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# Wide Distribution of a Novel *pmoA*-Like Gene Copy among Type II Methanotrophs, and Its Expression in *Methylocystis* Strain SC2

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**Experiments were conducted to determine if a novel *pmoA*-like gene (*pmoA2*) recently discovered in the methane-oxidizing bacterium *Methylocystis* strain SC2 (P. F. Dunfield, M. Tchawa Yimga, S. D. Dedysh, U. Berger, W. Liesack, and J. Heyer, FEMS Microbiol. Ecol. 41:17-26, 2002) is present in other methane-oxidizing bacteria (MOB), and if it is expressed. A newly developed primer combination (pmoA206f-pmoA703b) allowed a differential detection of *pmoA1* and *pmoA2*. By using this primer combination, we identified *pmoA2* in a wide range of type II MOB of the *Methylosinus-Methylocystis* group. However, screening by PCR and by Southern hybridization using a newly developed *pmoA2*-specific oligonucleotide probe also showed that closely related type II MOB, exhibiting 16S rRNA gene sequence identities of higher than 97%, may or may not harbor *pmoA2*. No *pmoA2* was detected in five type I MOB tested: *Methylococcus capsulatus* strain Bath, *Methylocaldum* strain E10A, *Methylobacter luteus*, *Methylomicrobium album*, and *Methylomonas* strain D1a. In comparative sequence analyses, all *pmoA2*-like sequences formed a coherent cluster clearly distinct from *pmoA1* sequences of type I and type II MOB, and from *amoA* sequences of the *Nitrosomonas-Nitrosospira* group. Phylogenetic analysis using the paml model suggested that *pmoA2* is subject to strong purifying selection and therefore has an important cellular function. We probed total RNA extracts of *Methylocystis* strain SC2 for gene expression of *pmoA*. A strong signal was observed for *pmoA1* in Northern hybridization, while the results obtained for *pmoA2* were ambiguous. However, reverse transcription-PCR confirmed that *pmoA2* was expressed, albeit at lower level than *pmoA1*. This provided experimental evidence that the gene product of *pmoA2* may be a functionally active enzyme.**

Methane-oxidizing bacteria (MOB) are able to utilize methane ($CH_4$) as their sole source of carbon and energy for growth (13). Their ability to oxidize $CH_4$ released at the interface of methanogenic environments and to act as sink for atmospheric $CH_4$ makes these organisms key players in balancing the global $CH_4$ budget and mitigating global warming due to $CH_4$ (4, 28). Phylogenies based on 16S rRNA genes show that MOB form distinct lineages in the *Gammaproteobacteria* (type I MOB) and *Alphaproteobacteria* (type II MOB) (2, 5, 6, 13, 16).

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 enzyme differ in their structures, kinetic properties, and ranges of substrates they utilize (26). Only a restricted number of MOB species harbor sMMO, while almost all MOB possess pMMO. The only MOB lacking pMMO is *Methylocella palustris* (5).

Cloning and sequence analysis of genes encoding pMMO revealed three consecutive open reading frames (*pmoC*, *pmoA*, and *pmoB*) in both type I (30, 31) and type II MOB (11). The *pmoA* gene, which encodes the 27-kDa subunit (PmoA) of pMMO, has been shown to be evolutionarily highly conserved among methanotrophs (17). The type I MOB *Methylococcus capsulatus* strain Bath and *Methylomicrobium album* strain BG8 (30, 31), as well as the type II species *Methylosinus trichosporium* strain OB3b and *Methylocystis* sp. strain M (11) contain duplicate *pmoA* gene copies. The sequences of these duplicate gene copies have been shown to be nearly identical.

However, we recently demonstrated that the type II MOB *Methylocystis* strain SC2 contains two very different *pmoA*-like genes (9). The first gene (*pmoA1* or conventional *pmoA*) exhibited very high sequence homology to *pmoA* genes of other type II MOB (even identical amino acid sequence to PmoA of some other *Methylocystis* strains). The second gene (*pmoA*2 or novel *pmoA*) possessed only 73% identity with *pmoA1* at the nucleotide level and 68.5% identity at the amino acid level. PmoA2 of *Methylocystis* strain SC2 was closely related to the deduced amino acid sequence of a *pmoA*-like gene retrieved in a previous study by cultivation-independent methods from rice field soil (86.3% identity) (19), indicating that the presence of multiple, diverse *pmoA* gene copies might not be unique to strain SC2. This was also demonstrated by the detection of a novel *pmoA2*-like copy in *M. trichosporium* strain KS21 (9).

Our study aimed to assess whether the presence of multiple, diverse *pmoA* gene copies is a common genotypic trait among methanotrophs or a phenomenon restricted to a few methanotroph strains. We developed PCR primers and hybridization probes for differential detection of *pmoA1* and *pmoA2* in single MOB genospecies. PCR-based screening by terminal restriction fragment length polymorphism (T-RFLP) analysis, Southern hybridization and comparative sequence analysis revealed that the presence of multiple, diverse *pmoA* genes in single genospecies is common among type II MOB, but not type I MOB. We also show that, contrary to previous results

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TABLE 1. List of methanotrophic strains screened for the presence of *pmoA2*

| Strain designation | Taxonomic assignment | T-RFs at temp[a]: | | Detection of *pmoA2* | Source or reference |
|---|---|---|---|---|---|
| | | 60°C | 66°C | | |
| SM16 | *Methylocystis* sp. | 245, 438 | 438 | + | 16 |
| LR1 | *Methylocystis* sp. | 245, 438 | 438 | + | 8 |
| B2/7 | *Methylocystis* sp. | 245, 438 | 438 | + | 16 |
| 62/12 | *Methylocystis* sp. | 245, 438 | 438 | + | 16 |
| KS9 | *Methylocystis* sp. | 245, 350 | 350 | + | 16 |
| O14c | *Methylocystis* sp. | 245 | 245 | − | 16 |
| IMET 10484 | *Methylocystis* sp. | 245, 350 | 350 | + | 16 |
| 21/1 | *Methylocystis* sp. | 245 | 245 | − | 16 |
| IMET 10499 | *Methylocystis* sp. | 245, 350 | 350 | + | 16 |
| F10V2a | *Methylocystis* sp. | 115, 245 | 115 | + | 16 |
| KS12 | *Methylocystis* sp. | 209 | 209 | − | 16 |
| IMET 10486 | *Methylocystis* sp. | 245, 438 | 438 | + | 16 |
| SC2 | *Methylocystis* sp. | 245, 438 | 438 | + | 16 |
| IMET 10491$^T$ | *Methylocystis echinoides* | 245, 438 | 438 | + | 16 |
| Pi5/4 | *Methylocystis* sp. | 245 | 245 | − | 16 |
| 81 | *Methylocystis parvus* | 245, 350 | 350 | + | 16 |
| SC8 | *Methylosinus sporium* | 245, 350 | 350 | + | 16 |
| SK13 | *Methylosinus sporium* | 245, 350 | 350 | + | 16 |
| 20/3 | *Methylosinus sporium* | 245, 159 | 159 | + | 16 |
| H1b | *Methylosinus sporium* | 245, 350 | 350 | + | 16 |
| SM6 | *Methylosinus trichosporium* | 245, 350 | 350 | + | 16 |
| H5 | *Methylosinus trichosporium* | 245 | ND[b] | − | 16 |
| M23 | *Methylosinus trichosporium* | 245 | 245 | − | 16 |
| 39/3 | *Methylosinus trichosporium* | 245 | 245 | − | 16 |
| NCIMB 11131$^T$, OB3b | *Methylosinus trichosporium* | 245 | ND | − | NCIMB[c] |
| SC10 | *Methylosinus trichosporium* | 130, 350 | 350 | + | 16 |
| KS21 | *Methylosinus trichosporium* | 245, 279 | 279 | + | 16 |
| NCIMB 11914$^T$ | *Methylobacter luteus* | ND | ND | − | NCIMB |
| NCIMB 11123$^T$, BG8 | *Methylomicrobium album* | ND | ND | − | NCIMB |
| NCIMB 11853, Bath | *Methylococcus capsulatus* | ND | ND | − | NCIMB |
| D1a[d] | *Methylomonas* sp. | ND | ND | − | This study |
| E10a[d] | *Methylocaldum* sp. | ND | ND | − | This study |

