

Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments

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Type II methane-oxidizing bacteria (MOB) were isolated from diverse environments, including rice paddies, pristine and polluted freshwaters and sediments, mangrove roots, upland soils, brackish water ecosystems, moors, oil wells, water purification systems and livestock manure. Isolates were identified based on morphological traits as either *Methylocystis* spp., *Methylosinus sporium* or *Methylosinus trichosporium*. Molecular phylogenies were constructed based on nearly complete 16S rRNA gene sequences, and on partial sequences of genes encoding PmoA (a subunit of particulate methane monooxygenase), MxaF (a subunit of methanol dehydrogenase) and MmoX (a subunit of soluble methane monooxygenase). The maximum pairwise 16S rDNA difference between isolates was 4.2%, and considerable variability was evident within the *Methylocystis* (maximum difference 3.6%). Due to this variability, some of the published 'specific' oligonucleotide primers for type II MOB exhibit multiple mismatches with gene sequences from some isolates. The phylogenetic tree constructed from *pmoA* gene sequences closely mirrored that constructed from 16S rDNA sequences, and both supported the presently accepted taxonomy of type II MOB. Contrary to previously published phylogenetic trees, morphologically distinguishable species were generally monophyletic based on *pmoA* or 16S rRNA gene sequences. This was not true for phylogenies constructed from *mmoX* and *mxaf* gene sequences. The phylogeny of *mxaf* gene sequences suggested that horizontal transfer of this gene may have occurred across type II MOB species. Soluble methane monooxygenase could not be detected in many *Methylocystis* strains either by an enzyme activity test (oxidation of naphthalene) or by PCR-based amplification of an *mmoX* gene.

Keywords: *Methylosinus*, *Methylocystis*, methane monooxygenase, *pmoA*, *mmoX*

INTRODUCTION

Methane-oxidizing bacteria (MOB) are a phylogenetically diverse group defined by the ability to use methane as a sole carbon and energy source. Eleven known

Abbreviations: MOB, methane-oxidizing bacteria; sMMO, soluble methane monooxygenase.

The GenBank accession numbers for the nearly complete 16S rRNA gene sequences for the isolates are AJ458466 to AJ458510. Partial sequences of the *pmoA*, *mxaf* and *mmoX* genes have been deposited under the accession numbers AJ458994–AJ459052, AJ459053–AJ459100 and AJ458511–AJ458535, respectively. Where multiple strains contained identical sequences, only one has been deposited.

genera of MOB are divided into two groups, type I (γ -*Proteobacteria*) and type II (α -*Proteobacteria*). These types differ in many characteristics, including carbon assimilation pathways and the arrangement of intracellular membranes (Bowman *et al.*, 1993; Bowman, 2000). The type I group is more phylogenetically diverse, but members of the type II group are as often isolated from natural systems, or detected using cultivation-independent molecular techniques (e.g. McDonald *et al.*, 1996; Costello & Lidstrom, 1999; Henckel *et al.*, 1999; Wise *et al.*, 1999; Auman *et al.*, 2000; Horz *et al.*, 2001; Steinkamp *et al.*, 2001). Recognized species within the type II group were until recently limited to *Methylosinus sporium*, *Methylosinus trichosporium*, *Methylo-*

cystis parvus and *Methylocystis echinoides*. Two newly described MOB, *Methylocella palustris* (Dedysh *et al.*, 2000) and *Methylocapsa acidiphila* (Dedysh *et al.*, 2002), also belong to the α -Proteobacteria line of descent. Presently these are grouped together with the *Methylocystis/Methylosinus* group as type II MOB. However they have several unique morphological and physiological characteristics, and based on 16S rRNA phylogeny are evolutionarily more closely related to some non-methanotrophic bacteria than to the previously known type II MOB.

Phylogenetic trees constructed based on 16S rRNA sequences often fail to reveal any clear organization of the type II *Methylocystis/Methylosinus* group. The different species are not always monophyletic (e.g. Bowman, 2000; Murrell & Radajewski, 2000). This may be due to the scarcity of public-domain sequences available from pure cultures, and possibly also to errors in the sequences which are available. The lack of a large, error-free 16S rRNA sequence database has also made difficult the task of designing oligonucleotide probes to target particular MOB types or species, probes which could be applied to the cultivation-independent study of these bacteria by such techniques as retrieval of environmental 16S rRNA gene sequences and fluorescence *in situ* hybridization.

