Identification of a New Gene, tmoF, in the Pseudomonas mendocina KR1 Gene Cluster Encoding Toluene-4-Monoxygenase

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Five genes, tmoABCDE, encoding toluene-4-monoxygenase (T4MO) were previously mapped to a 3.6-kb region of a 10.2-kb SacI DNA fragment isolated from Pseudomonas mendocina KR1 (K.-M. Yen, M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen, J. Bacteriol. 173:5315–5327, 1991). In this report, we describe the identification and characterization of a DNA region in the SacI fragment whose expression enhances the T4MO activity determined by the tmoABCDE gene cluster. This region was mapped immediately downstream of the putative transcription termination sequence previously located at the end of the tmoABCDE gene cluster (Yen et al., J. Bacteriol., 1991) and was found to stimulate T4MO activity two- to threefold when expressed in Escherichia coli or Pseudomonas putida. Determination of the nucleotide sequence of this region revealed an open reading frame (ORF) of 978 bp. Expression of the ORF resulted in the synthesis of an ~37-kDa polypeptide whose N-terminal amino acid sequence completely matched that of the product predicted from the ORF. The ORF thus defines a gene, which has now been designated tmoF. The TmoF protein shares amino acid sequence homology with the reductases of several mono- and dioxygenase systems. In addition, the reductase component of the naphtalene dioxygenase system, encoded by the nahA gene of plasmid NAH7 from P. putida G7, could largely replace the TmoF protein in stimulating T4MO activity, and TmoF could partially replace the NahA protein in forming active naphthalene dioxygenase. The overall properties of tmoF suggest that it is a member of the T4MO gene cluster and encodes the NADH:ferredoxin oxidoreductase of the T4MO system.

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A monoxygenase system in Pseudomonas mendocina KR1 allowing the hydroxylation of toluene and formation of p-cresol was recently reported (23). This toluene-4-monoxygenase (T4MO) system has been resolved into three functional components (13, 14). Properties of these three components suggested an electron flow in this enzyme system from NADH to a flavin-containing NADH:ferredoxin oxidoreductase, to a ferredoxin protein, and finally to a colorless iron-containing oxygenase (23). A similar functional arrangement of three components has been reported for the well-characterized naphthalene dioxygenase (NDO) system in Pseudomonas putida NCIB 9816 (5, 8, 9) and toluene dioxygenase system in P. putida F1 (19–21). Structurally, four polypeptides are required to assemble each of these two enzyme systems, in which each system the oxygenase component consists of two subunits (17, 29). Interestingly, five polypeptides are required for the assembly of the T4MO system in Escherichia coli (28). The complexity of the T4MO enzyme system, however, is not unique. Two other monoxygenase systems are known to consist of five polypeptides, namely, the methane monoxygenase (MMO) system in Methylococcus capsulatus (Bath), which catalyzes the oxidation of methane to methanol (4), and the phenol hydroxylase (DMP) system in Pseudomonas sp. strain CF600, which catalyzes the hydroxylation of phenol and some of its methylated derivatives to catechol and related compounds (13, 14). One of the T4MO proteins has been tentatively identified as a ferredoxin on the basis of its spectrophotometric properties (23) and its amino acid sequence homology with the ferredoxin proteins from the NDO, toluene dioxygenase, and benzene dioxygenase systems (28). Several other T4MO polypeptides have been shown to share amino acid sequence homology with some DMP components (28). Two of the T4MO polypeptides might be subunits of the oxygenase component of the T4MO system (28). The structure of the T4MO NADH:ferredoxin oxidoreductase was completely unknown (23, 28).

Five T4MO genes, tmoABCDE, were previously mapped to a 3.6-kb region of a 10.2-kb SacI DNA fragment (Fig. 1) isolated from P. mendocina KR1 (28). Downstream from this gene cluster, a DNA sequence reminiscent of a rho-independent transcription terminator was located (28). In this report, we describe the identification and characterization of a new gene, tmoF, whose expression enhances T4MO activity. We have mapped this gene immediately downstream of the putative transcription termination sequence at the end of the tmoABCDE cluster and determined its nucleotide sequence. The properties of tmoF suggest that it encodes the NADH:ferredoxin oxidoreductase component of the T4MO system.