[a] T-RFs observed in differential T-RFLP analysis. PCR at an annealing temperature of 60°C enabled simultaneous detection of both *pmoA1* and *pmoA2*, while PCR at 66°C led to the specific PCR amplification of *pmoA2* (if present).
[b] ND, not detected.
[c] NCIMB, National Collections of Industrial and Marine Bacteria Ltd.
[d] *Methylomonas* strain D1a and *Methylocaldum* strain E10a are novel isolates of type I MOB. Their taxonomic assignment is based on comparative 16S rRNA gene sequence analysis. Further details of the two strains will be published elsewhere.

(9), *pmoA2* of *Methylocystis* strain SC2 is expressed under standard growth conditions, although the level of expression is clearly lower than that of *pmoA1*.

## MATERIALS AND METHODS

**MOB strains.** The 32 strains used in this study are listed in Table 1. The conditions used for growth of the MOB were adapted from Heyer et al. (15, 16) or Leadbetter and Forster (22) and depended upon whether the cultures were intended for screening of *pmoA2* by PCR amplification or Southern blot analysis. Cultures to be used for PCR-based screening were grown, as described by Heyer et al. (16), on agar plates of mineral salts medium incubated at 30°C in closed glass chambers containing a gas mixture of 20% (vol/vol) $CH_4$, 5% $CO_2$, and 75% air. For Southern or Northern hybridization experiments, MOB were grown in liquid culture of medium 10 for *M. trichosporium* strain OB3b and *Methylocystis* strain SC2 or in medium 10 containing $NaNO_3$ instead of $NH_4Cl$ as the nitrogen source for *M. capsulatus* strain Bath and *Methylomonas* strain D1a. For growth of *Methylomonas* strain D1a, a supplement of vitamin solution was added to final concentrations of 1 μg/liter for vitamin $B_6$; 0.2 μg/liter for biotin and folic acid; 0.5 μg/liter for vitamin $B_1$, vitamin $B_2$, and DL-Ca-pantothenate; and 0.01 μg/liter for vitamin $B_{12}$. Liquid cultures were grown for 3 to 5 days at 30°C under a headspace of 20% (vol/vol) $CH_4$, 5% $CO_2$, and 75% air. Cells were pelleted at 4,000 × *g* for 20 min at 4°C and washed once with TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). The cell biomass was immediately used for extraction of DNA.

**DNA extraction.** DNA extraction for PCR-based studies was performed by a procedure based on mechanical agitation in a FastPrep FP120 cell disrupter (Savant, Holbrook, N.Y.) of 2-ml screw-cap reaction vessels filled with a mixture of culture, 0.1-mm-diameter silica-zirconium beads and phosphate buffer (pH 8.0) containing 2% sodium dodecyl sulfate (SDS) (14).

Cells grown in liquid culture (~1 g of fresh biomass) were suspended in 10 ml of TE buffer to which 25 mg of lysozyme and 20 μl of proteinase K (25 mg/ml) were added. The suspension was placed in a 37°C water bath for 2 h, after which a 10% (wt/vol) solution of SDS was added to a final concentration of 1%, followed by a 1.5-h incubation at 37°C. After centrifugation for 15 min at 4°C, the supernatant was collected, and 1 ml of 5 M potassium acetate (pH 7.5) was added. The suspension was centrifuged for 15 min at 4°C, and the resulting supernatant was transferred to a new vessel. Total nucleic acids were extracted from the supernatant twice with 1 volume of chloroform-isoamyl alcohol (24:1 [vol/vol]) and then precipitated from the aqueous phase with 1 volume of isopropanol. The nucleic acids were resuspended in 5 ml of TE buffer.

For removal of coextracted RNA, 30 μl of 100-mg/ml RNase A and 20 μl of 100,000-U/ml RNase $T_1$ were added, followed by incubation for 2 h at 37°C. The RNase treatment was stopped by extraction three times with 1 volume of chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated from the aqueous phase with 1 volume of isopropanol and then resuspended in 100 μl of TE buffer. The amount of DNA extracted was estimated by electrophoresis of aliquots on a 1% agarose gel and comparison to a *Pst*I digest of phage λ DNA.

**Extraction of total RNA from *Methylocystis* strain SC2.** Total nucleic acids were extracted as described above for strains grown in liquid culture, except that solutions for extracting RNA were prepared with diethyl pyrocarbonate (DEPC)-treated deionized water. For the removal of coextracted DNA, 1 volume of TMC buffer (10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 0.1 mM $CsCl_2$) (7) and 20 μl (5 U) of RNase-free DNase (Promega, Madison, Wis.) were added, followed by incubation for 2 h at 37°C. The reaction was stopped by extraction

TABLE 2. Oligonucleotide primers and probes used in this study

| Name[a] | Target | Strategy | Gene positions[b] | Sequence (5′→3′)[c] | Reference |
|---|---|---|---|---|---|
| A189f | *pmoA* | PCR, RT-PCR | 172–189 | GGNGACTGGGACTTCTGG | 17 |
| A682b | *pmoA* | PCR, RT-PCR | 702–685 | GAASGCNGAGAAGAASGC | 17 |
| pmoA206f | *pmoA1* and *pmoA2*[d] | PCR, RT-PCR | 172–206 | GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG | This study |
| pmoA703b | *pmoA1* and *pmoA2*[d] | PCR | 702–668 | GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT | This study |
| pmoA636b | *pmoA2* | S/N blot,[e] RT-PCR | 635–610 | ATCATGCGGATGTATTCMGGSGTGCC | This study |
| A593b | *pmoA1* (strain SC2) | S/N blot, RT-PCR | 615–593 | CATCGACGTGCGGACGAAGTGGA | 9 |
| Eub9f | 16S rRNA gene | PCR | 9–27 | GAGTTTGATCMTGGCTCAG | 21 |
| Eub1492b | 16S rRNA gene | PCR | 1512–1492 | ACGGYTACCTTGTTACGACTT | 34 |

[a] All (including labeled) oligonucleotides were purchased from Metabion (Martinsried, Germany).
[b] Numbering for *pmoA* refers to the *pmo* gene sequence of *Methylococcus capsulatus* strain Bath (30). Numbering for 16S rDNA refers to the 16S rRNA gene sequence of *E. coli* (3).
[c] N = A,T,C, or G; M = C or A; R = A or G; Y = C or T; and S = G or C.
[d] The use of the primer combination pmoA206f and pmoA703b in PCR at an annealing temperature of 60°C enabled simultaneous detection of both *pmoA1* and *pmoA2*, while use at 66°C led to the specific PCR amplification of *pmoA2* (if present).
[e] S/N blot, Southern or Northern blot.

three times with 1 volume of chloroform-isoamyl alcohol (24:1 [vol/vol]). The total RNA was precipitated from the aqueous phase with 1 volume of isopropanol and resuspended in 100 ml of DEPC-pretreated TE buffer. The integrity of the 16S and 23S rRNA fragments was checked by electrophoresis on a 1.2% agarose gel and comparison to an rRNA standard from *Escherichia coli* (Roche Diagnostics).

