Comparative Analysis of the Conventional and Novel pmo (Particulate Methane Monoxygenase) Operons from *Methylocystis* Strain SC2

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In addition to the conventional *pmo*4 gene (*pmo*4A1) encoding the active site polypeptide of particulate methane monoxygenase, a novel *pmo*4 gene copy (*pmo*4A2) is widely distributed among type II methanotrophs (methane-oxidizing bacteria [MOB]) (M. Tchawa Yimga, P. F. Dunfield, P. Ricke, J. Heyer, and W. Liesack, Appl. Environ. Microbiol. 69:5593–5602, 2003). Here we report that the *pmo*4A1 and *pmo*4A2 gene copies in the type II MOB *Methylocystis* strain SC2 are each part of a complete *pmo*CAB gene cluster (*pmo*CAB1, *pmo*CAB2). A bacterial artificial chromosome (BAC) library of strain SC2 genomic DNA was constructed, and BAC clones carrying either *pmo*CAB1 or *pmo*CAB2 were identified. Comparative sequence analysis showed that these two gene clusters exhibit low levels of identity at both the DNA level (67.4 to 70.9%) and the derived protein level (59.3 to 65.6%). In contrast, the secondary structures predicted for *Pmo*CAB1 and *Pmo*CAB2, as well as the derived transmembrane-spanning regions, are nearly identical. This suggests that *Pmo*CAB2 is, like *Pmo*CAB1, a highly hydrophobic, membrane-associated protein. A total of 190 of the 203 amino acid residues representing a highly conserved consensus sequence of the currently known *Pmo*CAB1 and *Amo*CAB sequence types could be identified in *Pmo*CAB2. The *amo*CAB gene cluster encodes ammonia monoxygenase and is evolutionarily related to *pmo*CAB. Analysis of a set of amino acid residues that allowed differentiation between conventional *Pmo*A and *Amo*A provided further support for the hypothesis that *pmo*CAB2 encodes a functional equivalent of *Pmo*CAB1. In experiments in which we used 5′ rapid amplification of cDNA ends we identified transcriptional start sites 320 and 177 bp upstream of *pmo*C1 and *pmo*C2, respectively. Immediately upstream of the transcriptional start sites of both *pmo*CAB1 and *pmo*CAB2, sequence motifs similar to *E. coli* σ70 promoters were identified.

Methane-oxidizing bacteria (MOB) (methanotrophs) are able to utilize methane (CH₄) as a sole source of carbon and energy for growth (15). These bacteria play an important role in the global methane cycle by oxidizing CH₄ released by methanogens in freshwater sediments and wetlands and thus mitigate the global warming effect of this greenhouse gas (6, 31). Phylogenies based on 16S rRNA genes show that MOB form distinct lineages in the gamma subclass of the class * Proteobacteria* (type I MOB) and the alpha subclass of the *Proteobacteria* (type II MOB) (3, 7, 8, 15, 18, 24). The two types of methanotrophs can be distinguished on the basis of biochemical and ultrastructural features (3, 15, 38).

The first step in CH₄ oxidation, the conversion of methane to methanol, is carried out by a methane monoxygenase (MMO). This enzyme exists in two forms, a particulate, membrane-associated form (pMMO) and a soluble form (sMMO). The two forms of the enzyme differ in structure, in kinetic properties, and in the range of substrates which are utilized (23). Only a restricted number of MOB species possess sMMO, while almost all MOB possess pMMO. The only MOB lacking pMMO are *Methylocella palustris* (8) and *Methylocella silvestris* (10). In MOB that harbor both forms of MMO, sMMO is synthesized under copper-deficient conditions, while in the presence of even a minuscule amount of available Cu(II) (0.85 to 1.0 µmol/g [dry weight] of cells) only pMMO is synthesized (15, 27).