MATERIALS AND METHODS

Construction of plasmids carrying the T4MO gene(s). Cloning vectors pCFM1146 and pKMY319 and plasmids pMY421, pKMY277, pKMY341, and pKMY336 (Fig. 1) have been described previously (26, 28). Construction of plasmid pKMY340 (Fig. 1) involved the use of plasmids pKMY277 (Fig. 1) and pMY421. Deletion of a 2.3-kb BamHI fragment of pKMY277 extending from coordinates 7.9 to 10.2 (Fig. 1) generated a plasmid designated pKMY280 (Fig. 1). Replacement of the 2.3-kb region of pMY421 extending from coordinates 3.1 to 5.1 (Fig. 1) in an Asp718-BamHI fragment with the 4.8-kb Asp718-BamHI fragment of
FIG. 1. Restriction map of a *P. mendocina* KR1 DNA fragment carrying the *tmo* genes, *tmoABCDEF*, and plasmid constructs containing various parts of this fragment. The sizes and transcriptional directions of the *tmo* genes are marked with arrows. The lines underneath the restriction map denote regions of the *P. mendocina* KR1 DNA fragment contained in the plasmids indicated. Plasmids pMY601 and pMY603 also contain the *nahA* gene at the locations indicated. The size of *nahA* is shown, and its transcriptional direction is indicated by an arrow.

pKMY280 extending from coordinates 3.1 to 7.9 (Fig. 1) produced pKMY340. Plasmid pKMY321 (Fig. 1) was constructed by cloning the entire insert of pMY421 as an *XbaI*-*XhoI* fragment into pKMY319. Plasmid pMY486 (Fig. 1) was generated by cloning the entire insert of pKMY341 (Fig. 1) as an *XbaI*-*SacI* fragment into pKMY319.

Construction of plasmid pMY617 involved a number of intermediate plasmids. Deletion of a *HindIII* fragment from the 5' end of the insert in pKMY341 generated plasmid pMY460 (Fig. 1). Cloning of the *SspI* fragment of pKMY336 extending from coordinates 4.8 to 5.2 (Fig. 1) into the *HpaI* site of the *E. coli* cloning vector pCFM4722 (1) produced plasmid pMY497 (Fig. 1). In pMY497, the nucleotide sequence shown in Fig. 2 was generated at the junction between the 5' end of the *tmoF* sequence derived from pKMY336 and the pCFM4722 sequence. This resulted in the replacement of the coding sequence for the ribosome binding site (RBS) of *tmoF* mRNA and the three N-terminal amino acids, MFN, of the *TmoF* polypeptide with a DNA sequence encoding a new ribosome binding site and the amino acid sequence MV (Fig. 2). Replacement of the 5' end of the *HindIII*-*XhoI* region of the insert of pMY460 as a *ClaI*-*XhoI* fragment with the ~0.3-emb *ClaI*-*XhoI* fragment of pMY497 containing the modified 5' end of *tmoF* generated a modified *tmoF* gene, *tmoF*+, in a plasmid designated pMY498 (Fig. 1). Elimination of the *EcoRI* site downstream of *tmoF*+ in pMY498 by *EcoRI* digestion, filling in the ends, and blunt-ending ligated generated plasmid pMY613. Insertion of an *EcoRI* linker at the *XbaI* site upstream of *tmoF*+ in pMY613 produced plasmid pMY614. Insertion of an *XbaI* linker at the *ClaI* site upstream of the created *EcoRI* site in pMY614 generated plasmid pMY615. Insertion of an *EcoRI* linker at the *PpuMI* site of pKMY336 (Fig. 1) between *tmoE* and *tmoF* generated plasmid pMY499. Insertion of the pMY499 region extending from coordinates 1.2 to 4.7 (Fig. 1) as an *XbaI*-*EcoRI* fragment into the *XbaI* and *EcoRI* sites of pMY615 produced plasmid pMY616 (Fig. 1). In pMY616, a new *tmoABCDEF*+ gene cluster was assembled in which the putative transcription terminator between *tmoE* and *tmoF* had been deleted and the 5' end of *tmoF* had been modified. Cloning of the *tmoABCDEF*+ cluster as an *XbaI*-*SacI* fragment of pMY616 into the *XbaI* and *SacI* sites of pKMY319 generated plasmid pMY617.

