY287 at the 5' end of the T4MO gene cluster (Fig. 2). Cloning of a 0.7-kb XbaI-Asp 718 fragment of pKMY332 extending from coordinates 2.4 to 3.1 and containing the tmoB gene (Fig. 2) into the XbaI and Asp 718 sites of pKMY319 produced pMY446. Further deletion of a 0.2-kb XbaI-Ndel fragment upstream of the tmoB gene in pMY446 (Fig. 2) generated pMY447.

Construction of plasmid pMY474 involved construction of intermediate plasmids pMY414, pMY426, pMY452, and pMY466. Cloning of a 0.4-kb Asp 718-ClaI fragment of pKMY282 extending from coordinates 3.1 to 3.5 and containing a major portion of the tmoC gene (Fig. 2) into the Asp 718 and AccI sites of pUC19 produced pMY414. Deletion of an ~0.1-kb BspMI-SphI fragment downstream of the tmoC gene (Fig. 2) generated pMY426. Ligation of a SacI linker to an end-filled HindIII site downstream of the tmoC gene in pMY426 and insertion of the Asp 718-ClaI fragment of this plasmid containing the tmoC gene into pKMY319 generated pMY452. Cloning of a 1.9-kb Clal-Asp 718 fragment of pKMY341 containing the tmoAB genes and the 5' end of the tmoC gene (Fig. 2) into the Clal and Asp 718 sites of pMY452 produced pMY466. Deletion of a 1.8-kb Clal fragment upstream of the tmoC gene in pMY466 generated pMY474.

Plasmids pMY404, pMY470, and pMY478 were used in the construction of plasmid pMY479. Cloning of the 4.7-kb BamHI-SphI fragment of pMY401 carrying the tmoABCDE
genes into plasmid pUC18 (40) produced pMY404. Deletion of the 3-kb BspMI fragment of pMY404 carrying the tmo-ABC genes (Fig. 2) generated pMY470. An approximately 0.4-kb HindIII fragment of pMY470 extending from coordinates 3.4 to 3.8 and carrying the tmoD gene (Fig. 2) was end filled and ligated along with another end-filled 0.8-kb HindIII fragment of pMY470 into the SmaI site of pUC19 to form plasmid pMY478. In pMY478, an Nhel site was generated upstream of the tmoD gene as a result of ligation of two end-filled 0.8-kb HindIII sites. Cloning of the 0.4-kb Nhel-SacI fragment of pMY478 carrying the tmoD gene into the XbaI and SacI sites of pKMY319 generated pMY479.

Plasmid pKMY327 was constructed from plasmid pKMY324. Cloning of the Xhol fragment of pKMY282 carrying the tmoABCDE genes into pKMY319 produced pKMY324. Deletion of a 3.1-kb ClaI fragment of pKMY324 extending from coordinates 0.4 to 3.5 generated pKMY327 (Fig. 2).

**Culture growth and induction conditions.** For heat-inducible strains carrying plasmid pCFM1146 or its derivatives, cells were initially grown in L broth containing kanamycin (50 μg/ml) at 30°C to an optical density at 550 nm (OD550) of 0.4 to 0.5. The cultures were then divided into two parts. One part was maintained at 30°C; the other part was transferred to a 42°C shaker and induced for 3 h. After induction, the culture was grown for 4 h at 30°C for the T4MO assay and for 17 h at the same temperature for the indigo assay.

For strains carrying a plasmid derived from pT7-5 and a second plasmid derived from pKMY319, the cultures were grown in L broth containing ampicillin (250 μg/ml), tetracycline (10 μg/ml), and sodium salicylate (0.35 mM) at 37°C to an OD550 of ~3.0 for the T4MO assay.

**Mutagenesis.** Mutants of *P. mendocina* KR1 able to grow on p-cresol and not on toluene were isolated after mutagenesis with N-methyl-N' -nitro-N-nitrosoguanidine (NG). KR1 cells were grown in 5 ml of L broth to an OD660 of ~0.7 and resuspended into 2 ml of 50 mM citrate buffer (pH 6.0) containing NG at a concentration of 0.1 mg/ml. After incubation at room temperature for 20 minutes, cells were washed twice with 2 ml of 1 M phosphate buffer (pH 7.0) and resuspended in L broth. They were then grown overnight and streaked on L-agar plates for single colonies. Colonies were picked and tested on TAS agar supplemented with toluene (70 μg/ml) for the desired mutants.