The pMMO gene cluster consists of three consecutive open reading frames (*pmo*C, *pmo*A, and *pmo*B) in both type I MOB (32, 35) and type II MOB (14). The *pmo* genes from *Methylococcus capsulatus* Bath are transcribed into a single 3.3-kb polycistronic mRNA (27). *Pmo*A is presumed to contain the active site because it has been shown to be specifically labeled by [14C]acetylene, a suicide substrate for MMO (30, 40).

The type I MOB *Methylococcus capsulatus* Bath and *Methylomonas album* BG8 (32, 35), as well as the type II organisms *Methylosinus trichosporium* OB3b and *Methylosps* strain M (14), have been shown to contain duplicate copies of the *pmo* operon. The sequences of the duplicate *pmo*CAB gene clusters are nearly identical (e.g., there are 13 differences in 3,183 bp in *M. capsulatus* Bath).

However, the type II MOB *Methylocystis* strain SC2 has recently been shown to contain two very different *pmo*A-like genes (11). A 495-bp fragment of one gene (conventional *pmo*A, *pmo*A1) exhibited very high levels of sequence homology to *pmo*A genes of other type II MOB (encoding PmOA amino acid sequences identical to those of some other *Methylocystis* strains). The corresponding fragment of the second gene (novel *pmo*A, *pmo*A2) exhibited only 73% identity with *pmo*A1 at the nucleotide level and 68.5% identity (83% similarity) at the deduced amino acid level. Genes closely related to *pmo*A2 of strain SC2 are widely but not universally present in type II MOB (36). No *pmo*A2-like sequences were detected in five representative type I MOB tested. Comparative se-
sequence analysis showed that all *pmoA2*-like sequences formed a coherent cluster that is clearly distinct from *pmoA1* sequences of type I and type II MOB and from *amoA* sequences of the *Nitrosomonas-Nitrosospira* group. Reverse transcription-PCR provided evidence that *pmoA2* was expressed in strain SC2 under standard laboratory growth conditions (36).

Here we show that both *pmoA1* and *pmoA2* are part of complete *pmoCAB* gene clusters in *Methylocystis* strain SC2. Although the deduced amino acid sequences of PmoCAB2 are very different from those of PmoCAB1, the putative secondary structure and regions of transmembrane-spanning helices seem to be highly conserved in the two PmoCAB2 variants. The biochemical equivalent of PmoCAB1 is the particulate methane hydratase (pMH), which is the main component of functionally active pMMO. The pMH complex consists of the following three subunits: α (45 kDa, PmoB1), β (27 kDa, PmoA1), and γ (23 kDa, PmoC1) (26, 40). The three polypeptides associate by noncovalent bonds and form a single complex with a stoichiometry of 1:1:1 (αβγ) (23). Since our data were derived from an analysis of *pmo* genes, here we mainly refer to PmoCAB1 and PmoCAB2 rather than to pMH.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The conditions used for growth of *Methylocystis* strain SC2 were adapted from the conditions described by Heyer et al. (18, 19). For extraction of high-molecular-weight (HMW) DNA, cells were grown in liquid cultures in medium 10 containing NaN₃ instead of NH₄Cl as the nitrogen source. The cultures were grown for 3 to 5 days at 30°C under a headspace containing 20% (vol/vol) CH₄, 5% (vol/vol) CO₂, and 75% (vol/vol) air. Cells were pelleted by centrifugation at 6,000 × g for 30 min at 4°C and washed once with washing buffer (WB) (20 mM Tris-HCl, 50 mM EDTA; pH 8.0). The cell biomass was immediately used for extraction of DNA. Cells of *Escherichia coli* strain DH10B were grown overnight in Luria-Bertani medium in a liquid culture at 37°C.

**Preparation of HMW DNA.** For extraction and further treatment of HMW DNA strain SC2, whole cells were embedded in agarose at a final density of 3 × 10⁹ cells ml⁻¹ by using protocols described previously (12, 34). The embedded cells were treated with lysis buffer (100 mM Tris-HCl, 100 mM EDTA, 1.5 M NaCl, 1% [wt/vol] hexadecyltrimethylammonium bromide, 2% [wt/vol] sodium dodecyl sulfate; pH 8.0) (9) for 20 h at 37°C, rinsed with 25 ml of WB, and incubated in 5 ml of proteinase K reaction buffer (100 mM EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg of proteinase K per ml; pH 8.0) at 50°C for 14 h. To remove residual proteins, agar plugs were washed four times for 1 h in WB. The DNA-containing agarose plugs were stored in 0.5 M EDTA (pH 8.0) at 4°C until they were used.