Construction of plasmids carrying the *NDO* gene(s). The *nahA*, *A*, *A*, and *nahA* genes were derived from recombinant plasmids pACG1 and pACG8 obtained from C. Serdar.
A NEW MEMBER OF P. MENDEOCINA KIRI TANGO GENES

FIG. 3. Restriction map of a region of plasmid PAC1 containing the NDO cluster derived from plasmid NAHT of P. paludis 67 and Plasmid NAHT of P. paludis 67 and plasmid contains containing various parts of the region. The sizes and transcripntional direction of the operons are marked by arrows. The three underlined restriction maps other regions are marked by arrows. The line underlined restriction maps other regions are marked by arrows. The two underlined restriction maps other regions are marked by arrows.

The size of this gene is shown, and the transcriptional direction is indicated with an arrow.
and D. Murdock at Amgen. pACG1 is an E. coli pACI plasmid (15) carrying the NDO gene cluster of plasmid NAH7 (17) (Fig. 3), and pACGS is a derivative of pACG1 with part of the nahA\textsubscript{4} gene and the nahA\textsubscript{4}A\textsubscript{4} genes located downstream of the XhoI site at coordinate 2.1 deleted (Fig. 3).

Construction of plasmid pMY603 (Fig. 1), which contains the nahA\textsubscript{4} gene, involved construction of plasmids pMY496 and pMY601. Deletion of the nucleotide sequence between coordinates 0.05 and 0.6 of pACG8 (Fig. 3) by digestion with SacII, blunting the end, attachment of an EcoRI linker, redigestion with EcoRI, and ligation generated plasmid pMY496 (Fig. 3). Replacement of the EcoRI-XhoI fragment of pMY499 extending from coordinates 4.7 to 5.1 (Fig. 1) with the EcoRI-XhoI fragment of pMY496 extending from coordinates 0.6 to 2.1 (Fig. 3) produced plasmid pMY601 (Fig. 1). Cloning of the region of pMY601 carrying the tmoABCDE-nahA\textsubscript{4} gene cluster as an XbaI-XhoI fragment into pKMY319 generated pMY603. Construction of plasmid pMY619 (Fig. 3), a derivative of pKMY319 (26) carrying the tmoF\textsuperscript{*}-nahA\textsubscript{4}A\textsubscript{4} gene cluster which can be induced by the P\textsubscript{G} promoter in pKMY319, involved construction of a number of intermediate plasmids. Cloning of the BamHII-XhoI fragment of pACG1 extending from coordinates 4.0 to 4.7 (Fig. 3) into the BamHI and SalI sites of the E. coli cloning vector pUC18 (25) produced plasmid pMY606 (Fig. 3). Cloning of the EcoRI-BamHI fragment of pACG1 extending from coordinates 0.05 to 4.0 (Fig. 3) into the EcoRI and BamHI sites of pMY606 produced plasmid pMY607 (Fig. 3). In pMY607, the entire NDO gene cluster was reassambled to contain appropriate 5' and 3'-flanking restriction sites for further manipulation. Insertion of a SacI linker at the HindIII site downstream of the NDO gene cluster in pMY607 generated plasmid pMY608 and insertion of an XbaI linker at the EcoRI site upstream of the NDO gene cluster in pMY608 generated plasmid pMY609 (Fig. 3). Replacement of the XbaI-Eco47III fragment of pMY609 extending from coordinates 0.05 to 1.8 (Fig. 3) with the XbaI-XmaI fragment of pMY498 containing the modified tmoF\textsuperscript{*} gene, tmoF\textsuperscript{*} (for details of the construction of pMY498, see above and Fig. 1), generated plasmid pMY611 (Fig. 3). Cloning of the XbaI-SacI fragment of pMY611 carrying the tmoF\textsuperscript{*}-nahA\textsubscript{4}A\textsubscript{4} gene cluster into the XbaI and SacI sites of pKMY319 completed construction of plasmid pMY619.