**Conjugation and transformation.** Plasmids were transferred from *E. coli* to *P. mendocina* cells by conjugation. *E. coli* HB101 carrying plasmid pRK2013 (11) was used as a helper strain to provide the transcribing function for the donor plasmid. L-broth suspensions of donor, recipient, and helper cells were washed to remove antibiotics and mixed in equal volumes. Small aliquots of the mixture were placed on L-agar plates to allow growth of all cell types. After overnight incubation at 30°C, the cells were plated on selection plates to select the desired transconjugates. Transformation of *E. coli* with plasmid DNA was done by the calcium chloride procedure originally described by Mandel and Higa (23).

**T4MO assay.** Late-log-phase cells were diluted in L broth to an OD660 of ~0.5 for the assay. The reactions were initiated by adding 15 nmol of 14C-toluene (40 to 60 mCi/ mmol; Sigma) to 0.5 ml of cells in L broth. After incubation at room temperature for 5 min, aliquots of 20 μl were spotted on small strips of a thin-layer chromatography plate. The plates were air dried at room temperature (22 ± 2°C) for 20 min and counted in a scintillation counter to determine the remaining radioactivity. T4MO activity is expressed as nanomoles of nonvolatile material produced from 14C-toluene per minute per milligram of whole-cell protein. The protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Cells were resuspended in 0.1 N NaOH and incubated in a boiling water bath for 20 min before protein determination.

Alternatively, the assays were performed on crude extracts of lysed cells as described by Whited and Gibson (36).

**Indigo assay.** The indigo assay was performed essentially as described previously (10) except that indole was added in each culture to a final concentration of 1 mM after induction.

**Isolation and manipulation of total KR1 DNA and plasmid DNA.** KR1 DNA was isolated as described by Dhaese et al. (8). Plasmid DNA was isolated as described by Johnston and Gunsalus (17). Enzymatic cleavage, modification, and ligation of DNA were done as specified by the enzyme suppliers.

**Determination of nucleotide sequences.** The nucleotide sequence of the 4.7-kb Xhol fragment carrying the T4MO genes was determined by the dyeoxy method of Sanger et al. (31) on double-stranded DNA, using the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). DNA samples were denatured in 0.2 M NaOH for 10 min and neutralized with 0.2 M ammonium acetate (pH 4.5) before use in the sequencing reactions. The 4.7-kb Xhol fragment and various deletion derivatives were cloned into the vector pUC19 or pUC18 for DNA sequencing. Both commercially available and synthetic primers were used for sequencing reactions.

**Purification and analysis of T4MO polypeptides.** T4MO polypeptides were partially purified from toluene-induced *P. mendocina* KR1 or temperature-induced *E. coli* FM5 carrying plasmid pMY421 by DEAE-cellulose chromatography as described by Whited and Gibson (36) except that TEDG buffer (50 mM Tris [pH 7.45], 10% glycerol, 10% ethanol, 1 mM dithiothreitol) instead of polyethylene glycol buffer was used in the column. Protein fractions collected from the column were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described below. Proteins that were overproduced or had molecular weights matching those of products predicted from the tmoABCDE gene cluster or those of T4MO components reported by Whited and Gibson (36) were further characterized by N-terminal amino acid sequence analysis to confirm their identities.

For determination of N-terminal amino acid sequences, partially purified T4MO polypeptides were further purified by SDS-PAGE and electroblotted onto polyvinyl difluoride membranes as described by Matsuda et al. (24), with slight modifications. The polypeptide bands immobilized on the polyvinyl difluoride membrane were visualized by Coomassie blue staining and destained with 50% methanol and 7% acetic acid. The stained bands were excised with a razor blade and sequenced in an Applied Biosystems model 477 automated protein sequencer as described elsewhere (22).

For isolation of TmoC protein and tryptic peptide mapping, partially purified TmoC protein was further purified by SDS-PAGE. The protein band was cut from the gel and eluted by electroelution as described previously (15). The eluted TmoC protein (approximately 10 μg) was subjected to trypsin digestion, and the obtained peptide mixture was separated by high-performance liquid chromatography (HPLC), using a C4 reverse-phase column (Vydac, 3-μm wide pore) and 0.1% trifluoroacetic acid acetonitrile elution gradient as described by Klein et al. (18). Peptide fractions were collected, and aliquots were loaded directly onto the
protein sequencer for N-terminal amino acid analysis as described above.

PAGE was performed essentially as described by Laemmli (20). Protein samples were heated at 65°C for 15 min in a loading buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8) before they were loaded on the gel.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *tmaABCDE* genes have been submitted to GenBank and assigned accession number M65106.

### RESULTS

**Cloning of the T4MO gene cluster.** The cloning strategy is based on complementation of cloned DNA fragments to a *P. mendocina* KR1 mutant that can utilize p cresol but not toluene as a carbon and energy source. Isolation of this mutant (Y4001) is described in Materials and Methods. In our enzyme assays, Y4001 exhibits no T4MO activity.