**Differential detection of *pmoA1* and *pmoA2*.** The *pmoA*-based diversity present in a single strain was assessed by determining the number and the size of terminal restriction fragments (T-RFs) observed in restriction digests of *pmoA* genes PCR-amplified with the primer combination pmoA206f-pmoA703b at two different annealing temperatures (Table 2). For T-RFLP analysis, the 5′ primer (pmoA206f) was labeled with the dye 5-carboxyfluorescein. The reaction mixture contained 1 μl of template DNA, 7.5 μl of 10× reaction buffer, 1.5 mM $Mg^{2+}$, 150 μM (each) deoxynucleoside triphosphate (dNTP), 0.125 μM (each) primer, and 2.5 U of *Taq* DNA polymerase (Promega, Mannheim, Germany). The thermal PCR profile was as follows: initial denaturation for 3 min at 94°C followed by 32 cycles consisting of denaturation at 94°C for 60s, primer annealing at 60 or 66°C for 60 s, and elongation at 72°C for 60s. The final extension step was extended to 7 min. Aliquots of the amplicons (10 μl) were checked by electrophoresis on a 1% agarose gel.

The digestion of purified amplicons (100 ng) and separation of the restriction digests on an ABI 373A automated sequencer (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) were carried out as described previously (24).

For direct sequencing of amplicons obtained at an annealing temperature of 66°C, PCR was carried out with primer pmoA206f without fluorescence label. Amplicons were purified by using the Qiagen PCR purification kit (Qiagen, Hilden, Germany) according to the recommendations of the supplier. Both strands of the PCR products were analyzed by using the dye terminator sequencing chemistry of PE Applied Biosystems and either pmoA206f or pmoA703b as the sequencing primer.

**Southern hybridization.** Aliquots of genomic DNA (10 μg) were digested overnight at 37°C with 50 U of restriction enzyme *Pst*I, *Eco*RI, or *Xho*I according to the protocols recommended by the supplier (Promega, Madison, Wis.). The restricted DNA was precipitated with ethanol, dried in a vacuum desiccator (Savant), resuspended in 20 μl of TE buffer, and separated on a 0.8% agarose gel at 25 V for 12 h. Southern blotting was carried out according to the procedure described in the *DIG Application Manual for Filter Hybridization* (Roche Diagnostics GmbH, Mannheim, Germany, 2000).

The digoxigenin (DIG)-labeled oligonucleotide probe pmoA636b (Table 2) was used for specific detection of *pmoA2*. A *pmo* gene probe was used for simultaneous detection of both *pmoA1* and *pmoA2*. This probe was based on a mixture of DIG-labeled amplicons of the different strains tested. PCR was carried out with either primer combination A189f-A682b (16) or pmoA206f-pmoA703b and an annealing temperature of 60°C. For direct labeling during PCR, DIG-dUTP was mixed with dTTP in the proportional relationship of 1:3.

Both oligonucleotide probe pmoA636b and the *pmo* gene probe were applied under the same stringency conditions. Hybridizations were carried out overnight at 42°C with a standard hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% blocking solution). Membranes were washed in 0.5× SCC at 60°C for 30 min, and hybridization signals were detected on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) after reaction with the ECF substrate for Western blotting (Amersham, Piscataway, N.J.).

**Northern hybridization.** Approximately 60 μg of total RNA from *Methylocystis* strain SC2 was first separated on a 1% formaldehyde agarose gel at 60 V for 3 h in presence of 1× MOPS (morpholinepropanesulfonic acid) buffer (10× MOPS buffer is 200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA [pH 7.0]). Northern blotting was carried out according to the procedure outlined in the *DIG Application Manual for Filter Hybridization* (Roche Molecular Diagnostics). Blotting was performed overnight on a Hybond-N-membrane (Amersham). The DNA was UV cross-linked using a UV Stratalinker 2400 (Stratagene, La Jolla, Calif.) at 266 nm for 3 min. The DIG-labeled oligonucleotide probes A593b and pmoA636b were used for specific detection of *pmoA1* and *pmoA2*, respectively (Table 2). Hybridizations were performed overnight at 40°C. Washing and visualization were carried out as described above for Southern hybridization.

**RT-PCR.** Copy DNA (cDNA) of mRNA from *pmoA* genes expressed in *Methylocystis* strain SC2 was synthesized with a Qiagen Omniscript kit according to the instructions of the manufacturer. The reverse transcription (RT) was carried out in a total volume of 20 μl at 37°C for 30 min. The reaction mixture contained 2 μg of total RNA from strain SC2, 0.5 mM (each) dNTP, reverse transcriptase buffer, 10 U of RNase inhibitor, 1.0 μM primer A682b (Table 2), and 4 U of Omniscript reverse transcriptase. Aliquots (1 μl) of the cDNA solution were used for subsequent PCR amplification. The primer combinations A189f-A593b and pmoA206f-pmoA636b were used to specifically amplify cDNA from either *pmoA1* or *pmoA2*. The reaction cocktail contained 1 μl of RT product, 10 μl of 10× reaction buffer, 1.5 mM $Mg^{2+}$, 200 μM each deoxynucleoside triphosphate (dNTP), 0.25 μM each primer, and 2.5 U of *Taq* DNA polymerase (Promega). The thermal profile was as follows: initial denaturation for 3 min at 94°C followed by 32 cycles of denaturation at 94°C for 40 s, primer annealing at 62°C for 40 s, and elongation at 72°C for 45s. The final extension step was extended to 7 min. Amplification was performed in a total volume of 100 μl in 0.2-ml reaction vessels and a DNA thermal cycler (model 2400; PE Applied Biosystems). Aliquots of the amplicons (10 μl) were checked by electrophoresis on a 1% agarose gel. Amplicons of the expected size were purified and sequenced as described above.

**Analysis of sequence data.** Trees were constructed for partial PmoA sequences (151 deduced amino acid positions) by using TreePuzzle and the ARB program package (developed by O. Strunk and W. Ludwig of the Technical University of Munich [http://www.arb-home.de]). TreePuzzle is a quartet maximum-likelihood method for reconstructing tree topologies (32, 33). The amino acid substitution model described by Adachi and Hasegawa (1) was used to construct the trees.

A tree was constructed for the same set of data on the nucleic acid level by the maximum-likelihood method FastDNAml (10).

In order to gain insight into the evolution and expression of the *pmoA2* gene, a phylogenetic tree of selected sequences was analyzed with the codeml function of the paml program of Ziheng Yang (http://abacus.gene.ucl.ac.uk/software/paml.html#GetPAML). This program calculates the relative rate of nonsynonomous (amino acid changing) to synonomous (non-amino acid changing) nucleotide substitutions ($d_N/d_S$, or ω) in a phylogeny (35). An ω value of <1 indicates that purifying natural selection is acting to prevent changes in a protein, while an ω value of >1 indicates diversifying selection. A ω value = 1 indicates neutral selection and would be expected for a pseudogene.