Construction of plasmid pMY618 (Fig. 3), a derivative of pKMY319 carrying the nahA\textsubscript{4}A\textsubscript{4}A\textsubscript{4} genes which can be induced from the P\textsubscript{G} promoter in pKMY319, involved construction of plasmid pMY610 (Fig. 3). Insertion of an XbaI linker at the Eco47III site at the 3' end of nahA\textsubscript{4} in pMY610 generated pMY610. Cloning of the region of pMY610 extending from coordinates 1.8 to 4.7 (Fig. 3) as an XbaI-SacI fragment into the XbaI and SacI sites of pKMY319 generated pMY612. Cloning of the entire insert of pMY612 extending from coordinates 0.6 to 4.7 (Fig. 3) as an XbaI-XhoI fragment produced pMY612. Cloning of the entire insert of pMY612 extending from coordinates 0.6 to 4.7 (Fig. 3) as an XbaI-SacI fragment into the XbaI and SacI sites of pKMY319 generated plasmid pMY620.

**Nucleotide sequence accession number.** The nucleotide sequence of the tmoF gene has been submitted to GenBank and assigned accession number M59045.

### TABLE 1. Effects of tmoF, tmoF\textsuperscript{*}, and nahA\textsubscript{4} genes on the tmoABCDE-determined T4MO activity

<table>
<thead>
<tr>
<th>Host(^a)</th>
<th>Plasmid(^b)</th>
<th>Pertinent genotype</th>
<th>Induced T4MO sp act (nmol min(^{-1}) mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli FM5</td>
<td>pCFM1146</td>
<td>tmoABCDE</td>
<td>0.1 ± 0.02 (2)</td>
</tr>
<tr>
<td></td>
<td>pMY421</td>
<td>tmoABCDE</td>
<td>10 ± 0.5 (3)</td>
</tr>
<tr>
<td></td>
<td>pKMY336</td>
<td>tmoABCDE</td>
<td>19 ± 4.2 (3)</td>
</tr>
<tr>
<td></td>
<td>pKMY340</td>
<td>tmoABCDE</td>
<td>20 ± 4.5 (3)</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>pKMY319</td>
<td>tmoABCDE</td>
<td>0.2 ± 0.1 (6)</td>
</tr>
<tr>
<td></td>
<td>pKMY321</td>
<td>tmoABCDE</td>
<td>5.8 ± 1.3 (5)</td>
</tr>
<tr>
<td></td>
<td>pMY486</td>
<td>tmoABCDE</td>
<td>15 ± 1.2 (6)</td>
</tr>
<tr>
<td></td>
<td>pMY603</td>
<td>tmoABCDE-nahA\textsubscript{4}</td>
<td>10 ± 3.3 (5)</td>
</tr>
<tr>
<td></td>
<td>pMY617</td>
<td>tmoABCDE\textsuperscript{*}</td>
<td>18 ± 3.5 (5)</td>
</tr>
<tr>
<td>P. putida KT2440</td>
<td>pKMY319</td>
<td>tmoABCDE</td>
<td>0.2 ± 0.1 (4)</td>
</tr>
<tr>
<td></td>
<td>pKMY321</td>
<td>tmoABCDE</td>
<td>4.7 ± 1.1 (5)</td>
</tr>
<tr>
<td></td>
<td>pMY486</td>
<td>tmoABCDE</td>
<td>16 ± 4.9 (4)</td>
</tr>
<tr>
<td></td>
<td>pMY603</td>
<td>tmoABCDE-nahA\textsubscript{4}</td>
<td>13 ± 5.3 (5)</td>
</tr>
</tbody>
</table>

\(^a\) All of the bacterial strains and growth and induction conditions (including temperature induction conditions of E. coli FM5 derivatives and sodium salicylate induction conditions of other strains) were described previously (28).

\(^b\) Construction of plasmids was described in Materials and Methods.

\(^c\) The T4MO assay was performed as described previously (28). Specific activities presented are means ± standard deviations, and the numbers of independent determinations are shown in parentheses.