Total DNA was isolated from strain KR1 and digested with the restriction endonuclease *Bgl*II. Libraries of the digested DNA were constructed in *E. coli* HB101 (3) by using the broad-host-range vector pRK290 (9). Transformants of each library were pooled and transferred by conjugation to strain Y4001. Transconjugants that could grow on toluene vapor were identified, and the plasmid in each of the *Tol* + transconjugates was characterized. By this method, a 9.2-kb *Bgl*II insert was identified to contain genes capable of complementing Y4001 for growth on toluene. However, no T4MO activity could be detected from the pRK290 plasmid carrying this insert (pBY300; Fig. 2) in either *E. coli* HB101 or *P. putida* KT2440. Subcloning of this insert into *E. coli* expression vectors pUC18 and pUC19 (40) did not lead to the detection of any T4MO activity. It was concluded that the 9.2-kb *Bgl*II fragment does not contain the entire T4MO gene cluster.

Two sites for the restriction endonuclease *Sca*I were mapped close to one end of the 9.2-kb *Bgl*II fragment, and deletion of the 1.2-kb *Sca*I-*Bgl*II fragment at this end (Fig. 2) did not affect complementation of Y4001. This observation prompted us to clone *Sca*I fragments for locating the entire T4MO gene cluster. We constructed a pRK290 derivative, pKMY235, which contains a *Sca*I cloning site (Materials and Methods). KR1 total DNA was partially digested with *Sca*I and fractionated by size in a 10 to 40% sucrose density gradient. To locate the fractions containing T4MO genes, sample DNA fragments from each of the fractions were tested for hybridization to radioactively labeled pUC19 carrying the 9.2-kb *Bgl*II fragment. Fractions that contained materials capable of hybridizing to the plasmid probe were identified. DNA fragments from these fractions were randomly cloned into the *Sca*I site of plasmid pKMY235 to construct libraries in *E. coli* HB101. Genetic complementation of the *Tol* + mutant Y4001 was again used to select recombinant plasmids carrying T4MO genes. In this experiment, we identified a plasmid containing a 20.4-kb insert, pKMY266, which allowed the utilization of toluene by Y4001. Despite the complementation pattern, no T4MO activity could be detected from pKMY266. The insert in pKMY266 consists of two different *Sca*I fragments of identical size. One of the 10.2-kb *Sca*I fragments, when cloned into pUC19 (pKMY277) and expressed in *E. coli*, led to the synthesis of a blue pigment which is chlorofom soluable and water insoluble and has maximum absorption of light at a wavelength of approximately 600 nm. Production of the blue pigment was also observed from *P. putida* cells harboring pKMY266 and was dependent on the presence of indole. This pigment was tentatively identified as indigo. A low level of T4MO enzyme activity (1 nmol/min/mg) was detected from *E. coli* JM109 carrying pKMY277. As shown below, indigo formation is catalyzed by the T4MO enzyme system.

**Mapping of the T4MO genes.** The region of the 10.2-kb *Sca*I fragment essential for T4MO activity was determined by deletion mapping. Deletion mapping was accompanied by DNA sequencing (Materials and Methods) to reveal restriction sites. Various regions of the *Sca*I fragment (Fig. 2) were cloned individually into *E. coli* expression vector pCFM1146 (Materials and Methods), which can express foreign genes from a heat-inducible phage lambda *pL* promoter. Each of the recombinant plasmids was introduced into *E. coli* FMI5 (Materials and Methods), and the resulting strains were assayed for T4MO activity under induced and uninduced conditions.

Inducible T4MO activity was observed from a strain carrying recombinant plasmid pKMY287, pMY437, or pMY421 but not from the strain carrying pMY429 or pMY448 (Fig. 2; Table 1). This result and the orientation of each insert in pKMY287, pMY437, or pMY421 with respect to the lambda *pL* promoter indicated that the T4MO genes are transcribed from left to right, as indicated on the map shown in Fig. 2. The nucleotide sequences of the T4MO genes and the N-terminus amino acid sequences of the gene products (see below) also confirmed the transcription direction of the T4MO genes. Among the three plasmids having T4MO activity, pMY437 carries the smallest insert, a 3.6-kb fragment defined by the recognition sites of HindIII and *Ssp*I (Fig. 2). The T4MO genes are therefore located between these two sites on the 10.2-kb *Sca*I fragment.