The recognition that several genes encoding key enzymes involved in methane oxidation could be used as phylogenetic markers has provided a useful toolbox for the cultivation-independent identification of MOB in various habitats. Those presently in use are: 1) the *pmoA* gene, which encodes the active-site-containing subunit of the particulate methane monooxygenase enzyme (pMMO) and is universal to all known MOB (Holmes *et al.*, 1995; McDonald & Murrell, 1997a), with the possible exception of *Methylocella palustris* [*pmoA* was not detected in *Methylocella palustris* using current primer sets or by hybridization with a *pmoA* fragment from *Methylococcus capsulatus* (Dedysh *et al.*, 2000)]; 2) the *mmoX* gene, which encodes a subunit of soluble methane monooxygenase (sMMO) and is present in only some MOB strains (McDonald *et al.*, 1995; Auman *et al.*, 2000); and 3) the *mxoF* gene, which encodes a subunit of methanol dehydrogenase. This last enzyme is universal to methylotrophic bacteria and therefore also universal to, but not unique to, MOB (McDonald & Murrell, 1997b). Phylogenetic trees constructed based on partial sequences of any of these three genes show similar topological patterns to the phylogenetic trees constructed from 16S rRNA sequences, indicating that there has been little horizontal transfer of these functional genes and that the methanotrophic phenotype probably evolved from a common ancestor. The phylogenies constructed based on *pmoA*, *mmoX* or *mxoF* gene sequences therefore reflect the evolution of species rather than simply of the genes, and sequencing of gene products amplified by PCR from environmental DNA extracts can provide some information on which MOB are present in that environment. It is, however, unclear to what scale of resolution these sequences are useful.

Most can easily distinguish type I from type II MOB, but more reference sequences from pure cultures are necessary to demonstrate whether particular species can be mapped to particular monophyletic clusters of these genes.

The aim of the present work was to develop better databases of 16S rRNA, *pmoA*, *mmoX* and *mxoF* sequences derived from pure cultures of type II MOB. Recently a similar exercise was performed for ammonia oxidizers (Purkhold *et al.*, 2001). This provided a better phylogenetic framework for this group and, for example, dispelled the impression that most sequences retrieved from environmental samples stemmed from novel uncultivated bacteria. Developing a better database for MOB should help to: 1) determine at what scale of taxonomic resolution the morphologically recognized species correlate with taxa recognized by molecular sequence data; 2) provide a better database to correlate environmental gene sequences retrieved in cultivation-independent studies with strains characterized in pure culture; and 3) facilitate the design and evaluation of oligonucleotide probes targeting specific taxonomic or physiological groups, for use in cultivation-independent molecular ecology studies. To these ends a large collection of MOB was first isolated from diverse environments, and selected cultures identified based on morphological characteristics and on molecular phylogenies. The present paper presents the results of the type II MOB. The type I MOB in the collection will be described in a later study.

METHODS

Isolation. Except where indicated, all strains were isolated by the authors and co-workers in the past 30 years. Soil and water samples were obtained from diverse environments as listed in Table 1. The enrichment procedure for MOB was as described by Heyer *et al.* (1984). Briefly, a serial 10 \times dilution series of the samples was made in a mineral salts medium ('medium 10') containing (per l): 0.1 g MgSO₄·7H₂O; 0.5 g NH₄Cl; 0.7 g Na₂HPO₄·2H₂O; 0.3 g KH₂PO₄; 0.01 g CaCl₂·6H₂O; 0.005 g FeSO₄·7H₂O; and 1 ml trace elements solution (see below). The final pH was 7.0. One millilitre of trace elements solution contained: 0.44 mg ZnSO₄·7H₂O; 0.20 mg CuSO₄·5H₂O; 0.17 mg MnSO₄·2H₂O; 0.06 mg Na₂MoO₄·2H₂O; 0.10 mg H₃BO₃; and 0.08 mg CoCl₂·6H₂O. In some cases 10 ml l⁻¹ of a sterile-filtered (0.2 μ m) vitamin solution was added to the medium after autoclaving. This 10 ml aliquot contained: 20 μ g folic acid; 20 μ g biotin; 100 μ g pyridoxine hydrochloride (vitamin B₆); 50 μ g thiamin hydrochloride (vitamin B₁); 50 μ g DL-Ca-pantothenate; 50 μ g riboflavin (vitamin B₂); and 1 μ g vitamin B₁₂.

