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

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In addition to the conventional *pmoA* gene (*pmoA1*) encoding the active site polypeptide of particulate methane monooxygenase, a novel *pmoA* gene copy (*pmoA2*) 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 *pmoA1* and *pmoA2* gene copies in the type II MOB *Methylocystis* strain SC2 are each part of a complete *pmoCAB* gene cluster (*pmoCAB1*, *pmoCAB2*). A bacterial artificial chromosome (BAC) library of strain SC2 genomic DNA was constructed, and BAC clones carrying either *pmoCAB1* or *pmoCAB2* 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 PmoCAB1 and PmoCAB2, as well as the derived transmembrane-spanning regions, are nearly identical. This suggests that PmoCAB2 is, like PmoCAB1, 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 PmoCAB1 and AmoCAB sequence types could be identified in PmoCAB2. The *amoCAB* gene cluster encodes ammonia monooxygenase and is evolutionarily related to *pmoCAB*. Analysis of a set of amino acid residues that allowed differentiation between conventional PmoA and AmoA provided further support for the hypothesis that *pmoCAB2* encodes a functional equivalent of PmoCAB1. In experiments in which we used 5′ rapid amplification of cDNA ends we identified transcriptional start sites 320 and 177 bp upstream of *pmoC1* and *pmoC2*, respectively. Immediately upstream of the transcriptional start sites of both *pmoCAB1* and *pmoCAB2*, sequence motifs similar to *Escherichia coli* σ^{70} promoters were identified.

Methane-oxidizing bacteria (MOB) (methanotrophs) are able to utilize methane (CH_4) as a sole source of carbon and energy for growth (15). These bacteria play an important role in the global methane cycle by oxidizing CH_4 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 CH_4 oxidation, the conversion of methane to methanol, is carried out by a methane monooxygenase (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 (*pmoC*, *pmoA*, and *pmoB*) 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). PmoA is presumed to contain the active site because it has been shown to be specifically labeled by [^{14}C]acetylene, a suicide substrate for MMO (30, 40).

The type I MOB *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8 (32, 35), as well as the type II organisms *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (14), have been shown to contain duplicate copies of the *pmo* operon. The sequences of the duplicate *pmoCAB* 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 *pmoA*-like genes (11). A 495-bp fragment of one gene (conventional *pmoA*, *pmoA1*) exhibited very high levels of sequence homology to *pmoA* 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 *pmoA*, *pmoA2*) exhibited only 73% identity with *pmoA1* at the nucleotide level and 68.5% identity (83% similarity) at the deduced amino acid level. Genes closely related to *pmoA2* of strain SC2 are widely but not universally present in type II MOB (36). No *pmoA2*-like sequences were detected in five representative type I MOB tested. Comparative se-

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quence 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 PmoCAB variants. The biochemical equivalent of PmoCAB1 is the particulate methane hydroxylase (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 $NaNO_3$ instead of NH_4Cl as the nitrogen source. The cultures were grown for 3 to 5 days at 30°C under a headspace containing 20% (vol/vol) CH_4, 5% (vol/vol) CO_2, and 75% (vol/vol) air. Cells were pelleted by centrifugation at $6,000 \times g$ 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 from strain SC2, whole cells were embedded in agarose at a final density of 3×10^9 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 (pIndigoBAC-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 digestions (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, Wis.) was replaced by 1% low-melting-point agarose (Bio-Rad). DNA size fractionation was performed in relation to a low-range PFG marker (New England Biolabs, Beverly, Mass.) with a contour-clamped homogeneous electric field apparatus (CHEF-DR III; Bio-Rad) by using three distinct separation steps, as described by Osoegawa et al. (28).

Ethidium bromide-stained gel slices containing both electrophoresed HMW DNA and the PFG marker allowed us to cut slices from the unstained agarose gel containing genomic DNA that was approximately 50 to 150 kb long. HMW DNA was extracted immediately by agarase digestion with β-agarase I as described by the manufacturer (New England Biolabs). To prevent shearing of HMW DNA, subsequent steps were carried out without removing the digested agarose.