Under induced conditions, plasmids pKMY287 and pMY437 gave similar levels of T4MO activity and plasmid pMY421 gave slightly higher T4MO activity (Table 1). Under uninduced conditions, a significantly higher residual T4MO activity was observed from pKMY287 than from pKMY437 and pMY421 (Table 1). We cannot explain the slightly higher

| Table 1. T4MO and indigo-forming activities of recombinant *E. coli* plasmids carrying different KR1 DNA fragments |
|-------------------------------------------------|-----------------|-----------------|
| Plasmid*                                      | T4MO sp act‡ (nmol/min/mg of protein) | Indigo formation (µg/mg of protein) |
| pCFM1146, induced                             | 0.1             | 0.1             |
| pMY429                                        | 0.1             | 0.1             |
| Uninduced                                     | 0.1             | 0.2             |
| Induced                                       | 0.1             | 0.1             |
| pMY448                                        | 0.1             | 0.1             |
| Uninduced                                     | 0.1             | 0.1             |
| Induced                                       | 0.1             | 0.1             |
| pKMY287                                       |                |                 |
| Uninduced                                     | 2.0             | 8.4             |
| Induced                                       | 7.0             | 21              |
| pMY437                                        |                |                 |
| Uninduced                                     | 0.5             | 2.1             |
| Induced                                       | 7.3             | 26              |
| pMY421                                        |                |                 |
| Uninduced                                     | 0.9             | 2.6             |
| Induced                                       | 10              | 29              |

* Each plasmid except pCFM1146 is the *E. coli* expression vector pCFM1146 carrying T4MO genes. The different inserts in these plasmids are shown in Fig. 2.
‡ Values in toluene-induced and uninduced KR1 cells were 30 and 0.5 nmol of nonvolatile material formation from toluene per min per mg of protein, respectively.
T4MO activity observed for pMY421 than for pMY437. The T4MO activity associated with pKMY287 might be the result of two opposing forces operating simultaneously on expression of the T4MO genes from this plasmid. A DNA fragment of 800 bp is present between the promoter and the T4MO genes in pKMY287 but not in pKMY437 or pKMY421 (Fig. 2). This fragment might reduce transcription or translation efficiency of the T4MO genes on one hand and provide a secondary promoter for constitutive expression of the T4MO genes on the other hand.

There is a correlation between the presence of T4MO activity and the indigo-producing capability among the strains shown in Table 1. Indigo was produced only in strains with T4MO activity; the amounts of indigo produced could be approximately correlated with the levels of T4MO activity in indigo-producing strains (Table 1). As shown below in Fig. 2, the indigo-plus strains all contain the intact T4MO gene cluster, and each of the indigo-minus strains is lacking a T4MO component gene. Indigo production can therefore serve as a good indicator for the presence of the T4MO gene cluster when these genes are investigated.

**Nucleotide sequences of the T4MO genes.** The XhoI-XhoI DNA region extending from coordinates 0.4 to 5.1 on the map shown in Fig. 2 was sequenced (Materials and Methods) in its entirety in both orientations. The nucleotide sequence corresponding to the HindIII-SspI region demonstrated to give T4MO activity is presented in Fig. 3. Five open reading frames were identified in this region. Each of the open reading frames was confirmed by determining the N-terminal amino acid sequence of the corresponding gene product produced in *E. coli* from pMY421 and by cloning each of the regions containing an open reading frame and demonstrating
corresponding activity (see below). The genes defined by these open reading frames were tentatively designated (in order of transcription) tmoA, tmoB, tmoC, tmoD, and tmoE (Fig. 2). Comparison of amino acid sequences between some of the polypeptides encoded by the tmo genes and the products of other known genes is presented in Discussion.

Immediately upstream from the tmo gene cluster are two putative open reading frames running in the same direction as the tmo genes (sequence not shown). The first one begins with the trinucleotide ATG, extends a potential coding distance of 157 amino acids, and ends 29 base upstream of the tmoA gene. The second one begins presumably upstream of the sequenced region, covers a potential coding region of 225 amino acids within the sequenced region, and ends 14 base upstream of the first putative open reading frame. A computer search of GenBank has detected no significant homology between the two putative opening reading frames and any known gene. Whether these open reading frames encode protein products is not yet known. Downstream of the tmo gene cluster is a G+C-rich region of dyad symmetry followed by a series of thymidine residues (Fig. 3), a structure characteristic of a rho-independent transcription terminator (29).

The base composition of the tmoABCDE cluster is unusual for P. mendocina genes. The G+C content of the DNA fragment presented in Fig. 3 is 48.9%. This low value is significantly different from the reported G+C content of 62.8 to 64.3% for the P. mendocina genome (28). Consistent with the low G+C content, there is no codon usage preference for guanine and cytosine at the third position of each codon in the tmo genes (data not shown). The P. mendocina KR1 strain used in this study was initially typed in D. T. Gibson's laboratory. We have retypied and confirmed the identity of this strain. A simple explanation of the low G+C content of the tmo genes is that these genes originated from another bacterial species and were later transferred into P. mendocina on a plasmid. Consistent with such a hypothesis is the observation that the toluene-utilizing property can be transferred from P. mendocina KR1 to P. putida KT2440 (42).