### RESULTS

**Identification of the T4MO-enhancing activity.** Previously we demonstrated that a plasmid (pMY421) carrying an insert which extends from the HindIII site at coordinate 1.2 to the XhoI site at coordinate 5.1 in the SacI fragment shown in Fig. 1 contains all of the five genes, tmoABCDE, required for the synthesis of the T4MO system in E. coli (28). However, we subsequently found that extension of the insert to the XmaI site at coordinates 5.9 (Fig. 1) included a DNA sequence which upon expression, consistently enhanced the tmoABCDE-determined T4MO activity. Further extension of the insert beyond the XmaI site did not produce higher T4MO activity. These results were obtained from cells carrying plasmids pKMY336 and pKMY340. These two plasmids, like pMY421, are derivatives of the E. coli plasmid vector pCFM1146 and contain the tmoABCDE gene cluster which can be induced by heat from a phage lambda promoter (28). Unlike pMY421, pKMY336 carries an insert extending from the HindIII site at coordinate 1.2 to the XmaI site at coordinate 5.9 (Fig. 1) and pKMY340 carries an insert extending from the same HindIII site to the BamHI site at coordinate 7.9 (Fig. 1). Under induction conditions, plasmids pKMY336 and pKMY340 both produced twice as much T4MO activity as did plasmid pMY421 in E. coli FM5 (28) (Table 1). This T4MO-enhancing activity was not simply an oddity attributable to E. coli FM5 and could be observed in other bacterial strains as well. When the inserts in pMY421 and in pKMY336 were cloned into the broad-host-range expression vector pKMY319 (26) and the resulting plasmids pKMY321 and pMY486 (Fig. 1), respectively, were induced with sodium salicylate in both E. coli HB101 (28) and P. putida KT2440 (28), the T4MO-enhancing activity from pMY486 was observed in both hosts. Compared with the
level of T4MO activity produced from plasmid pKM332, an approximately threefold stimulation of the T4MO activity was observed in *E. coli* HB101 and *P. putida* KT2440 carrying plasmid pMY486 (Table 1). Since the *tmoF* gene ends immediately upstream of the putative transcription terminator (28), the results in Table 1 indicated that the DNA region between the putative transcription terminator and the *XmaI* site at coordinate 5.9 (Fig. 1) determined a T4MO-enhancing activity.

Identification of a new gene, *tmoF*, encoding T4MO-enhancing activity and determination of its nucleotide sequence. To determine whether the T4MO-enhancing activity detected from pKM336 was specified by a new gene, a plasmid, pMY440, was constructed by deleting a 3.4-kb HindIII fragment extending from coordinates 1.2 to 4.6 (Fig. 1) from the 5′ end of the insert in pKM336 (Fig. 1) and the remaining *P. mendocina* KR1 DNA fragment in pMY440 extending from coordinates 4.6 to 5.9 (Fig. 1) was sequenced in its entirety by both orientations. A complete open reading frame consisting of 978 bp and running with the same transcriptional direction as that of the *tmoABCDE* cluster was identified immediately downstream of the putative transcription terminator at the end of the *tmoABCDE* cluster (Fig. 4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude extracts of induced *E. coli* cells carrying pMY440 revealed the synthesis of a ~37-kDa polypeptide from the insert in pMY440 (Fig. 5). The polypeptide band in the gel was electroblotted onto a polyvinyl difluoride membrane, stained, excised, and sequenced in a protein sequencer as described previously (28). The determined N-terminal amino acid sequence, MFN1Q5DDLLH HFE, completely matched the N-terminal amino acid sequence of the protein predicted from the open reading frame (Fig. 4). The open reading frame thus defines a gene. This gene was designated *tmoF*. The nucleotide sequence of *tmoF* predicted a polypeptide product with a molecular weight of 35,983 which approximately matched the estimated molecular weight of the polypeptide produced from pMY440. The G+C content of the *tmoF* sequence is 47.8%.

**FIG. 4.** Nucleotide sequence of the *tmoF* gene and the inferred amino acid sequence of its product. The sequence was determined by a method previously described (28). The sequenced region extends from coordinates 4.6 to 5.9 shown in Fig. 1 and overlaps the sequence reported previously (28). The putative transcription termination sequence determined previously (28) is underlined. The bases that are complementary to the 3′ end of *E. coli* 16S rRNA and appear to determine the ribosome binding site (16) are marked with open circles. The sequence presented here is numbered in relation to the *tmoABCDE* sequence previously reported (28).