Eluted HMW DNA was ligated with 100 ng of pIndigoBAC-5. The molar ratio of insert to vector DNA was approximately 1:10. Ligation was performed in a 100-μl (total volume) mixture with 800 U of T4 ligase (New England Biolabs) for 24 h. The reaction was stopped by heat inactivation at 70°C for 30 min. The

ligation mixture was spotted onto the middle of a microdialysis filter (pore size, 0.025 μm; Millipore, Bedford, Mass.) and was desalted by floating the filter on sterile 20% (vol/vol) polyethylene glycol for 3 h. Subsequently, *E. coli* cells were transformed with desalted DNA (ElectroMax DH10B competent cells; Invitrogen Life Technologies; Carlsbad, Calif.) at 2.5 kV, 25 μF, and 100 Ω in a precooled 1-mm electroporation cuvette. A total of 960 single clones were picked and transferred into 96-well microtiter plates.

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 $8,000 \times g$ for 5 min. Microtiter plates that contained at least one clone carrying target DNA were identified by PCR-based amplification of *pmoA* 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.

Primers A189f and 682b and an annealing temperature of 50°C were used to specifically detect *pmoA1*, while primers PmoA206f and PmoA703b and an annealing temperature of 66°C were used to screen for *pmoA2* (Table 1). Each reaction mixture contained 1 μl of template, 10 μl of 10× reaction buffer, 1.5 mM Mg^{2+}, each deoxynucleoside triphosphate at a concentration of 200 μM, 2.5 U of *Taq* polymerase (Promega, Madison, Wis.), and each primer at a concentration of 0.25 μM. Amplifications were performed in 100-μl (total volume) mixtures in 0.2-ml reaction tubes by using a DNA thermal cycler (model 2400; 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 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 identification 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-IIX-C7) were selected for further analysis.

DNA sequencing of BAC clones. The primary structures of the *pmoCAB1* (clone SC2-VII-C1) and *pmoCAB2* (clone SC2-IIX-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) used according to the manufacturer's instructions. Each sequencing reaction mixture contained 13 μl (approximately 200 to 800 ng) of BAC DNA, 30 pmol of primer, and 6 μl of BigDye terminator mixture (PE Applied Biosystems). After initial denaturation at 94°C for 3 min, the following profile was repeated 99 times: rapid thermal ramp to 94°C and denaturation for 30 s, primer annealing at 50°C for 20 s, and elongation at 60°C for 4 min. After completion of the reaction, cycle sequencing products were purified with AutoSeq G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). Sequences determined by primer walking were edited manually and assembled by using EditSeq and Seqman II (DNAstar Inc., Madison, Wis.). In addition, the *pmoA2*-carrying clone SC2-IIX-C7 was fully sequenced by a shotgun cloning approach with a minimal sequence quality of phred 30.

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% CH_4 approximately 20 h before RNA extraction. Total RNA was stabilized before cell lysis by application of the RNAprotect bacterial reagent (Qiagen). Aliquots containing 1×10^9 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 produced by using the ThermoScript reverse transcription-PCR system (Invitrogen) and a primer (Table 1) with target specificity for either *pmoC1* (ConvC100r) or *pmoC2* (NovC313r). ConvC100r and NovC313r were selected

TABLE 1. Oligonucleotide primers used in this study

Primer	Position	Sequence (5′→3′)[a]	Direction
Primers for screening			
A189f[b]	1486–1504[c]	GGN GAC TGG GAC TTC TGG	Forward
PmoA206f[d]	1486–1521[e]	GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG	Forward
682b[d]	2026–2009[c]	GAA SGC NGA GAA GAA SGC	Reverse
PmoA703b[b]	2026–1992[e]	GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT	Reverse
RACE primers			
NovC40r	236–216[e]	GAT CGT ATC GGT CTC GCC GGC	Reverse
NovC100r	293–273[e]	GCC GAC GTA GAA GAC CAG CAT	Reverse
NovC313r	511–492[e]	CAG CCG ATG AGC GTC AGG AT	Reverse
ConvC-60r	269–289[c]	TGC GCG TGA CCG GTA TCG CTC	Reverse
ConvC40r	372–352[c]	CAG CTT CCG TGC CAG CAG CAG	Reverse
ConvC100r	445–425[c]	TCG TAA ATG CGA ACG ACC AGA	Reverse
RACE kit primers[f]			
Oligod(T)Anchored	−38−−1[c,e]	GAC CAC GCG TAT CGA TGT CGA C(T)$_{16}$V	Forward
Anchor primer	−38−−17[c,e]	GAC CAC GCG TAT CGA TGT CGA C	Forward

[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).