Characterization of polypeptides encoded by the tmoABCDE gene cluster. To confirm the open reading frames identified from nucleotide sequences, the products of the tmoABCDE genes synthesized from plasmid pMY421 in E. coli were partially purified and their N-terminal amino acid sequences were determined (Materials and Methods). Following fractionation of total proteins by DEAE-cellulose chromatography, the polypeptides encoded by the tmo gene cluster were further purified by SDS-PAGE. The five polypeptides contained in the appropriate fractions collected from the DEAE-cellulose column and the total proteins produced in temperature-induced E. coli cells carrying plasmid pMY421 are shown in Fig. 4. The five tmo gene products were transferred from the SDS-polyacrylamide gel to a polyvinyl difluoride membrane, and their N-terminal amino acid sequences were determined (Materials and Methods). The N-terminal amino acid sequence for each of the polypeptides agrees completely with that predicted from the corresponding open reading frame (Table 2). The five open reading frames thus define five genes. In addition, this result indicated that other than being stripped of the initiating methionine residue, none of the tmo gene products is processed at the N terminus after synthesis. The close match between the observed molecular weights of the tmo gene products and the molecular weights of polypeptides predicted from the tmoABCDE sequences
also suggested a lack of major processing at the other parts of each tmo gene product (Table 2; see below).

The nucleotide sequence of the tmoC gene has the capacity to encode a polypeptide with a molecular weight of approximately 12,000 (Table 2). However, on SDS-PAGE the observed molecular weight of the TmoC protein is approximately 25,000 (Table 2; Fig. 4), almost exactly twice the size of the expected tmoC product. A 25-kDa protein was also isolated from P. mendocina KR1 (Materials and Methods) and identified as being the same 25-kDa TmoC protein isolated from the recombinant E. coli by comparing the N-terminal amino acid sequences. Further characterization of the 25-kDa protein demonstrated that it is encoded solely by the tmoC gene. This protein was partially purified from E. coli FM5 carrying plasmid pMY421 and further purified from an SDS-polyacrylamide gel (Fig. 4). It was subsequently eluted from the gel and subjected to trypsin digestion. The resulting peptide mixture was separated into individual peptides by HPLC. Aliquots from each peptide fraction were directly loaded onto the protein sequencer for sequence analysis. A total of seven peptide fragments were isolated (Fig. 5) and sequenced. As shown in Table 3, the amino acid sequences of the seven peptides completely match the amino acid sequences predicted from the various parts of the tmoC sequence. Peptides 4 and 5 gave identical amino acid sequences, as did peptides 6 and 7 (Table 3). In each case, the observed difference in retention time between the peptides (Fig. 5) could result simply from different oxidation states of labile amino acids, such as methionine and tryptophan, present in the otherwise identical peptides. The result shown in Table 3 demonstrated that all of the analyzed tryptic peptides of the 25-kDa protein were the cleavage products of the TmoC protein. We concluded that the observed 25-kDa protein was a dimer of the tmoC product not fully reduced under the conditions used for SDS-PAGE. In addition, the tryptic peptide data (Table 3) and the N-terminal acid sequence of the tmoC product (Table 2) completely established the amino acid sequence of the TmoC protein and confirmed the nucleotide sequence of tmoC gene.

Identification of tmoABCDE as the T4MO genes. To determine whether each of the tmoABCDE genes is essential for T4MO activity, a single mutation was introduced into each
of the genes and its effect on T4MO activity was studied. Plasmid pKMY341 was initially constructed by cloning the tmoABCDE genes into the E. coli plasmid pT7-5 (Materials and Methods). In pKMY341, the tmoABCDE genes were expressed from the promoter of β-lactamase gene in pT7-5. Plasmids pMY459, pMY458, pMY482, pMY484, and pMY472, which contain mutations in the tmoA, tmoB, tmoC, tmoD, and tmoE genes, respectively, were subsequently constructed from pKMY341 (Materials and Methods). Enzyme assays revealed that each of the mutations completely eliminated T4MO activity in E. coli cells (Table 4). If any of the tmoABCD genes does not encode a T4MO polypeptide and is thus not a T4MO gene, a mutation in this gene with a polar effect still could affect the expression of any downstream T4MO gene(s) and thus abolish T4MO activity. To determine whether this is the case, complementation tests between plasmids pMY459, pMY458, pMY482, pMY484, and pMY472 and plasmids carrying only one of the tmoABCDE genes were conducted. Plasmids pMY438, pMY447, pMY474, pMY479, and pKMY327 were constructed to contain the tmoA, tmoB, tmoC, tmoD, and tmoE genes, respectively, in the broad-host-range expression vector pKMY319 (41). Each of these plasmids was introduced by transformation into a strain that contained a corresponding member of plasmids pMY459, pMY458, pMY482, pMY484, and pMY472, and the T4MO activity in each of the resulting strains was determined after induction. A plasmid carrying a mutation in any member of the tmoABCDE gene cluster was complemented by the plasmid that carries that particular