**Nucleotide accession numbers.** The *pmoA2* gene sequences obtained in the course of this study from environmental samples and cultured MOB have been deposited in the EMBL, DDBJ, and GenBank databases under the accession no. AJ543418 to AJ543423 and AJ544093 to AJ544102, respectively. The *pmoA1*

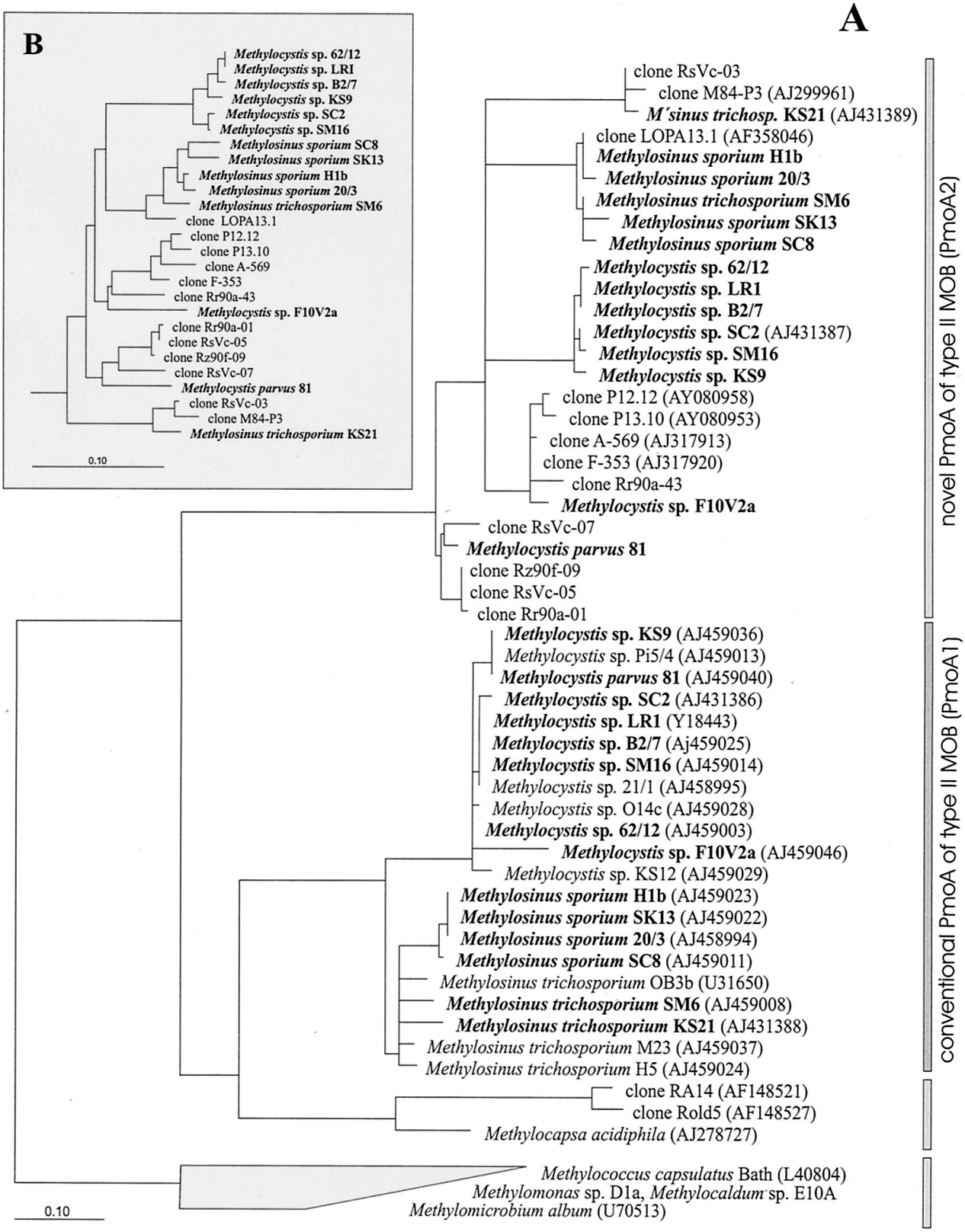


FIG. 1. Phylogenetic trees constructed for partial *pmoA* sequences and deduced amino acid sequences (PmoA1 and PmoA2). (A) PmoA tree showing the relationship of PmoA1 and PmoA2 of type II MOB of the *Methylosinus-Methylocystis* group to environmental clones as well as to *Methylocapsa acidiphila* and selected type I MOB. The environmental sequences were retrieved by cultivation-independent methods from the following habitats: forest soil (clones RA14, Rold5 [18], and P12.12 and P13.10 [27]), peat soil (clone LOPA13.1 [25]), natural and manipulated organic soils (clones A-569 and F-353), as well as bulk soil (clones RsVc-03, RsVc-05, and RsVc-07) and rice roots (clones M84-P3 [19], Rr90a-01,

sequences obtained for reference from *Methylocaldum* strain E10a and *Methylomonas* strain D1a have been deposited under accession no. AJ5441091 and AJ544092, respectively.

## RESULTS

**Differential detection of *pmoA1* and *pmoA2* by T-RFLP analysis.** The first aim of the present study was the development of an appropriate method for detection of multiple, divergent *pmoA* genes present in a single organism. Using the *pmoA2*-like environmental clone sequence M84-P3 (19) and the *pmoA2* sequence of *Methylocystis* strain SC2 as the starting point, we designed several forward and reverse primers for specific retrieval of *pmoA2* from both environmental samples and cultured MOB. According to the *pmoA* database available, none of these primers perfectly matched *pmoA1* or *amoA*, suggesting that these primers should be specific for *pmoA2*. Environmental DNA from rice paddy soil is composed of genomic DNA from a complex community of phylogenetically diverse microorganisms, including various type I and II methanotrophs (19). Consequently, to assess the target specificity and the range of *pmoA2*-like sequence diversity detectable, we tested various primer combinations in first- and second-round PCRs using environmental DNA extracted from flooded rice microcosms in a previous study (19). This led to the retrieval of various *pmoA2*-like sequences from bulk soil and rice roots of flooded rice microcosms. Six representatives of these environmental clone sequences have been deposited in the EMBL, DDBJ and GenBank databases (see Materials and Methods) and are shown in Fig. 1. The best performance with regard to both target specificity and range of *pmoA2*-like sequence diversity detectable was exhibited by the primer combination pmoA206f-pmoA703b (Table 2).