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 56°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 ConvC40r; for *pmoC2*, primer NovC100r) allowed us to amplify tailed cDNA.

A second PCR was performed with a nested *pmoC*-targeting primer (for *pmoC1*, primer ConvC-60r; 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 mixture (PE Applied Biosystems) as described by the manufacturer. The starting point of the poly(A) sequence indicated the 3′ end of the cDNA, which corresponded to the putative transcriptional start site.

Computational analysis. Homology searches were carried out with standard software tools and the BLAST server of the National Center for Biotechnology Information by using the BLAST algorithm (1) with a nonredundant DNA database, as well as with a nonredundant protein database compiled for this study from SWISSPROT, TREMBL, and PIR.

Putative factor-independent terminators were calculated by using the program TERMINATOR of the Genetics Computer Group software package (4). Similarity and identity values for deduced amino acid sequences were calculated by using the stretcher program (http://bioweb.pasteur.fr/seqanal/interfaces/stretcher.html) based on global pairwise alignments (25) computed with a BLOSSUM65 (17) substitution matrix. Alternatively, similarity and identity values were calculated with the ARB program package (developed by O. Strunk and W. Ludwig of the Technical University of Munich [http://www.arb-home.de]), based on manually edited multiple alignments.

Hydrophobicity plots of PmoCAB1 and PmoCAB2 were calculated by using the comparison tool of the Protein Hydrophilicity/Hydrophobicity Search and Comparison Server (http://bioinformatics.weizmann.ac.il/hydroph/) of the Weizmann Institute of Science, Rehovot, Israel. The locations of putative transmembrane-spanning regions were computed by using the toppred transmembrane topology prediction program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

Nucleotide sequence accession numbers. The sequences of the *pmoCAB* gene clusters plus flanking regions from *Methylocystis* strain SC2 have been deposited in the EMBL, DDBJ, and GenBank databases under accession numbers AJ584611 and BX649604 for *pmoCAB1* and *pmoCAB2*, respectively. The information on the flanking regions of *pmoCAB2* includes the annotation results for the fully sequenced 18.3-kb insert of clone SC2-IIX-C7.

RESULTS AND DISCUSSION

***Methylocystis* strain SC2 BAC clones carrying *pmo* genes.** Gilbert et al. (14) reported that in accordance with previous studies (32), cloning of *pmo* 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 *pmo* operon sequence by cloning of overlapping fragments involves the risk that sequence data obtained originate from different, multiple *pmo* operons. This problem is negligible if the sequences of multiple *pmo* operons are virtually identical, as is the case for the duplicate *pmo* operons of *M. trichosporium* OB3b (14). However, reconstruction of the primary operon structure from a genome containing multiple *pmo* operons with divergent sequences could be much more problematic. Thus, in order to avoid cloning of overlapping *pmo* fragments and to obtain full-length *pmo* 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 *pmoA1* and *pmoA2*, respectively, in a set containing 960 BAC clones. To ensure that complete *pmo* 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-IIX-C7) were selected for further analysis. The complete sequences of the two *pmo* 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-IIX-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 *pmoA1* was present in a defined *pmoCAB* gene cluster. This gene arrangement agrees

<pre>
                                       -35   -10

M. trichosporium OB3b   TATTGTCATTGAAGACGCGTACGGAAATGGTTGCGGCTCGCCGGACGAGG

                         ++++++++++   + +   ++ +++++++ + + +++   +++ ++

Methylocystis sp. M   TATTGTCATTATGTTGGTGAGAGGGAATGGTTGGGCCACGCT-AACGGGG

                         +++++ + +++   ++   ++++++ + + + ++++++ + + +++

strain SC2, pmoCAB1   TATTGTGAATATTGGGGGTTATTGGAATGTT-GCGGCACGCTGAGCTGGG

                         + +++   + +   + ++   +   + + +++++ +

strain SC2, pmoCAB2   TCTTGACGTCGACAGTGTTCGCCGTGATAGG----GTTCCCCCAGCTGTG
</pre>

FIG. 1. Sequence alignment of experimentally determined (*Methylocystis* sp. strain M, *pmoCAB1* and *pmoCAB2* of *Methylocystis* strain SC2) and computationally predicted (*M. trichosporium* OB3b) promoter regions of *pmo* operons. The alignment was computed by using a ClustalW algorithm. The shading indicates the −35 and −10 sequence motifs. Nucleotides identical to the corresponding nucleotides of the *E. coli* σ^{70} promoter consensus sequences (−35 sequence, TTGACA; −10 sequence, TATAAT) are indicated by boldface type, and the putative transcriptional start sites (positions A1 and C1) are underlined.