![FIG. 5. Reverse-phase HPLC peptide map obtained from a tryptic digest of electroeluted TmoC protein. A sample of approximately 10 µg was digested, and the resulting peptide mixture was separated by HPLC. Peptide fractions 1 to 7 were collected and sequenced.](http://jb.asm.org/)

TABLE 3. Amino acid sequences of tryptic peptides of the 25-kDa TmoC protein produced from E. coli FM5 carrying plasmid pMY421

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GDDIYVSTK</td>
</tr>
<tr>
<td>2</td>
<td>GILPNKAHS</td>
</tr>
<tr>
<td>3</td>
<td>AYQAMCPHEILLSESGYEGWITCR</td>
</tr>
<tr>
<td>4 and 5</td>
<td>AHLWTGNDTGHGINPOCCLAEYPVEVK</td>
</tr>
<tr>
<td>6 and 7</td>
<td>ICSLDDEWVDEMTFEDTSGDTEVEVNSEEHGVK</td>
</tr>
</tbody>
</table>

TABLE 4. Complementation between individually cloned tmo genes and the tmo gene clusters carrying corresponding mutations

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Plasmid</th>
<th>tmo gene(s)</th>
<th>T4MO sp act (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>In vivo</td>
</tr>
<tr>
<td>Y5246</td>
<td>pKMY341</td>
<td>ABCDE</td>
<td>10.7</td>
</tr>
<tr>
<td>Y5283</td>
<td>pMY459</td>
<td>A B C D E</td>
<td>0.06</td>
</tr>
<tr>
<td>Y5282</td>
<td>pMY458</td>
<td>A B D E</td>
<td>0.04</td>
</tr>
<tr>
<td>Y5286</td>
<td>pMY482</td>
<td>A B C D E</td>
<td>0.08</td>
</tr>
<tr>
<td>Y5287</td>
<td>pMY484</td>
<td>ABC D E</td>
<td>0.05</td>
</tr>
<tr>
<td>Y5285</td>
<td>pMY482</td>
<td>ABC D E</td>
<td>0.10</td>
</tr>
<tr>
<td>Y5258</td>
<td>pMY438</td>
<td>A</td>
<td>0.09</td>
</tr>
<tr>
<td>Y5265</td>
<td>pMY447</td>
<td>B</td>
<td>0.03</td>
</tr>
<tr>
<td>Y5288</td>
<td>pMY474</td>
<td>C</td>
<td>0.04</td>
</tr>
<tr>
<td>Y5289</td>
<td>pMY479</td>
<td>D</td>
<td>0.02</td>
</tr>
<tr>
<td>Y5224</td>
<td>pKMY327</td>
<td>E</td>
<td>0.04</td>
</tr>
<tr>
<td>Y5289</td>
<td>pMY459</td>
<td>A B C D E</td>
<td>2.0</td>
</tr>
<tr>
<td>Y5290</td>
<td>pMY447</td>
<td>B A B C D E</td>
<td>3.9</td>
</tr>
<tr>
<td>Y5297</td>
<td>pMY474</td>
<td>C A B C D E</td>
<td>7.4</td>
</tr>
<tr>
<td>Y5298</td>
<td>pMY479</td>
<td>D A B C D E</td>
<td>2.0</td>
</tr>
<tr>
<td>Y5292</td>
<td>pKMY327</td>
<td>A B C D E</td>
<td>10.3</td>
</tr>
</tbody>
</table>

a Each strain was constructed by introducing an appropriate plasmid or plasmids into E. coli HB101. All cultures were grown in the presence of 0.35 mM sodium salicylate, which induced the T4MO genes cloned into pKMY319 (Materials and Methods).

b Construction of all plasmids is described in Materials and Methods.

c Genes designated with a minus sign are inactivated by mutations.