Using *Methylocystis* strain SC2 as the model organism, this primer combination was therefore chosen to establish for cultured type II MOB a tool for differential detection of *pmoA1* and *pmoA2* by T-RFLP analysis. The T-RF size distribution of *pmoA1* and *pmoA2* was assessed by computer simulation. The T-RF sizes (5′ or 3′ termini) were predicted for as many as 10 different restriction endonucleases. For *Msp*I, we identified two distinct 5′-T-RFs, which corresponded to *pmoA1* (245 bp) and *pmoA2* (438 bp), respectively. Based on the in silico analysis, we concluded that PCR with primer pair pmoA206f-pmoA703b followed by digestion with *Msp*I was the simplest way to classify the two different *pmoA* sequence types present in *Methylocystis* strain SC2. To test our predictions experimentally, PCR amplification of *pmoA* was conducted for subsequent T-RFLP analysis. The effect of annealing temperature used in PCR on the *pmoA* sequence type detectable via T-RFLP analysis was investigated by performing the amplifications at various annealing temperatures. Two different T-RFs with sizes of 245 and 438 bp were observed at an annealing temperature of 60°C, whereas only one distinct T-RF was observed at an annealing temperature of 66°C (Fig. 2). Thus, no discrepancy was observed between the results expected by in silico analyses and the empirical data obtained. However, to verify that only *pmoA2* was amplified at 66°C, another PCR was carried out with the annealing temperature of 66°C, and the resulting PCR product was sequenced. Comparative sequence analysis confirmed that the product was derived from *pmoA2*. This finding indicated that T-RFLP analysis in combination with comparative sequence analysis could be a rapid and reliable method for screening methanotrophic pure cultures for the presence of multiple, divergent *pmoA* gene copies.

**Screening for *pmoA2* in single MOB strains by T-RFLP analysis.** In addition to *Methylocystis* strain SC2 and *M. trichosporium* strain KS21, 30 pure cultures, including 25 type II MOB and 5 type I MOB, were screened for the presence of *pmoA2* by differential T-RFLP analysis. Prior to *pmoA*-based analysis, the identity of each strain was confirmed by comparative sequence analysis of its 16S rRNA gene (16).

Except for *M. trichosporium* strains OB3b and H5, PCR products were obtained for all type II MOB at both annealing temperatures (60 and 66°C). The five type I MOB tested failed to give a positive PCR with the primer combination pmoA206f-pmoA703b (Table 1). The T-RFLP patterns obtained for the type II MOB species revealed multiple, distinct T-RFs within single strains, suggesting that some degree of *pmoA* sequence diversity was present. For all of the strains that tested positive at an annealing temperature of 66°C, a single T-RF was observed. Depending upon the strain, these T-RFs were 115, 159, 209, 245, 279, 350, or 438 bp in size (Table 1). Representatives of each of these different T-RFs were further investigated by direct sequencing of the *pmoA* amplicon generated at an annealing temperature of 66°C. Translated sequences of PCR products were aligned and compared with a set of representative PmoA sequences. It was confirmed that the 209- and 245-bp T-RFs were derived from typical *pmoA1* sequences of type II MOB (*Methylosinus-Methylocystis* group) that could still be amplified with the presumed *pmoA2*-specific primer set at 66°C. However, T-RFs with sizes of 115, 159, 279, 350, and 438 bp were indicative of *pmoA2* (Table 1). The treeing analysis showed that all PmoA2 sequences obtained from type II MOB of the *Methylosinus-Methylocystis* group (Table 1) formed a coherent cluster clearly distinct from that of PmoA1 (Fig. 1). In addition, cloned sequences retrieved by cultivation-independent approaches from various environments, including forest soil, peat soil, natural and manipulated organic soils, and flooded rice microcosms grouped within the PmoA2 cluster.

The intercluster PmoA identity values between sequence

Rr90a-43, and Rz90f-09) of flooded rice microcosms. In the present study, the retrieval of environmental *pmoA2*-like sequences from the bulk soil and rice roots of flooded rice microcosms was based on total DNA extracted in a previous study (19). Type II MOB for which both PmoA1 and PmoA2 sequences could be detected are shown in boldface. GenBank accession numbers are given in parentheses after the species or clone names for sequences that were not obtained in this study. The tree was constructed by using TreePuzzle, a quartet maximum-likelihood method (32, 33). Lineages for which, based on 25,000 puzzling steps, the exact branching order could not be unambiguously determined are shown by multifurcation. The scale bar represents 0.1 change per amino acid position. (B) *pmoA* tree showing the *pmoA2* intracluster relationship at the nucleic acid level. The tree was constructed with the same data set as that on the PmoA level and by the maximum-likelihood method (FastDNAml [10]); however, the presentation of the tree is restricted to the *pmoA2* cluster. The distance bar represents 0.1 substitution per nucleotide position.

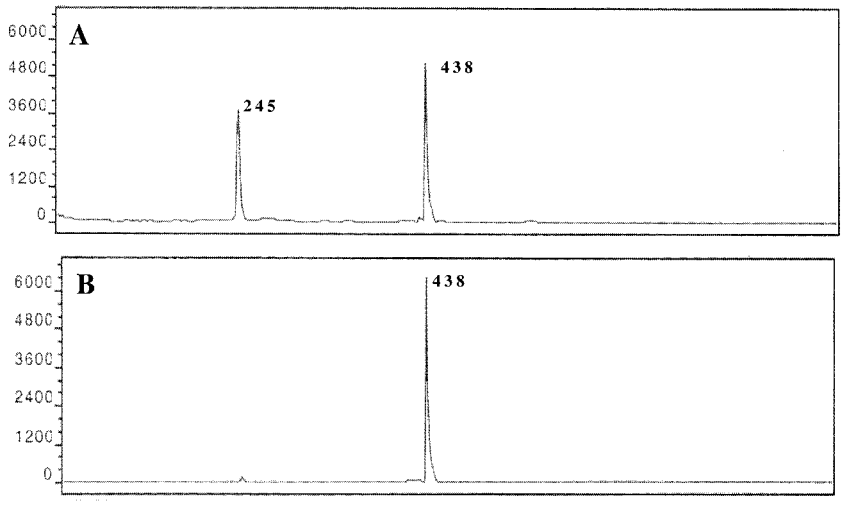


FIG. 2. *pmoA*-based T-RFLP profiles of *Methylocystis* strain SC2. PCRs were carried out with primers pmoA206f and pmoA703b at two different annealing temperatures: 60°C (A) and 66°C (B). On the *y* axis, the intensities of fragments are given in arbitrary units. Numbers indicate the lengths of T-RFs corresponding to *pmoA1* (245 bp) and *pmoA2* (438 bp).

types of PmoA1 and PmoA2 ranged from 64 to 74.5%. The lowest *pmoA2* and PmoA2 intracluster identity values were 18.2 and 21.5%, respectively. For comparison, the corresponding values for *pmoA1* and PmoA1 were 14.7 and 12.4%, respectively. The PmoA2 cluster was divided into five sublineages. These sublineages were characterized by *Methylocystis parvus* strain 81, *Methylocystis* strain F10V2a, *M. trichosporium* strain KS21, or by a set of either *Methylocystis* or *Methylosinus* spp.

**Screening for *pmoA2* by Southern hybridization.** In order to provide further evidence for the absence of *pmoA2* in *M. trichosporium* strain OB3b and three type I MOB by a second, PCR-independent approach, a 26-bp oligonucleotide probe was formulated for the specific detection of *pmoA2* (termed pmoA636b) (Table 2) by Southern hybridization. Based on the matching target region of *pmoA2*-like sequences initially available for the design of this probe, including environmental clone sequences directly retrieved from flooded rice microcosms, *Methylocystis* strain SC2 (one weak G:T mismatch) and *M. trichosporium* KS21 (Fig. 3), we concluded that pmoA636b should target a wide range of diverse *pmoA2* sequence types (compare Fig. 1 and 3). The probe exhibited various mismatches to the corresponding target sites of *pmoA1* of the MOB investigated by Southern hybridization. Southern blots of digested chromosomal DNA were hybridized, respectively, with the oligonucleotide probe pmoA636b and a *pmoA* gene probe (generated as a mixture of DIG-labeled *pmoA* amplicons of the different strains tested). All hybridization experiments were carried out with *Methylocystis* strain SC2 as a positive control. Under the hybridization and washing conditions used, probe pmoA636b did not detect *pmoA1* for any of the MOB tested. As expected, no *pmoA2* signal was observed for the type I MOB *M. capsulatus* strain Bath, *Methylocaldum* strain E10a, or *Methylomonas* strain D1a (Fig. 4). A *pmoA2* signal was also not obtained for *M. trichosporium* strain OB3b, but the presence of *pmoA2* was confirmed in another *M. trichosporium* strain (SM6) and in *Methylocystis* strain SC2. These results agreed well with those obtained by PCR. Full-length gene probes of *pmoA* detected multiple *pmoA* copies in several of the strains. Three gene copies, including *pmoA2*, were detected in the genomic DNA of *M. trichosporium* strain SM6 and *Methylocystis* strain SC2, while two *pmoA* copies were observed for *M. trichosporium* strain OB3b and the three type I MOB (Fig. 4).