**Construction of BAC library.** Cloning of HMW DNA into a bacterial artificial chromosome (BAC) vector (pLpIndoBAC-5 Cloning-Ready; Epicentre, Madison, Wis.) required partial digestion with restriction endonuclease HindIII. To obtain the maximum percentage of genomic DNA fragments in the desired size range, a time series of partial restriction digests (29) was performed for each extract of HMW DNA prior to final mass digestion. To enable subsequent agarase digestion, the center part of a 1% pulse-field agarose gel (Bio-Rad, Madison, Calif.) was transferred immediately by agarase digestion with gelase (Promega, Madison, Wis.) and transferred into 96-well microtiter plates. Row pools of test-positive plates were screened in the same way. Finally, clones of single rows that tested positive were analyzed separately.

**PCR-based screening for BAC clones carrying *pmo* genes.** For identification of clones carrying either *pmoA1* or *pmoA2*, 10-µl aliquots of BAC clone-positive *E. coli* cells, which were grown in 12 wells (one row) of a 96-well microtiter plate, were combined. An aliquot (20 µl) of each row pool was combined to generate plate pools. Pooled cells were lysed by boiling for 15 min, and the cell debris was subsequently pelleted by centrifugation with a microcentrifuge at 8,000 × g for 5 min. Microtiter plates that contained at least one clone carrying target DNA were identified by PCR-based amplification of *pmo* genes by using 1 µl of the supernatant of each plate pool as the template. Row pools of test-positive plates were screened in the same way. Finally, clones of single rows that tested positive were analyzed separately.

**DNA sequencing of BAC clones.** The primary structures of the *pmoCAB* (clone SC2-VII-C1) and *pmoA2* (clone SC2-IX-C7) gene clusters and their flanking regions were determined by direct sequence analysis of BAC DNA by using a primer walking approach and oligonucleotide primers designed for accurate sequencing. The known partial sequences of *pmoA1* and *pmoA2* (11) were used as the starting points to formulate novel oligonucleotide primers. BAC DNA was extracted with a Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Each sequencing reagent (up to 50 pmol of primer, and 6 µl of BigDye terminator mix (PE Applied Biosystems, Weiterstadt, Germany). The thermal profile was as follows: initial denaturation for 3 min at 94°C, followed by 30 cycles consisting of denaturation at 94°C for 40 s, primer annealing at 50°C for 45 s at the temperatures indicated above, and elongation at 72°C for 75 s. The final elongation step was extended to 7 min. Aliquots of the amplicons were checked by electrophoresis on 1% agarose gels. Amplicons of the expected size were purified and sequenced to verify identity of test-positive clones. To ensure that complete *pmo* operons were located on the selected clones, the 5’ and 3’ termini of the cloned inserts were sequenced by using vector-specific primers. The data obtained were compared with sequences of known *pmoB* and *pmoC* genes. Two clones that carried either *pmoA1* (clone SC2-VII-C1) or *pmoA2* (clone SC2-IX-C7) were selected for further analysis.

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**Isolation of total RNA.** Cells of strain SC2 were grown in batch cultures to an optical density at 600 nm of 0.5 to 0.7 (mid-exponential growth phase). Expression of *pmo* genes was promoted by ensuring that the gas phase contained 10% CH₄, approximately 20 h before RNA extraction. Total RNA was stabilized before cell lysis by application of the RNAProtect bacterial reagent (Qiagen). Aliquots containing 1 × 10⁹ cells were lysed by mechanical disruption (bead beating at 2,500 rpm for 90 s), and RNA was extracted with an RNEasy mini kit (Qiagen). The manufacturer’s protocol was modified slightly. Instead of the recommended on-column DNAse digestion, which failed to remove DNA completely, the RNA preparations were treated with RNase-free DNase (RQ1; Promega) for 40 min at 37°C. The concentration of nucleic acids was determined by photometric measurement at 260 nm.