**FIG. 5.** SDS-PAGE analysis of TmoF protein produced in *E. coli* FM5 carrying plasmid pMY440. The conditions for induction of strain FM5 and for electrophoresis were as described previously (28). Before SDS-PAGE was performed, the induced cells were heated at 65°C in the loading buffer, as was previously done with the protein samples (28). The lysates generated by this treatment were directly loaded on the gel for protein analysis. Total proteins of induced *E. coli* FM5 carrying plasmid pMY440 (lane 3) (Materials and Methods) and plasmid vector pCFM1146 (lane 2) (28) are shown. The low-range molecular weight standards (lane 1) (Bio-Rad Laboratories, Richmond, Calif.) were used to estimate the size of the TmoF protein.
FIG. 6. Homology of TmoF protein with other reductases. The amino acid sequence of TmoF is compared with those of the reducec tase components of the Dmp system (DmpP) from Pseudomonas sp. strain CF600 (13, 14); the MMO system (MmoC) from M. capsulatus (Bath) (18), the xylene monoxygenase system (XylA) from P. putida PaW1 (22), the NDO system (NahA) from P. putida G7 (17), the benzoate dioxygenase system (BenC) from A. calcoaceticus BD413 (12), and the toluate dioxygenase system (XylIz) from P. putida PaW1 (10). Amino acids identical to those in TmoF are shown in capital letters and amino acids similar (as defined previously) (28) to those in TmoF are shown in lowercase letters. In reference to TmoF, dissimilar amino acids are represented by dashes. All proteins have an N-terminal region resembling chloroplast-type ferredoxins and a C-terminal region resembling oxidoreductases of various origins (see text). The amino acids completely conserved in these reductions, as in chloroplast-type ferredoxins (11), are marked with asterisks. The highly but not completely conserved positions that exhibit some amino acid variations either in the reductases or in chloroplast-type ferredoxins (11) are marked with open circles. Highly conserved regions in the C-terminal portion of the reductases are underlined.

and is similar to the G+C content of the tmdABCDE cluster (49.1%).

Function of the tmoF gene. To investigate the role of tmoF in the T4MO system, the deduced amino acid sequence of the TmoF polypeptide was compared with that of other gene products. It was found that TmoF shares homology with the oxidoreductase components of several mono- and dioxygenase systems, including the reductase components of the DMP, MMO, and xylene monoxygenase systems encoded by the dmpP gene of plasmid pV1150 from Pseudomonas sp. strain CF600 (13, 14), the mmoC gene of M. capsulatus (Bath) (22), and the xylA gene of TOL plasmid pWWO from P. putida PaW1 (10) and the reductase components of naphthalene, benzoate, and toluene dioxygenase systems encoded by the nahA gene of plasmid NAH7 from P. putida G7 (17), the benC gene of Acinetobacter calcoaceticus BD413 (12), and the xylIz gene of TOL plasmid pWWO (10). An alignment of the amino acid sequences of these reductases with that of TmoF showing identical and similar amino acids is presented in Fig. 6. These reductase proteins all have molecular weights similar to that of TmoF and share a significant number of identical and similar amino acids with TmoF (Table 2).

The DmpP, BenC, XylA, XylIz, and MmoC proteins were each shown to have an N-terminal region resembling chloroplast-type ferredoxins and a C-terminal region resembling oxidoreductases of bacterial, yeast, plant, animal, and human origins (12, 14, 18). As shown in Fig. 6, these properties are also shared by the TmoF and NahA proteins. In the N-terminal portion of each of the proteins shown in Fig. 6, many amino acids either completely or highly conserved in chloroplast-type ferredoxins (11) are also completely or mostly conserved in these proteins. The amino acids completely conserved, as in chloroplast-type ferredoxins, in-
include two glycine residues and the four cysteine residues at positions 53, 58, 61, and 96 (Fig. 6), which may be involved in the coordination of the two iron atoms in the [2Fe-2S] cluster. In the C-terminal portion of these reductase proteins, regions of significant homology can be identified (Fig. 6). Region I contains sequences previously proposed to be involved in the binding of flavin adenine dinucleotide and region III contains sequences proposed to be involved in the binding of NAD (12). The proteins BenC, XylA, XylZ, and MmcC, each of which was presumably formed during evolution by the fusion of a ferredoxin to a reductase, are all capable of transferring electrons from NADH directly to the oxygenase component in their respective enzyme system. Unlike these reductases, NahA has functions with a separate ferredoxin protein in carrying out this same task (8, 9). In this respect, TmoF is similar to NahA, since a ferredoxin is also present in the T4MO system (28).