**Expression of *pmoA2* in *Methylocystis* strain SC2 (theoretical considerations).** The paml model (35) was applied to a phylogenetic tree incorporating selected *pmoA1* and *pmoA2* gene sequences as shown in Fig. 5. The phylogeny was fit to a model incorporating eight separate ω values. All estimated ω values were <1. The ω values within the *pmoA1* and *pmoA2* clusters were <0.1, indicating that in recent evolutionary history, strong purifying selection has been acting on both genes. This is strong evidence that both genes are expressed and have important cellular functions. Interestingly, the branch connect-

| | |
|---|---|
| Probe pmoA636b | 5′...CCGTGSGGMCTTATGTAGGCGTACTA...3′ |
| Target | |
| Clone M84-P3 | 3′...GGCACGCCTGAATACATCCGCATGAT...5′ |
| Strain SC2, *pmoA2* | ...GGCACCCCGGAATACATCCGCATG**G**T... |
| Strain SC2, *pmoA1* | ...**TCG**A**T**GCCGGAATA**T**ATCCGCATGAT... |
| Strain OB3b$^T$, *pmoA1* | ...**TCG**A**T**GCCGGAATA**T**ATCCGCATG**G**T... |
| Strain SM6, *pmoA2* | ...GGCACGCCGGAATACATCCGCATG**G**T... |
| Strain SM6, *pmoA1* | ...**TCG**A**T**GCC**C**GA**G**TA**T**ATCCGCATG**G**T... |
| Strain D1A, *pmoA1* | ...GG**T**AC**A**CCTGAATACATCCG**T**ATG**G**T... |
| Strain E10A, *pmoA1* | ...GG**T**ACCCC**C**GA**G**TACATCCGCATG**G**T... |
| Strain Bath, *pmoA1* | ...GG**T**ACGCCTGA**G**TACATCCGCATG**G**T... |

FIG. 3. Alignment showing the targeting region of probe pmoA636b among *pmoA2* and corresponding *pmoA1* sequences of those MOB investigated by Southern hybridization. The clone M84-P3 is shown as a representative of a set of diverse environmental *pmoA2* sequences that perfectly match in the target region probe pmoA636b, including clones RsVc-03, Rr90a-43, RsVc-07, Rz90f-09, RsVc-05, and Rr90a-01 (Fig. 1). Letters shown in boldface indicate weak G:T mismatching nucleotide positions, while those shown in boldface and underlined indicate stronger mismatches (e.g., C:T, C:C, or G:G). Strains shown in the alignment are *Methylocystis* strain SC2, *M. trichosporium* strains OB3b and SM6, *Methylomonas* strain D1a, *Methylocaldum* strain E10a, and *M. capsulatus* strain Bath.

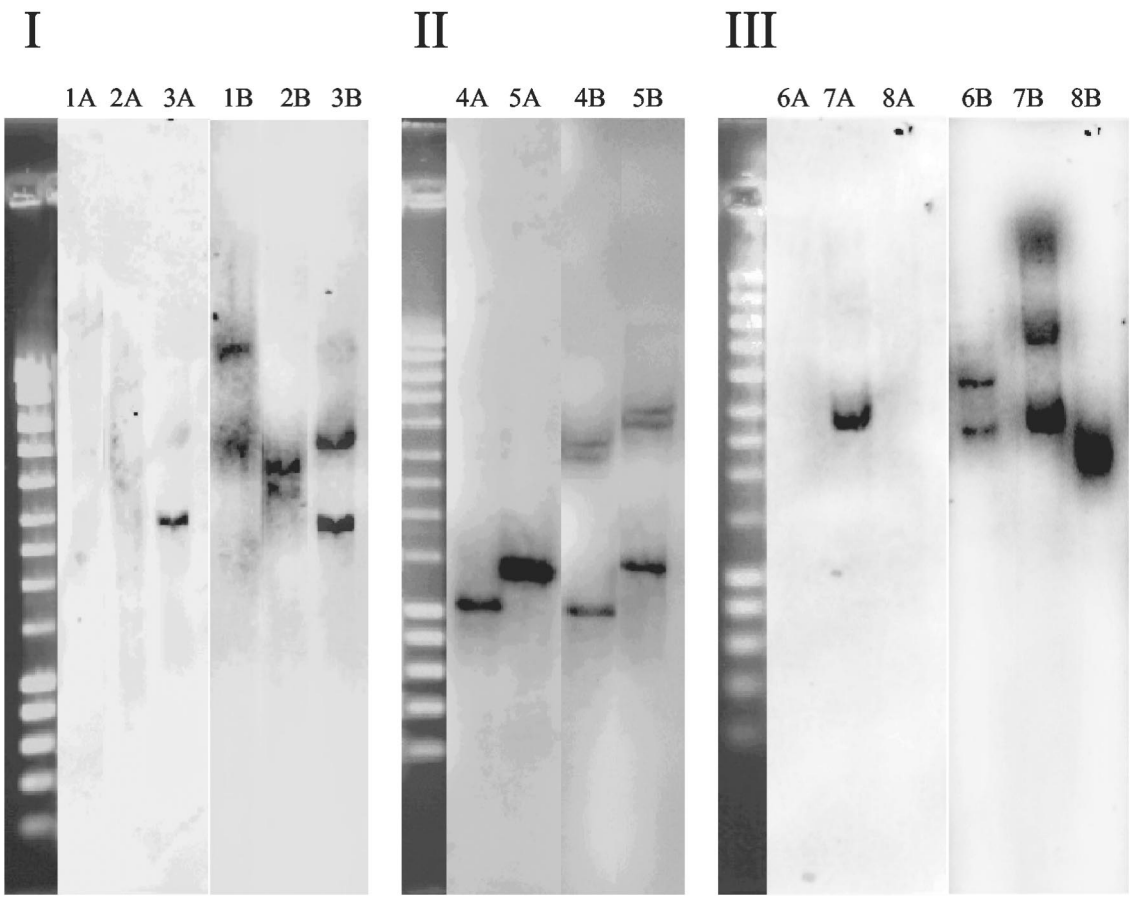


FIG. 4. Southern hybridization of genomic DNA to oligonucleotide probe pmoA636b (A lanes) or PCR-generated universal *pmoA* gene probe (B lanes). Under the hybridization conditions used in this study, probe pmoA636b is specific for *pmoA2* (Fig. 3), while the universal *pmoA* gene probe detects both *pmoA1* and *pmoA2*. (I) *Eco*RI digests. Lanes: 1, *M. trichosporium* strain OB3b; 2, *Methylocaldum* strain E10A; 3, *Methylocystis* strain SC2. (II) *Xho*I digests. Lanes: 4, *Methylosinus trichosporium* strain SM6; 5, *Methylocystis* strain SC2. (III) *Pst*I digests. Lanes: 6, *Methylococcus capsulatus* strain Bath; 7, *Methylocystis* strain SC2; 8, *Methylomonas* strain D1a. *Methylocystis* strain SC2 was used as positive control (9). The *Pst*I digest of DNA of phage λ was used as a size marker.