**Identification of transcriptional start sites of *pmoCAB* gene clusters.** cDNA was synthesized by using the oligo dT reverse transcriptase Kit (Invitrogen, Carlsbad, Calif.) and 100 ng of RNA. A primer (Table 1) with target specificity for either *pmoC1* (ConvC100r) or *pmoC2* (NovC313r), ConvC100r and NovC313r were selected...
for analysis from a set of newly designed primers because they exhibited the best performance in cDNA synthesis. Aliquots (0.5 to 1 µg) of DNA-free RNA were used as templates and were transcribed at 35°C for 1 h. The cDNA was purified with a Qiaquick PCR purification kit (Qiagen).

The transcriptional start sites were determined by rapid amplification of cDNA ends (RACE) by using a 5′/3′ RACE kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. The oligonucleotide primers used are shown in Table 1. Briefly, a poly(A) tail was synthesized on the 3′ end of the first-strand cDNA by using terminal transferase. The use of an anchored oligo(dT) primer and a primer targeting a region of pmoc (for pmoc1, primer ConvC100r; for pmoc2, primer NovC100r) allowed us to amplify tail DNA.

A second PCR was performed with a nested pmoc-targeting primer (for pmoc1, primer ConvC60r; for pmoc2, primer NovC40r) and an anchored forward primer; a 1:50 dilution of the first PCR product was used as the template. First- and second-round PCR were carried out as described above with an annealing temperature of 61°C. PCR products were purified with a Qiaquick PCR purification kit and were sequenced by using the BigDye terminator sequencing kit and were sequenced by using the BigDye terminator mix (PE Applied Biosystems) as described by the manufacturer.

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### RESULTS AND DISCUSSION

*Methylocystis* strain SC2 BAC clones carrying pmoc genes. Gilbert et al. (14) reported that in accordance with previous studies (32), cloning of pmoc operons in multicopy vectors, such as pUC plasmids, is possible only in overlapping fragments because parts of the genes might be toxic to *E. coli*. Analysis of a pmoc operon sequence by cloning of overlapping fragments involves the risk that sequence data obtained originate from different, multiple pmoc operons. This problem is negligible if the sequences of multiple pmoc operons are virtually identical, as is the case for the duplicate pmoc operons of *M. trichosporium* OB3b (14). However, reconstruction of the primary operon structure from a genome containing multiple pmoc operons with divergent sequences could be much more problematic. Thus, in order to avoid cloning of overlapping pmoc fragments and to obtain full-length pmoc operons on the cloned fragments, we used a single-copy BAC vector to construct a genomic library of strain SC2.

Using a coordinated PCR-based screening approach, we identified six and seven clones carrying pmocA1 and pmocA2, respectively, in a set containing 960 BAC clones. To ensure that complete pmoc operons were located on the BAC clones, the sequences of the terminal regions of the inserts were compared to known pmoc and pmob sequences. One clone that carried pmocAB1 (clone SC2-VII-C1) and another clone that carried pmocAB2 (clone SC2-IX-C7) were selected for further analysis. The complete sequences of the two pmoc gene clusters plus their flanking regions were determined by a primer walking strategy. In addition, the complete sequence of the pmocAB2 cluster was confirmed by applying shotgun sequencing to clone SC2-IX-C7. Comparison of the sequences determined for the pmocAB2 operon and flanking regions either by primer walking or by shotgun cloning did not reveal any ambiguous nucleotide positions.