The structural and functional homologies between TmoF and the well-characterized NahA prompted us to test the functional interchangeability between tmoF and nahA in stimulating the tmoABCDE-determined T4MO activity and in directing the formation of active NDO. For the evaluation of nahA function in stimulating the tmoABCDE-determined T4MO activity, a tmoABCDE-nahA gene cluster was constructed and cloned into the expression vector pKM319 (26). The resulting plasmid pMY603 (Fig. 1) was used in a bioassay to give an induced level of T4MO activity in E. coli HB101 or P. putida 2440 only slightly lower than that given by plasmid pMY846, which carries the wild-type gene cluster tmoABCDE-DEF (Fig. 1; Table 1). This result demonstrated that the NADH:ferredoxin oxidoreductase of the NDO system encoded by plasmid NAH7 can largely replace the TmoF protein in stimulating T4MO activity in both E. coli and P. putida.

The nahA and nahA genes of the NDO system encode ferredoxin and the two subunits of the terminal oxygenase component, respectively (17). In the construction of a tmoF-nahA-nahA gene cluster for the evaluation of the functional similarity of tmoF to nahA in the formation of NDO, it was desirable to remove the putative transcription termination sequence upstream of tmoF to increase the sensitivity of the test. A restriction site for the SspI enzyme (AATTATT) is located immediately downstream of the tmoF sequence encoding the first three amino acids of the TmoF protein

<table>
<thead>
<tr>
<th>Reductase</th>
<th>Mol wt</th>
<th>% of amino acid residues in TmoF homologous to those in other reductases</th>
<th>Identical amino acids</th>
<th>Similar amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmpP</td>
<td>38,500</td>
<td>32.5</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>BenC</td>
<td>38,800</td>
<td>28.8</td>
<td>19.3</td>
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<tr>
<td>MmoC</td>
<td>38,600</td>
<td>27.0</td>
<td>21.2</td>
<td></td>
</tr>
<tr>
<td>XylA</td>
<td>38,200</td>
<td>26.7</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>NahAa</td>
<td>35,200</td>
<td>25.2</td>
<td>18.4</td>
<td></td>
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<tr>
<td>XylZ</td>
<td>36,200</td>
<td>25.2</td>
<td>21.8</td>
<td></td>
</tr>
</tbody>
</table>

* aThe mono- and dioxygenase systems in which these reductases play an electron-transferring role are described in the text.

bThe molecular weights of the reductase proteins are determined from nucleotide sequences (10, 12, 13, 17, 18, 22).

The percentages were calculated on the basis of amino acid alignment presented in Fig. 6. Similar amino acids are as defined previously (28).

The putative transcription termination sequence and the region encoding the ribosome binding site of tmoF mRNA and the three N-terminal amino acids, MFN, of the TmoF protein were removed at the SspI site and were replaced with a DNA sequence encoding a new ribosome binding site and the amino acids MV (Materials and Methods) (Fig. 2). These sequence changes did not lead to a significantly higher TmoF activity and nevertheless resulted in a modified tmoF gene, tmoF*, that remained active in stimulating the tmoABCDE-determined T4MO activity. As shown in Table 1, plasmid pMY617, which carries the gene cluster tmoABCDE-DEF* that can be induced from the Pg promoter (Materials and Methods), expressed T4MO activity in E. coli at a level slightly higher than that expressed by plasmid pMY486, which carries the wild-type gene cluster tmoABCDE-DEF (Fig. 1).

To determine whether tmoF could replace nahA in directing NDO synthesis, the assembled tmoF-nahA-nahA cluster, the wild-type nahA-nahA-nahA cluster, and a derivative of the latter cluster lacking nahA, were individually cloned into the expression vector pKM319, and the induced NDO activities from the resulting plasmids pMY619, pMY620, and pMY618, respectively, were compared. As shown in Table 3, induction of NDO from all these plasmids could be detected in E. coli HB101. The low-level induction from plasmid pMY618 (Table 3), which does not carry the nahA gene, reflected the presence of a nonspecific reductase activity in the E. coli host and has also been observed by other researchers (6, 17). The induced NDO activity from plasmid pMY619, although representing only 1/10th that obtained from plasmid pMY620, was more than twofold the level obtained from plasmid pMY618 (Table 3). This result thus demonstrated that the tmoF product can partially replace the NahA protein in the formation of NDO. In conjunction with the observations that TmoF shares amino acid sequence homology with reductases of several oxygenase systems and that NahA can largely replace TmoF in stimulating T4MO activity, this result suggested that TmoF serves as an NADH:ferredoxin oxidoreductase in the T4MO
system. The nonspecific reductase activity in the E. coli host could account for the tmoABCDE-specified T4MO activity observed in the absence of tmoF (Table 1) (28). The presence of a similar nonspecific reductase activity in P. putida could also make TmoF dispensable in the formation of T4MO in this host (Table 1).