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). *pmoA2* 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 σ^{70} 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 σ^{70} *pmo* promoters in the latter two organisms led to the conclusion that *pmo* operons are recognized by σ^{70} 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 *pmoCAB1* (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* σ^{70} 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

TABLE 2. Comparison of *pmo* genes at the DNA and amino acids levels

Organisms	*pmoC*		*pmoA*		*pmoB*	
	% DNA identity	% Amino acid similarity (identity)	% DNA identity	% Amino acid similarity (identity)	% DNA identity	% Amino acid similarity (identity)
Strain SC2 *pmo2*-strain SC2 *pmo1*	69.0	74.8 (59.3)	70.9	78.9 (65.6)	67.4	75.4 (63.5)
Strain SC2 *pmo2*-*Methylocystis* sp. strain M	71.7	77.1 (64.0)	71.1	79.7 (65.2)	67.0	74.7 (61.4)
Strain SC2 *pmo2*-*M. trichosporium* OB3b	71.6	77.1 (63.6)	70.7	80.1 (64.5)	69.2	75.8 (64.1)
Strain SC2 *pmo2*-*M. capsulatus* Bath	67.5	71.6 (57.6)	65.5	74.6 (57.0)	60.5	67.1 (46.9)
Strain SC2 *pmo1*-*Methylocystis* sp. strain M	83.9	91.8 (85.9)	91.7	97.6 (95.2)	86.1	94.5 (89.5)
Strain SC2 *pmo1*-*M. trichosporium* OB3b	82.8	91.0 (80.5)	85.2	94.0 (87.3)	78.8	88.9 (81.2)
Strain SC2 *pmo1*-*M. capsulatus* Bath	68.8	72.9 (59.2)	66.6	76.2 (58.3)	59.2	61.2 (45.6)
Methylocystis sp. strain M-*M. trichosporium* OB3b	85.5	93.0 (85.9)	84.2	94.4 (86.5)	80.5	86.8 (78.7)
Methylocystis sp. strain M-*M. capsulatus* Bath	78.7	74.4 (60.3)	66.4	75.4 (57.5)	61.2	60.9 (45.2)
M. trichosporium OB3b-*M. capsulatus* Bath	69.2	73.3 (69.2)	67.4	76.2 (57.9)	60.6	61.8 (44.0)

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 PmoCAB of the type I MOB *M. capsulatus* Bath resulted in moderately lower levels of identity and similarity for PmoC and PmoA and clearly lower values for PmoB.

(ii) Putative secondary structures. The polypeptides predicted for both PmoCAB1 and PmoCAB2 are highly hydrophobic and contain several transmembrane-spanning regions (Fig. 2 and 3). All N-terminal sequences were predicted to be located in the cytosol. Comparison with putative transmembrane helices of PmoCAB subunits from *M. trichosporium* OB3b, *Methylocystis* sp. strain M, and *M. capsulatus* Bath revealed very high levels of structural similarity. The high degree of structural conservation of PmoCAB2 suggests that it clearly resembles PmoCAB1 in terms of its physicochemical properties. This allowed us to conclude that, like *pmoCAB1*, *pmoCAB2* encodes a membrane-associated protein (Fig. 3). As described by Gilbert and coworkers (14), the first PmoB transmembrane-spanning region (PmoB [helix 1]) (Fig. 3) has to be regarded hypothetically only, because the N-terminal region of PmoB is considered to constitute a leader sequence (26, 37, 40).