### Structural organization of pmocAB1 and pmocAB2

Comparative sequence analysis revealed that pmocA1 was present in a defined pmocAB gene cluster. This gene arrangement agrees

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#### TABLE 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5′→3′)</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A189^d</td>
<td>1486–1504^a</td>
<td>GGN GAC TGG GAC TTC TGG</td>
<td>Forward</td>
</tr>
<tr>
<td>PmoA206fd</td>
<td>1486–1521^a</td>
<td>GGNAGCTGGACCTCTGGATGCTACCTTAAAGATCG</td>
<td>Forward</td>
</tr>
<tr>
<td>682f</td>
<td>2026–2009^a</td>
<td>GAA SGC NGA GAA SGC</td>
<td>Reverse</td>
</tr>
<tr>
<td>PmoA703fb</td>
<td>2026–1992^a</td>
<td>GAASCGNAGAGAAGSAGCGACCGGACCGACGT</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

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^a N = A, C, G, or T; S = C or G; V = A, C, or G.
^b Data from reference 20.
^c Primer position in relation to the transcriptional start site of pmocAB1.
^d Data from reference 36.
^e Primer position in relation to the transcriptional start site of pmocAB2.
^f Obtained from Roche Diagnostics (Mannheim, Germany).

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well with the structural organization reported previously for pmo operons of type I MOB (32, 35) and type II MOB (14). In autotrophic nitrifiers, the amo genes are also arranged in the order amoCAB. The amoCAB cluster encodes ammonia monooxygenase (AMO) and is believed to be an evolutionary homolog of pmoCAB (20, 21, 23), pmaA2 also corresponds to a defined pmoCAB gene cluster, but the primary structure of this cluster is clearly distinct from that of pmoCAB1 (see below).

**pmoCAB1.** The pmoCAB1 genes have putative lengths of 771, 759, and 1,263 bp, respectively. The intergenic sequence regions cover 276 bp (pmoC1-pmoA1) and 161 bp (pmoA1-pmoB1), respectively. Putative Shine-Dalgarno sequence motifs very similar to the E. coli Shine-Dalgarno consensus sequence (5′-TAAGGAG-3′) (33) could be identified in the upstream region of each of the three pmo1 genes.

By using a 5′ RACE approach, the putative transcriptional start site was determined to be located 320 bp upstream of pmoC1 (designated position A1). Upstream of position A1, putative σ70 promoter-like sequence motifs were identified. PCR amplification of cDNA by using primer ConvC100r and a primer targeting a region directly upstream or downstream of the predicted start site confirmed the data obtained by 5′ RACE. Only primers targeting the cDNA downstream of position A1 resulted in PCR products (data not shown). The putative −35 and −10 hexamer promoter motifs agreed with the corresponding E. coli consensus sequences at four and three nucleotide positions, respectively (16). The putative pmo1 promoter in strain SC2 also exhibited high levels of similarity in both the −35 and −10 regions to the promoter sequences predicted for the pmo operons of *M. trichosporium* OB3b and *Methylocystis* sp. strain M (14) (five identical nucleotide sequence positions for both the −35 and −10 regions) (Fig. 1). Detection of putative σ70 pmo promoters in the latter two organisms led to the conclusion that pmo operons are recognized by σ70 and are negatively regulated under low-copper conditions (14).

A factor-independent terminator was identified 121 bp downstream of pmoB1. In addition, a factor-independent terminator was predicted to occur 172 bp upstream of position A1, probably terminating an operon that is localized upstream of pmoB1 (the sequence of an acid phosphatase from *Mesorhizobium loti* exhibited the best E value in a tBLASTx analysis [4e-34]).

**pmoCAB2.** The pmoCAB2 genes have putative lengths of 777, 771, and 1,290 bp, respectively. They are separated by two intergenic sequence regions that are 229 bp (pmoC2-pmoA2) and 95 bp (pmoA2-pmoB2) long. As in the pmo1 operon, putative Shine-Dalgarno sequences were identified upstream of the start codons of all three pmo2 genes.