Tubes were incubated at 30 °C in closed glass chambers containing a gas mixture of 20% (v/v) CH₄, 5% CO₂ and 75% air. Samples of the terminal positive dilution steps were streaked on plates of medium 10 solidified by adding 1.5% Bacto agar (Difco), and grown under the same gas mixture. Single colonies growing on plates were selected with the aid of a stereo microscope and streaked on new plates, and this repeated until pure cultures were obtained. Purity was verified by phase-contrast microscopy of liquid cell culture, by streaking onto Nutrient Agar (Difco) diluted 1:1 with H₂O,

Table 1. MOB isolated in this study, with strain designation, location and date of sampling, and species characterization

Species identification is based on morphological traits. Species are: *Methylosinus sporium* (Mss), *Methylosinus trichosporium* (Mst) and *Methylocystis* spp. (Mc). Morphological or cultural characteristics which are atypical compared to the standards outlined in the Methods are indicated. The *mmoX* gene, indicating the presence of sMMO, was detected based on a PCR assay and primer set published by Auman *et al.* (2000).

Strain	Species	Location and source for enrichment culture	Sampling date	Unusual cultural and morphological traits	<i>mmoX</i>
1	Mst	Hiddensee (German Baltic coast), eelgrass in brackish water	08/70		+
20/1	Mst	Wittenhagen (Germany), surface soil near oil extraction plants	08/70		+
33/1	Mst	Hiddensee (Germany), water from fen lake	08/70		+
39/3	Mst	Rügen, Wiek (Germany), water near oil extraction plants	08/70		+
55/8	Mst	Rügen, Hagen (Germany), surface sediment of dystrophic bog lake	10/79		+
60	Mst	Hiddensee (German Baltic coast), surface sediment of shallow water area	10/79		+
62/2b	Mst	Neuglobsow (Germany), water from oligotrophic Lake Stechlin	10/79		+
493/3	Mst	Jena-Lobeda (Germany), sludge from sewage treatment plant	06/76		
I4/1, I3/4	Mst	Vercelli (Italy), surface rice field soil	06/92	Red-pigmented colonies	+
SM6 (IMV B-3060)	Mst	Wichmar (Germany), water from polluted river Saale	08/78	Red-pigmented colonies (Strauss & Berger, 1983)	+
SC10	Mst	Wichmar (Germany), water from polluted river Saale	08/78		+
H3, H5	Mst	Feldberg (Germany), surface sediment of eutrophic Lake Haussee*	07/00		+
M23	Mst	Coleroon Delta (India), coast of Indian Ocean, mangrove roots	04/00		+
O19/1	Mst	Baltic Sea, Gotland Deep, Station 271, surface sediment at 240 m depth‡	07/00		+
KS18	Mst	Lake Kinneret (Israel), oxic surface sediment†	11/00		+
KS21	Mst	Lake Kinneret (Israel), oxic surface sediment†	11/00	Long (>4 µm) slender (<1 µm) rods, frequently swollen at one end	+
KS24b	Mst	Lake Kinneret (Israel), oxic surface sediment†	11/00	Yellow-pigmented colonies	+
IMET 10561	Mst	Hiddensee, Kloster (Germany), liquid manure	08/70		+
D15a	Mst	Neuglobsow (Germany), surface sediment of eutrophic Lake Dagow*	07/00	Long (>4 µm) slender (<1 µm) straight rods	+
430/2, 434	Mss or Mst	Wittenhagen (Germany), surface soil near oil extraction plants	06/75	Weakly reniform rods	+
8	Mss	Hiddensee, Bessin (German Baltic coast), lagoon water	08/70		+
9/1	Mss	Hiddensee, Bessin (German Baltic coast), water from shallows	08/70		+
20/3	Mss	Wittenhagen (Germany), surface soil near oil extraction plants	08/70		+
44/2	Mss	Rügen, Nardevitz (Germany), water from spring bog	08/70		+
SC8	Mss	Wichmar (Germany), water from polluted river Saale	09/78		+
SD56	Mss	Döbritschen (Germany), water from polluted river Saale	05/80		+
SE2	Mss	Eichicht (Germany), water from polluted river Saale	09/78		+
SK13	Mss	Weißén (Germany), water from polluted river Saale	09/78		+
SM27a	Mss	Maua (Germany), water from polluted river Saale	05/78		+
H1b, H4a	Mss	Feldberg (Germany), surface sediment of eutrophic Lake Haussee*	07/00		+
F10/1b	Mss	Neuglobsow, Fuchskuhle (Germany), surface sediment of dystrophic peat bog lake*	07/00		+