(iii) Highly conserved amino acid residues. Recently, Tukhvatullin et al. (37) described a structural and functional model for pMH from *M. capsulatus* Bath. This model was predicted based on amino acid residues found to be almost completely conserved in a data set consisting of 3 sequence types for PmoB1 and 12 sequence types for AmoB, 112 sequence types for PmoA1 and 349 sequence types for AmoA, and 5 sequence types for PmoC1 and 5 sequence types for AmoC. Because of the high degree of conservation, these amino acid positions are

believed to include various functionally important residues. Of the 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

```
        10           20           30           40           50           60
MNQTTEKAVS    AAQAGETDTI   VNYKPAYITM   GVMLVFYVGI   RVYEQYFGWK   AGLDSFAPEF

        70           80           90          100          110          120
QTYWMNLMWT    ELPLEFVAFC   GIGGYLWKTR   DRNIDAVAPR   EEMRRILTLI   GWLAVYAFSV

       130          140          150          160          170          180
YWGASYFTEQ    DGTWHQTVIR   DTDFTPSHIL   EFYLSYPIYI   ICGWGAFMYA   HTRIPQFAKR

       190          200          210          220          230          240
ISLAFLMFFA    GPFMIFPNIG   LNEWGHTFWF   MEELFTAPLH   WGFVFFGWFA   LAVFGVARQV

       250          258
LDRVIELSKE    YEKDALAL                                            ┌─────────┐
                                                                  │  PmoC   │
                                                                  └─────────┘
```

```
        10           20           30           40           50           60
MSASIETGSP    TGKTWKSKEE   FLGCVILTDW   ILLVILFAVL   LGSFHIHYML   LAGDWDFWID

        70           80           90          100          110          120
FKDRRMWPTV    TPIVAMCFAA   AAQSFFWTRF   RLPIGATTVV   LALLIGEWIN   RYDNFWGWTF

       130          140          150          160          170          180
FPINLVFPSA    LIPMGFWLDI   VLMISGSWLV   TALLGGLGWG   LLFYPINWPV   LAQYHQAAEI

       190          200          210          220          230          240
DGVLLTLADL    IGFNYVRTGT   PEYIRMVERG   TLRTFGKDVV   PVAAFFSGFI   SMLVYFLWWN

       250          256
MGKWFSTTKY    LEIEEV                                              ┌─────────┐
                                                                  │  PmoA   │
                                                                  └─────────┘
```

```
        10           20           30           40           50           60
MKSLNLHSMA    KCATARVVRL   WILGLAVAGA   LTTLPAATPA   AAHGERSQQA   FLRMRTLNWY

        70           80           90          100          110          120
DVKWSKTELN    VNDEMELTGK   VHVFSGWPQA   VARPGESFLN   VGEPGPVLIR   KSAFVGEVPV

       130          140          150          160          170          180
PRTFSMDVGN    DYEYKIVLKA   RRQGRYHVHV   QINVKDGGPI   VGPGQWITIK   GDMKDFTNPV

       190          200          210          220          230          240
TLLEGSTIDL    ETYGITWTYI   YHFFWMAAAA   AWILYWFMTK   GIIVRYWQVK   AGKGRELIGQ

       250          260          270          280          290          300
DEKRIGAVTL    AAVLLAVLVF   YASANREFPR   TLPMQAGLLT   GISPIDEPRS   TVAVQFKGGS

       310          320          330          340          350          360
YTVPGRELRI    RAKVTNNGKE   PIRLGEFTSA   GLRFLNPDVF   TTRPDFPDYL   LADRGLSVSD

       370          380          390          400          410          420
PNPIAPGETR    DLEVIVQDAR   FDIERLSDLA   YDTDSQFGGL   LFFFSPSGER   QRAEIGGPVI

       429
PKFQAGATL                                                        ┌─────────┐
                                                                 │  PmoB   │
                                                                 └─────────┘
```

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).