DISCUSSION

In this study, we have identified and characterized a new P. mendocina KR1 gene, tmoF, whose expression enhanced the T4MO activity determined by the tmoABCDE cluster (28). This gene was not identified initially in our study of the T4MO gene cluster because of the nonessential role it played in T4MO synthesis in E. coli (28). In fact, tmoF is not essential in tmoABCDE-directed T4MO synthesis in any of the bacterial hosts (including three E. coli, three P. putida, and two P. mendocina strains) we have examined to date (Table 1) (27, 28). Several lines of evidence presented in Results nevertheless suggest that tmoF is a member of the T4MO gene cluster and encodes the NADH:ferrodoxin oxidoreductase of the T4MO system: (i) a two- to threefold stimulation of the TmoABCDE-determined T4MO activity by TmoF (Table 1), (ii) homology of TmoF with the oxidoreductases of other mono- and dioxygenase systems (Fig. 6), (iii) partial interchangeability in function between TmoF and NADH:NADP+ oxidoreductases of other mono- and dioxygenase systems (Table 1), and (iv) close linkage of tmoF with the tmoABCDE cluster (Fig. 4). The TmoABCDE-determined T4MO activity in the absence of TmoF is most likely due to the presence of a nonspecific reductase activity in the host (see Results). However, the determination of whether TmoF is absolutely essential for T4MO activity must await further in vitro analysis using purified T4MO functional components.

Expression of tmoF from plasmids pKMY336, pMY486, and pMY440 indicated that tmoF is in the same transcription unit as the tmoABCDE cluster and that the putative transcription terminator downstream of the tmoABCDE cluster allows transcriptional read-through. In the construction of plasmid pMY617, not only was the putative transcription terminator upstream of tmoF deleted, but also the nucleotide sequence encoding the ribosome binding site of TmoF mRNA and the first three amino acids of TmoF protein was changed (see Materials and Methods). The effect on the expression of tmoF or the activity of T4MO in the absence of the transcription terminator still cannot be effectively evaluated in cells carrying pMY617. The function of the putative transcription terminator remains unclear.

The presence of tmoF indicates that the T4MO gene cluster consists of six genes. We have presented suggestive evidence for the roles of TmoA, TmoC, TmoE, and TmoF in T4MO synthesis (Tables 1 and 3) (28). The functions of TmoB (molecular weight, 9,600) and TmoD (molecular weight, 12,000) remain completely unknown. However, clues for a function of TmoB or TmoD might be provided by the MMO system of M. capsulatus. The MMO system consists of three components and catalyzes the oxidation of methane to methanol (2). Among its many other activities is the catalytic conversion of toluene into cresol (3), a reaction also catalyzed by the T4MO system (23). The substrate-binding oxygenase component of the MMO system is a colorless iron-containing protein whose absorption spectrum shows a maximum at around 280 nm and a shoulder at around 406 nm (24). These properties are similar to those of T4MO oxygenase component, which is also a colorless iron-containing protein with maximum absorption at 280 nm and a shoulder at around 390 nm (23). The oxygenase component of the MMO system consists of three subunits with molecular weights of 54,000, 42,000, and 17,000 (24).

The putative oxygenase component of the T4MO system was shown to consist of at least two subunits with molecular weights of 50,000 and 32,000 (23) or 58,000 and 38,500 as predicted from gene sequences (28). The possibility exists that the T4MO oxygenase contains an additional subunit encoded by the tmoB or tmoD gene.

In addition to the oxygenase component, the MMO system contains a reductase component and a regulator component. The reductase is essential for mono-oxygenase activity and capable of converting the enzyme from an oxidase to an oxygenase (7). It is a single-subunit protein, with a molecular weight of ~17,000 (7). It will be interesting to determine whether TmoB or TmoD can regulate T4MO activity.

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