The putative transcriptional start site of pmoCAB2 was determined by 5′ RACE to be located 177 bp (at a position designated C1) upstream of pmoC2. As in the pmo1 operon, PCR amplification of pmo2 cDNA with a primer set consisting of NovC100r and a primer targeting the promoter region directly upstream or downstream of position C1 confirmed that C1 was the putative start site (data not shown). However, it should be mentioned that when cDNA synthesis was carried out at 37°C instead of 56°C, partial polyadenylation occurred 40 bp downstream of position C1. This may indicate that there is an interruption of cDNA synthesis due to mRNA secondary structure elements.

The promoter sequences predicted for pmoCAB2 matched the −35 and −10 hexamer sequence motifs of the E. coli σ70 consensus sequence at five and three nucleotide positions, respectively. The comparison of the sequence motifs predicted for pmoCAB1 and pmoCAB2 revealed a lower degree of identity (Fig. 1).

A factor-independent terminator was identified 75 bp downstream of pmoB2. In addition, a putative factor-independent terminator was identified 368 bp upstream of position C1. This indicated that the 3′ end of an unidentified operon (characterized by ORPHEUS [13] as a hypothetical protein) is localized upstream of the pmo2 operon.

**Comparative molecular analysis of pmoCAB1 and pmoCAB2.** (i) Identity and similarity values. In silico translation of pmoCAB1 resulted in derived amino acid sequences with 256 residues (PmoC1), 252 residues (PmoA1), and 420 residues (PmoB1). The corresponding data for pmoCAB2 were 258 residues (PmoC2), 256 residues (PmoA2), and 429 residues (PmoB2). Despite the fact that N-terminal amino acids were predicted to constitute leader sequences (26), all derived...
amino acids were included in a further comparative analysis. The pmo operons determined for M. trichosporium OB3b and Methylocystis sp. strain M (14), as well as one of the two nearly identical pmo operons of the type I MOB M. capsulatus Bath (32, 35), were used for comparison (Table 2). Comparative analysis of the amino acid sequences deduced from pmoC and pmoB revealed that PmoC1 and PmoC2, as well as PmoB1 and PmoB2, have identity and similarity values which are in the same range as the values for partial PmoA1 and PmoA2 sequences (11). The levels of identity between polypeptides predicted for PmoCAB2 of strain SC2 and polypeptides predicted for the conventional PmoCAB proteins of type II MOB ranged from 59.3 to 65.6%, while the levels of similarity were in the range from 74.7 to 80.1%. Comparison of PmoCAB2 with PmoCAB1 of strain SC2 differed at only 85 residues determined by Tukhvatullin et al. (37) to be highly conserved in PmoB1 and AmoB (α-peptide), 79 were also identified in PmoB2. Likewise, PmoA2 (β-peptide) and PmoC2 (γ-peptide) possessed 34 of 36 and 77 of 82 highly conserved residues, respectively.

In total, the PmoCAB2 sequence had 13 deviations from the consensus sequence. PmoCAB1 of strain SC2 differed at only two positions (P86→A97 and I249→L254 of PmoC1; the numbering of the consensus sequence is that of Tukhvatullin et al. [37], who did not consider putative leader sequences). However, it should be noted that of the 13 residues at which PmoCAB2 differed from the PmoCAB1-AmoCAB consensus sequence, only a single residue is thought to have a different chemical property than the corresponding residue of the consensus sequence (R180 of PmoC2, compared with the consensus residue G167 in PmoC1 of M. capsulatus Bath). Each of the other 12 residues belonged to the same amino acid similarity group (as defined empirically by the BLOSUM65 matrix [17]) as its counterpart in the consensus sequence. Thus, the similarity value for the consensus sequence of PmoCAB1 and AmoCAB and the corresponding positions in PmoCAB2 (>99%) provides support for the assumption that pmoCAB2 encodes a pMMO-like (or AMO-like) enzyme.