ing the *pmoA2* cluster to the other branches of the phylogeny has an elevated ω value of 0.28. While still <1, this increase may indicate a relaxation of purifying selection or even an increase in diversifying selection forces acting at particular times or on particular codons. Although purely speculative, a functional differentiation of the *pmoA1* and *pmoA2* genes may have occurred. Two different experimental strategies (i.e., Northern hybridization and RT-PCR) were applied to assess whether *pmoA2* was expressed when strain SC2 was grown under standard laboratory growth conditions (see Materials and Methods section "MOB strains").

**Expression of *pmoA2* in *Methylocystis* strain SC2 (experimental assessment).** Total RNA of *Methylocystis* strain SC2 was extracted and separated on a 1% formaldehyde agarose gel and blotted onto a nylon membrane for Northern hybridization. Probing was carried out with oligonucleotide A593b or pmoA636b specific for either *pmoA1* or *pmoA2*. In comparison to a strong signal observed for *pmoA1*, no conclusive signal was obtained for *pmoA2* (Fig. 6).

Consequently, we applied RT-PCR as a second, methodologically more sensitive, approach. Initially, we used the primers A593b and pmoA636b (Table 2) for RT. The primer combinations A189f-A593b and pmoA206f-pmoA636b were used to specifically amplify cDNA from either *pmoA1* or *pmoA2*. Sequencing of RT-PCR products confirmed strong expression of *pmoA1*. PCR of the cDNA generated with probe pmoA636b gave a product of nearly the size we expected. Sequencing of that product determined that it was not *pmoA*; it corresponded to a portion of 23S rRNA. We therefore assumed that nonspecific cDNA was formed, perhaps because the primer may have bound to a nonspecific target at the lower temperature of RT. A similar problem occurred in our earlier attempts to perform RT-PCR (9) and may have contributed to our initial failure to detect *pmoA2* expression in strain SC2. In an attempt to overcome the problem, we used the universal *pmoA* primer A682b to generate the cDNA. Using the cDNA as template, we carried out specific PCR assays in which we applied either A593b or pmoA636b as the reverse primer. In both cases, products of the expected size were obtained (Fig. 7), and comparative sequence analysis of the RT-PCR product showed that sequences corresponded either to *pmoA1* or to *pmoA2*.

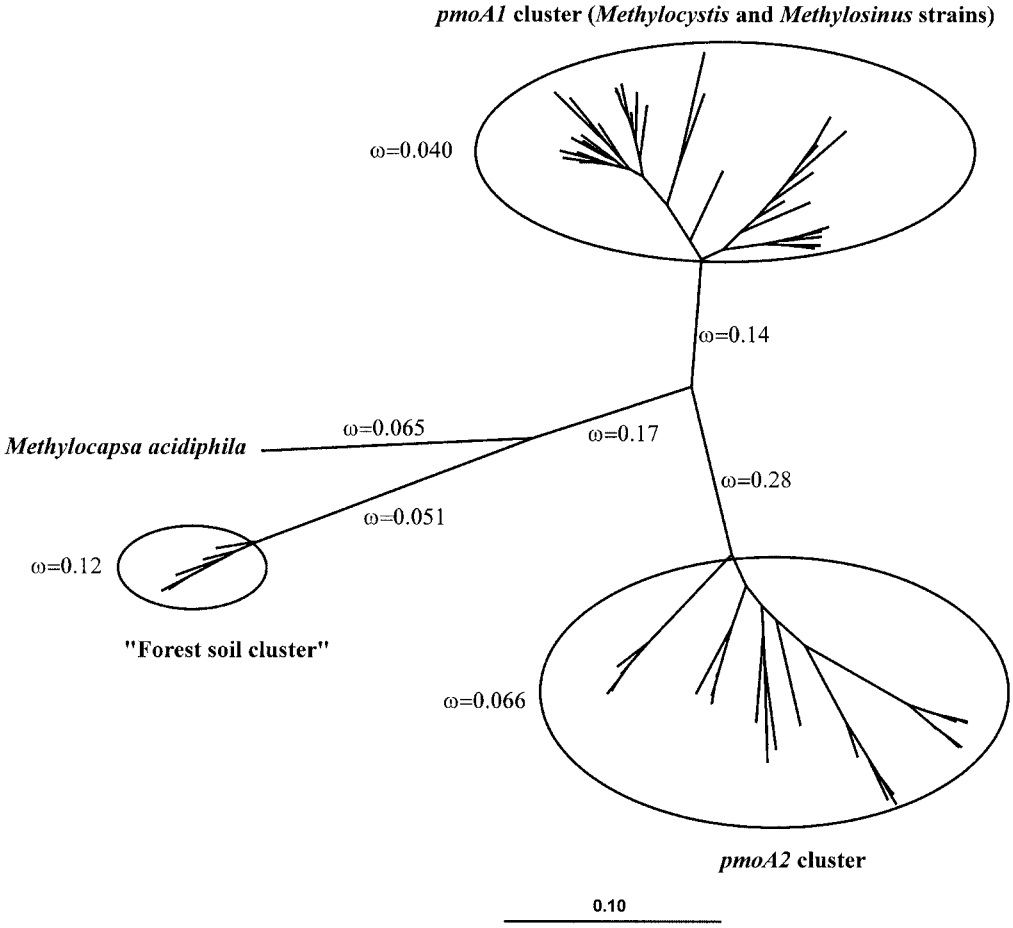


FIG. 5. Analysis of ω values within an unrooted phylogenetic tree incorporating 61 selected *pmoA* nucleotide sequences. The branching topology was calculated by using a neighbor-joining algorithm (29) with a Jukes-Cantor correction (20) (2,000 data resamplings), and branch lengths were adjusted by the paml program. A maximum-likelihood algorithm produced nearly the same tree topology. The distance bar indicates 0.1 nucleotide substitution per position. The paml model (model 2 in codeml) was fit with eight separate ω values as indicated: one for each of the five longest branches and one each for the *pmoA1* cluster, the *pmoA2* cluster, and the "forest soil cluster," respectively, which are enclosed by circles. The *pmoA1* cluster included sequences from various *Methylocystis* and *Methylosinus* strains (accession no. U31569, U31651, AJ431386, AJ431388, AJ458994, AJ458998, AJ459000-AJ459003, AJ459008, AJ459011, AJ459013, AJ459014, AJ459019, AJ459021-AJ459025, AJ459027-AJ459029, AJ459034, AJ459036, AJ459038, AJ459046-AJ459048, and AJ459052). The *pmoA2* cluster included sequences from pure cultures of *Methylocystis* and *Methylosinus* and sequences retrieved from soils by cultivation-independent methods (accession no. AJ431389, AJ299961, AY080950, and AJ459013 and sequences from the present study). The "forest soil cluster" contains sequences retrieved by Holmes et al. (18) using cultivation-independent methods (accession no. AF148525, AF148521, AF148527, AF148528, and AF148523).