d ND, not determined.
gene in synthesizing the T4MO enzyme (Table 4). Plasmid pairs pMY474-pMY482 and pKMY327-pMY472 gave higher levels of complementation than did other complementing pairs (Table 4). The underlying basis for the differences was not investigated. Several possibilities exist. For example, in the construction of plasmids carrying a single \textit{tmo} gene, a secondary promoter or DNA sequence affecting translation of mRNA might be fortuitously introduced upstream of the \textit{tmo} gene in some plasmids. This would affect the expression level of a particular \textit{tmo} gene, which in turn could lead to a higher or lower level of complementation. Despite the different levels of complementation for different plasmid pairs, the data in Table 4 demonstrated that in each case the two complementing plasmids together gave a T4MO activity 20- to 100-fold higher than the background level given by each plasmid of the complementing pair. This result demonstrated that the mutation in each of the \textit{tmo} genes did not abolish the expression of the downstream \textit{tmo} gene(s). Since each of the mutations prevented the synthesis of a functional T4MO system, each of the \textit{tmoABCD} genes plays an essential role in directing the synthesis of the T4MO enzyme system. The fact that expression of each \textit{tmo} gene from a foreign promoter in \textit{E. coli} is essential for T4MO synthesis indicated that none of the \textit{tmo} genes functions solely to regulate the expression of the \textit{tmo} gene cluster.

To determine whether any of the \textit{tmoABCD} genes controls the transport of substrate for the T4MO enzyme system, an alternative method was used to measure the T4MO activity from \textit{E. coli} strains carrying each of the plasmids pKMY341, pMY459, pMY458, pMY482, pMY484, and pMY472. Crude extracts instead of whole cells of each strain were assayed for T4MO activity (Materials and Methods). As shown in Table 4, the in vitro (crude extract) T4MO activity of the strain carrying plasmid pKMY341 was in the same order of magnitude as that of strain KR1 measured by the same method (36) and was an order of magnitude lower than its in vivo (whole-cell) activity. Nevertheless, the effect of mutation within each of the \textit{tmoABCD} genes in abolishing T4MO activity remained unchanged in the in vitro T4MO assay (Table 4). This result demonstrated that each of the \textit{tmoABCD} genes remained essential for synthesis of the T4MO enzyme system even in the absence of substrate transport across the cell envelope. It was thus clear that none of the \textit{tmo} genes functions to control the transport of substrate for the T4MO enzyme system. Since none of the \textit{tmo} genes functioned to regulate the expression of the \textit{tmoABCD} cluster or control major processing of the products of this gene cluster (see above), it was tentatively concluded that each of the \textit{tmoABCD} genes encodes a structural polypeptide of the T4MO enzyme system.

**DISCUSSION**

In this study, we have cloned and sequenced five genes, \textit{tmoABCD}, from \textit{P. mendocina} KR1 and demonstrated that each of the genes is essential for the synthesis of T4MO in \textit{E. coli}. None of these genes functioned to control the expression or control transport of substrates across the cell envelope, nor was any of the genes involved in major processing of the products of the \textit{tmo} gene cluster (see Results). It was tentatively concluded that these five genes encode structural polypeptides of the T4MO enzyme system.

The definitive function of each of the \textit{tmo} genes is not known. However, there is evidence at least to suggest a role for the \textit{tmoC} gene. Comparison of the amino acid sequence deduced from the \textit{tmoC} gene with those of known ferredoxin proteins reveals homology between the \textit{tmoC} product and several other ferredoxins functioning in dioxygenase systems. Among the 114 amino acid residues in the \textit{tmoC} protein, 36 residues (31.6%) are identical to those of the benzene dioxygenase ferredoxin protein at corresponding positions and 14 residues (12.3%) are represented by evolutionarily related amino acids in the benzene dioxygenase ferredoxin at corresponding positions (Fig. 6). Similar homology exists between the \textit{tmoC} protein and the naphthalene dioxygenase ferredoxin protein (Fig. 6). The ferredoxin component of the toluene dioxygenase system from \textit{P. putida} F1 differs from the benzene ferredoxin protein by only six amino acid residues (46). It therefore shares similar homology with the \textit{tmoC} protein. The region of maximum homology between the \textit{TmoC} protein and the other ferredoxins is located between positions 53 and 77 (Fig. 6). Among the 23 amino acid residues in this region, the \textit{TmoC} protein shares 10 (43%) with the benzene dioxygenase ferredoxin and 9 (39%) with the naphthalene dioxygenase ferredoxin (Fig. 6). In addition, the two dioxygenase ferredoxins share 13 (56.5%) amino acids in this region (Fig. 6). It is interesting to note that this region contains two conserved cysteine residues (at positions 53 and 74, respectively), each of which is followed by a conserved histidine in the vicinity. Benzene dioxygenase ferredoxin (12) and toluene dioxygenase ferredoxin (34) each contain a single [2Fe-2S] cluster with a redox potential significantly higher than those of [2Fe-2S] clusters coordinated to four cysteine residues. Cline et al. (6) have suggested that in addition to cysteines, histidine residues may provide nitrogen ligands to the [2Fe-2S] cluster, which may contribute to the higher redox potential of the cluster. It is likely that this region of maximum homology between the two dioxygenase ferredoxins is involved in the binding of the [2Fe-2S] cluster. The fact that the \textit{TmoC} protein shares overall homology with these
two ferredoxins and does so especially in this region suggests that it is a ferredoxin of the toluene monoxygenase system.