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 and may suggest that the novel pMMO-like enzyme has a different functional role in strain SC2 than the conventional pMMO has. To obtain further evidence for this hypothesis, we are currently assessing in cultivation studies the response of the level of *pmoCAB2* expression to various environmental conditions (e.g., different concentrations of methane and competitive
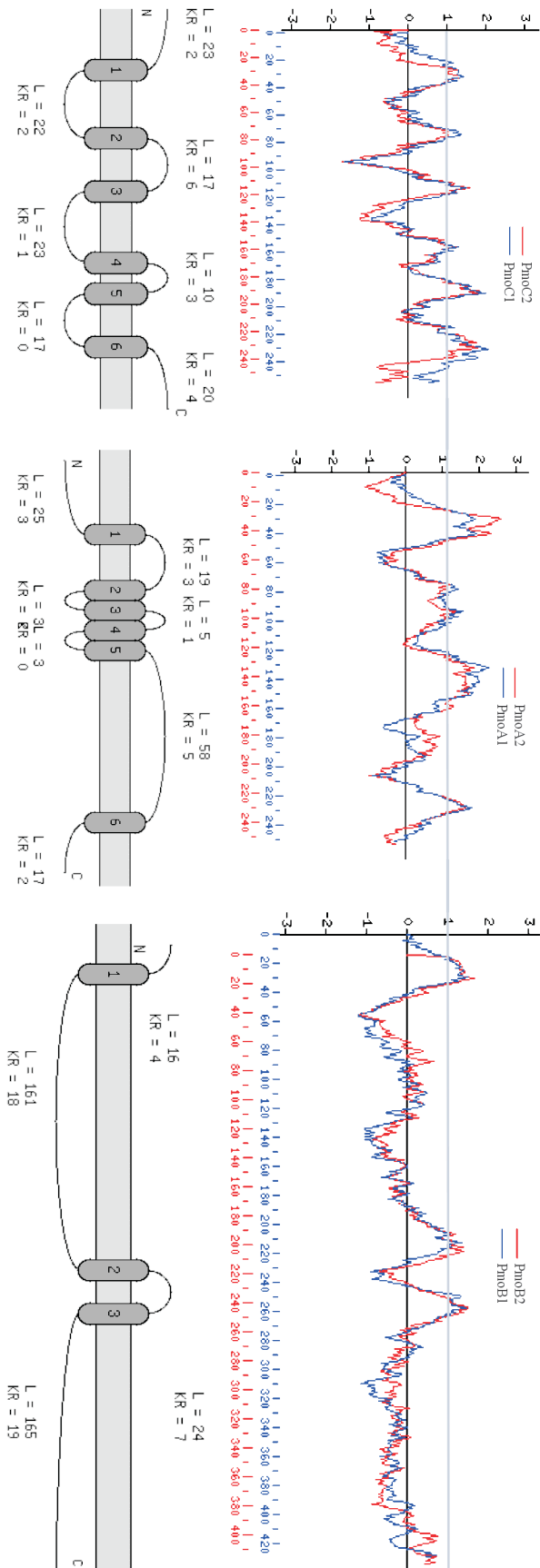
FIG. 3. Predicted topologies of derived PmoCAB1 and PmoCAB2 from *Methylocystis* strain SC2. (Top) Hydrophobicity plot of PmoCAB1 versus PmoCAB2. The *y* axis indicates the relative hydrophobicity value at a given position in PmoCAB. (Bottom) Schematic presentation of predicted transmembrane helices of PmoCAB2. N-terminal sequences are predicted to be located in the cytosol. Exact locations of transmembrane-spanning regions are shown in Fig. 2.

TABLE 3. Amino acid residues enabling differentiation of AmoA, PmoA1, and PmoA2

Position[a]	Residue in:		
	AmoA[b]	PmoA1	PmoA2
142	M[c]	1	L
145	T	s	S
147	n (d)[d]	S (A)	S (A)
157[e]	(a)	(i)	(I)
171	F (F)	I (I)	l (i)
172	G	a	A
179	V (I)	e (d)	E (D)
185	L	m	l
190	(f)	(i)	(I)
204	v	i	I
206	l	m	M
207	I	v	v (I)
209	(d)	(h)	(H)
211	S	t	T
217	G	k	K
218	H (H)	d (d)	D (D)
219	T (A)	v (i)	V (I)
221	V (I)	p (a)	P (A)

[a] The positions are the positions in PmoA2, as shown in Fig. 2.

[b] Identification of signature residues indicative of either AmoA or PmoA was based on 919 AmoA sequences, 315 PmoA1 sequences, and 40 PmoA2 sequences.

[c] Amino acid residues are indicated by the one-letter codes. Uppercase letters indicate residues which are conserved in at least 95% of the reference data set, while lowercase letters indicate residues which are conserved in at least 80% of the sequences compared.

[d] The letters in parentheses represent amino acid similarity groups, as follows: A, PAGST; D, QNEDBZ; H, HKR; I, LIVM; and F, FYW.

[e] A highly conserved amino acid residue could not be identified for positions 157, 190, and 209. However, the majority of residues identified for each individual data set at these positions belonged to the same amino acid similarity group.

substrates) and trying to generate mutants defective in either *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). This observation may be a further indication that the novel enzyme has a different functional role than the conventional pMMO has.

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