## DISCUSSION

The newly developed PCR assay enabled the differential detection of *pmoA1* and *pmoA2* in single MOB strains by T-RFLP analysis. The *pmoA*-based T-RFLP fingerprinting and comparative sequence analysis of 27 different type II MOB strains revealed that *pmoA2* is widely distributed among type II MOB, including strains of *Methylosinus sporium*, *M. trichosporium*, *Methylocystis echinoides*, and *M. parvus*, as well as a set of other *Methylocystis* strains (16). The MOB strains that tested positive for *pmoA2* can be considered to reflect the full phylogenetic diversity known within the *Methylosinus-Methylocystis* group. The treeing analysis clearly showed that *pmoA2* and deduced PmoA2 sequences form a coherent cluster clearly distinct from that of *pmoA1* and deduced PmoA1, respectively. It is noteworthy that PmoA1 sequences of the *Methylosinus-Methylocystis* group clustered in the maximum-likelihood tree (Fig. 1) more closely to the PmoA sequence from *Methylocapsa acidiphila* than to their own corresponding PmoA2 sequences (Fig. 1).

The data obtained also suggested that *pmoA2* is not present in all type II MOB investigated. *M. trichosporium* strains OB3b and SM6 exhibit an overall 16S rRNA gene sequence identity of 98.2%. PCR-based screening led to the identification of *pmoA2* in strain SM6, but *pmoA2* could not be detected in strain OB3b. Further evidence that, in contrast to strain SM6, *M. trichosporium* strain OB3b does not harbor a *pmoA2* gene copy was provided by Southern hybridization using probe pmoA636b (Fig. 3 and 4). It should also be noted that the gene probe of PCR-amplified *pmoA* (containing both the conventional *pmoA1* and the novel *pmoA2*) led to the detection of two *pmoA* gene copies in *M. trichosporium* strain OB3b (Fig. 4). This is consistent with previous reports that strain OB3b har-

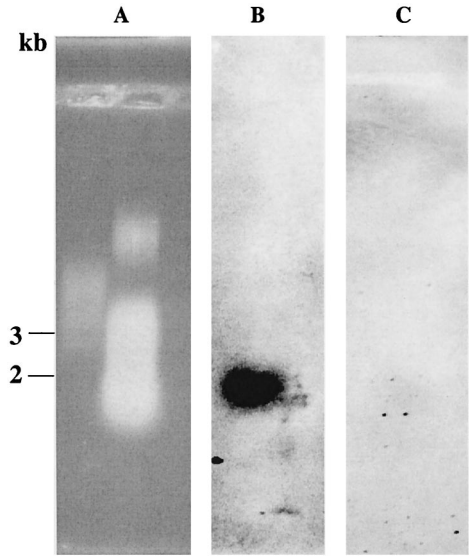


FIG. 6. Northern hybridization of total RNA from *Methylocystis* strain SC2 to *pmoA*-targeted oligonucleotide probes. (A) Agarose gel electrophoresis of total RNA in presence of an RNA marker (RNA ladder; New England Biolabs). (B) Hybridization with probe A593b to specifically detect mRNA expressed from *pmoA1*. (C) Hybridization with probe pmoA636b to specifically detect mRNA expressed from *pmoA2*. Lanes contain approximately 60 μg of total RNA from strain SC2.

bors two nearly identical *pmoCAB* operons that correspond to *pmoA1* (11). Thus, the two different experimental approaches (PCR-based screening and Southern blot analysis) taken together provide strong evidence that closely related type II MOB genospecies may or may not harbor *pmoA2*. Interestingly, a similar observation has been made for the distribution of *mmoX* genes among type II MOB. Nearly identical species appear to differ in whether they possess or do not possess the *mmoX* gene (13, 16).

However, we cannot completely rule out the possibility that variations in the target site of the primer combination pmoA206f-pmoA703b may be the reason why *pmoA2* was not detected in all type II MOB tested. Thus, the distribution of *pmoA2* among type II MOB might still have to be addressed in further studies. This may also be true for type I MOB, although in all five strains tested, the detection of a *pmoA2*-like gene copy by PCR (Table 1)—and in some cases by Southern hybridization (Fig. 4)—failed.

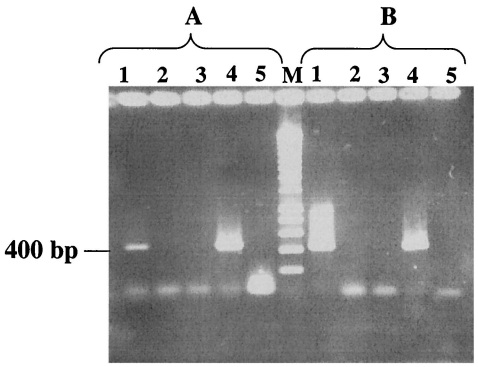


FIG. 7. Detection of *pmoA*-like mRNA in *Methylocystis* strain SC2 by RT-PCR. RT was carried out with primer A682b. (A) Use of the primer combination pmoA206f-pmoA636b for specific detection of functional activity of *pmoA2*. (B) Use of the primer combination A189f-A593b for specific detection of functional activity of *pmoA1*. Lanes: 1, total RNA plus RT-PCR (positive detection); 2, total RNA plus PCR without the RT step (negative control); 3, RNase-treated RNA sample plus PCR (negative control); 4, genomic DNA plus PCR (positive control); 5, no DNA plus PCR (negative control).

The second major aim of the present study was to determine whether *pmoA2* is expressed and encodes a functionally active protein or is instead an unexpressed pseudogene. Proteins with important basic cellular functions are often subject to strong purifying selection. Under such selection, a synonomous (non-amino acid changing) nucleotide mutation in the encoding gene has a much greater chance of becoming fixed than does a nonsynonomous (amino acid changing) mutation. Nonsynonomous/synonomous substitution rate ratios (ω) can typically be 0.1 or less for genes under strong purifying selection (36). Using the paml model (35), our phylogenetic analysis indicated that the *pmoA1* and *pmoA2* genes have both displayed similarly low ω values in recent evolutionary history. This is strong evidence that both genes are expressed and have important cellular functions. In contrast to our previous study (12), experimental evidence that *pmoA2* of *Methylocystis* strain SC2 is expressed could be obtained by RT-PCR (Fig. 7). However, as concluded from Northern hybridization experiments (Fig. 6), the level of *pmoA2* expression under the laboratory standard growth conditions applied was clearly lower than that of *pmoA1*.

Further studies will need to focus on the functional role of the enzyme expressed by the *pmo* operon that corresponds to *pmoA2*. Multiple pMMOs with different substrate specificities would provide MOB with a means of adapting to changing concentrations of methane and competitive substrates such as ammonia (13). There is some evidence for such a mechanism. Increasing Cu concentration has been shown to allow *M. capsulatus* to form a pMMO with a higher affinity for $CH_4$ (23). *Methylocystis* strain LR1, which possesses a *pmoA2*, displays a variable affinity for methane depending on the growth conditions (7), although this may be due to physiological conditions rather than to genetic changes (8). Ammonia does not appear to affect all soil MOB communities in the same way, suggesting that different MOB have different sensitivities to this cosubstrate (12). Thus, further investigation will require various cultivation conditions and the use of knockout mutations.

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