The complete amino acid sequence of the tmoC product was determined in this study (Tables 2 and 3). This polypeptide has a molecular weight of ~12,000. The TmoC protein was isolated in our experiments as a dimer of ~25 kDa which remained unaltered by SDS-PAGE. The TMO system of P. mendocina K1 has been resolved into three functional components by White and Gibson (36). One of the components (component C) was a 23-kDa protein that had spectroscopic properties similar to those of ferredoxins purified from toluene dioxygenase and benzene dioxygenase and was proposed to function as a ferredoxin in the TMO system (36). From the characteristics of the TmoC protein and its gene sequence, it is almost certain that the 23-kDa protein is a dimer of the tmoC product.

The properties of the three partially purified functional components of the TMO system allowed White and Gibson (36) to propose an electron flow in this enzyme system from NADH to a flavin-containing NADH:ferredoxin oxidoreductase, to a ferredoxin protein, to a colorless iron-containing oxygenase. The putative oxygenase component (component B) was observed to contain at least two subunits with molecular weights of 50,000 and 32,000 (36). The molecular weights of these two subunits approximately match the molecular weights of the tmoA and tmoE products (Table 2). The two subunits of the putative oxygenase component of the TMO system thus could be encoded by the tmoA and tmoE genes.

The least-characterized functional component of the TMO system (component A) is the putative NADH:ferredoxin oxidoreductase (36). The molecular weight of this component is not known, nor is it clear whether it contains subunits.

Despite the similarity between the TmoC protein and the ferredoxin proteins from the benzene dioxygenase, toluene dioxygenase, and naphthalene dioxygenase systems, no significant sequence homology was detected between tmoA and tmoE and the TMO system genes encoding other components in these dioxygenase systems (16, 32, 46). Recently, a multicomponent monooxygenase system from Pseudomonas sp. strain CF600 which consists of five polypeptides (DmpLM-NOP) and catalyzes hydroxylation of phenol to form cate-
chol (phenol hydroxylase) was described (27, 30). Several polypeptides of this phenol hydroxylase system share homology with the polypeptides of T4MOO system. Comparison of amino acid sequences between polypeptides TmoA and DmpN, TmoD and DmpM, and TmoE and DmpL is shown in Fig. 7. In TmoA, 26% of the amino acid residues are found at corresponding positions in DmpN and another 24% of the amino acid residues are represented by related amino acids at corresponding positions in DmpN. In both TmoA and DmpN, hydrophilic regions between positions 1 and 100 and between positions 350 and 400, consisting mainly of charged amino acids, and a hydrophobic region centering around position 200, consisting mainly of uncharged amino acids, can be detected. A higher degree of homology was observed between polypeptides TmoD and DmpM. The percentages of amino acid residues in TmoD which are identical and similar to those of DmpM are 30.4 and 30.4%. The degrees of complete homology and similarity of polypeptide TmoE to polypeptide DmpL are 22.3 and 19.9%. In both TmoE and DmpL, a large hydrophilic region extending from the N terminus to slightly beyond position 100 can be easily seen. A number of small hydrophilic regions scattered between position 100 and the C terminus also occur at corresponding positions in TmoE and DmpL. The functions of polypeptides DmpL, DmpM, and DmpN are unknown. If TmoA and TmoE indeed represent two subunits of the oxygenase component in the T4MOO system, DmpN and DmpL polypeptides could play a similar role in the phenol hydroxylase system. We have not detected significant homology between other gene products of these two enzyme systems.

Expression of the tmoABCDE genes from a foreign promoter (Table 1) and the nucleotide sequences of these genes (Fig. 2) indicated that these five genes are in a single transcription unit. Whether there are other genes of the same pathway in this transcription unit is not clear. We have not investigated the nature of the two putative open reading frames upstream of the tmoA gene. The putative transcription termination sequence located downstream of the tmoABCDE gene cluster does not necessarily define the end of a transcription unit. DNA sequences similar to that found in a transcription terminator are also located downstream of the nahA gene cluster on the NAH7 plasmid (32). However, nahBCDEF genes are expressed coordinately with the nahA gene cluster in the same transcription unit (43), presumably by transcriptional read-through.

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REFERENCES

24. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electrophoblted onto polyvinylidene difluoride mem-
42. Yen, K.-M. Unpublished data.