While the structure of pMMO and its content of metal centers is still under discussion (2, 5, 22), it is widely accepted that pMMO requires copper as a cofactor for both functional activity and structural integrity (2, 5). Tukhvatullin et al. (37) analyzed the set of highly conserved amino acid residues described above to examine their possible roles in the formation of complexes with metal ions. This analysis took into account the physicochemical characteristics of particular amino acid residues, as well as experimental data for pMMO and pMH obtained by electron spin resonance, electron nuclear double resonance, Mössbauer, and atomic absorption spectroscopy. The analysis aimed to elucidate elements of pMMO-AMO structure and to reveal amino acid residues which are involved in the formation of the active sites. Thirty-nine residues which have the potential to act as ligands were identified. Thirty-eight of these residues could also be identified in PmoCAB2. The only exception, Y26 (PmoA1 and AmoA), is replaced in PmoA2 by F (PmoA2 in Fig. 2 at position F44). Y26 is thought to be involved in formation of the catalytic center only in a
subset of the structural-functional models predicted. However, in the majority of suggested models, Y26 is replaced as a ligand by other residues. Thus, the replacement of tyrosine by phenylalanine does not necessarily have an impact on the functional properties of PmoA2.

Among the PmoCAB1 and AmoCAB sequences, the largest data set is available for PmoA1 and AmoA, which include the active sites of the enzymes. To define the putative cellular function of PmoCAB2 more precisely, we identified in 315 PmoA1 and 919 AmoA sequences a set of 18 amino acid positions that can be used to differentiate between conventional pMMO and AMO (Table 3). Analysis of these signature positions in 40 PmoA2 sequences clearly showed that PmoA2 agrees with PmoA1 at all but one position.

Taken together, the analysis of a set of amino acid residues that are highly conserved among all pMMO and AMO and of a set that can be used to differentiate between the two enzymes clearly suggests that pmoCAB2 encodes a functional equivalent to conventional pMMO. Calculation of the ratio of nonsynonymous nucleotide substitutions to synonymous nucleotide substitutions in a phylogeny (36, 39) suggested that in recent evolutionary history pmoA2 was subject to strong purifying selection and therefore has an important cellular function. However, the analyses also suggested that pmoA2 may have been subject to diversifying selection forces acting at particular times or on particular codons. The elevated number of deviations in the PmoCAB2 sequence from the consensus sequence of PmoCAB1 and AmoCAB supports the hypothesis that there have been diversifying selection forces acting at particular times or on particular codons. The elevated number of deviations in the PmoCAB2 sequence from the consensus sequence of PmoCAB1 and AmoCAB supports the hypothesis that there have been diversifying selection forces acting at particular times or on particular codons. The elevated number of deviations in the PmoCAB2 sequence from the consensus sequence of PmoCAB1 and AmoCAB supports the hypothesis that there have been diversifying selection forces acting at particular times or on particular codons.

FIG. 2. Primary structures of derived PmoCAB2 expressed in one-letter code. Predicted transmembrane helices are indicated by shading. Residues that are highly conserved in PmoCAB1 and AmoCAB (consensus sequence) are indicated by boldface type (37). Amino acids that are located at conserved positions but differ from the amino acids in the consensus sequence determined by Tukhavatullin et al. (37) are underlined. The N-terminal helix of PmoB, as predicted by toppred and shown in Fig. 3, is not indicated because the residues are thought to constitute a leader sequence (26, 37).
FIG. 3. Predicted topologies of derived PmoCAB1 and PmoCAB2 from Methylocystis strain SC2. (Top) Hydrophobicity plot of PmoCAB1 versus PmoCAB2. The y axis indicates the relative hydrophobicity value at a given position in PmoCAB. N-terminal sequences are predicted to be located in the cytosol. Exact locations of transmembrane-spanning regions are shown in (Bottom). Predicted locations of derived PmoCAB1 and PmoCAB2 from Methylocystis strain SC2.
zyme has a different functional role than the conventional pMMO. This observation may be a further indication that the novel enzyme has a different level of expression is clearly lower than that of pmoCAB1 or pmoCAB2. The pmoCAB2 operon is expressed under standard laboratory growth conditions (36; this study), but its level of expression is clearly lower than that of pmoCAB1 (36).

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