Table 1. (Cont.)

Strain	Species	Location and source for enrichment culture	Sampling date	Unusual cultural and morphological traits	<i>mmoX</i>
KS16a, KS17	Mss	Lake Kinneret (Israel), oxic surface sediment†	11/00		+
21/1	Mc	Wittenhagen (Germany), water near oil extraction plants	08/70		-
33/26, 42/22	Mc	Hiddensee (German Baltic coast), water from shallows	09/78		-
45/7a	Mc	Zingst, Grabow (German Baltic coast), surface sediment in eutrophic backwater†	08/79		+
50/42a, 50/54	Mc	Hiddensee (German Baltic coast), surface sediment from shallows	08/79		+
51	Mc	Rügen (Germany), water from Devil's Bog	08/70		+
62/12	Mc	Neuglobsow (Germany), water from oligotrophic Lake Stechlin	08/79		-
62/38a	Mc	Neuglobsow (Germany), littoral sediment from oligotrophic Lake Stechlin	10/79		+
494	Mc	Maua (Germany), water from polluted river Saale	06/76		+
495/4	Mc	Reinkenhagen (Germany), soil near oil extraction plants	07/76		+
Pi5/4, Pi6/2	Mc	Los Banos (Philippines), surface rice field soil	08/93	Large (1–2 µm), highly refractive coccoid cells	-
Ch22	Mc	Suzhu (China), surface rice field soil	10/93	Rods (1.5–4 µm)	-
IMET 10484	Mc	Mannhäger Moor (Germany), soil near oil extraction plants	06/71		+
IMET 10486	Mc	Wittenhagen (Germany), water near oil extraction plants	08/70		+
IMET 10489	Mc	Hiddensee, Kloster (Germany), liquid manure	08/70		-
IMET 10491	Mc	Jena-Lobeda (Germany), sludge from sewage treatment plant	08/76	<i>Methylocystis echinoides</i> (Bowman <i>et al.</i> , 1993)	-
SC2	Mc	Wichmar (Germany), water from polluted river Saale	10/78		-
SD5	Mc	Döbritschen (Germany), water from polluted river Saale	10/78		+
SE12	Mc	Eichicht (Germany), water from polluted river Saale	09/79		+
SK28	Mc	Weißén(Germany), water from polluted river Saale	09/79		+
SM16	Mc	Maua (Germany), water from polluted river Saale	10/79		+
B2/7	Mc	Gießen (Germany), wet meadow soil, 0–10 cm	06/00		-
O14c	Mc	Baltic Sea, Bornholm Deep, Station 213, surface sediment at 89 m‡	07/00		-
M31, M42/1	Mc	Coleroon Delta (India), coast of Indian Ocean, mangrove roots	04/00		-
H9a	Mc	Feldberg (Germany), surface sediment of eutrophic Lake Haussee*	07/00		-
D28	Mc	Neuglobsow (Germany), surface sediment of eutrophic Lake Dagow*	07/00	Rods, frequently swollen at one end	-
KS7	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00	Large (1–2 µm) coccoid cells and rods (1.5–4 µm), sometimes motile	-
KS3	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00	Large (1–2 µm) highly refractive coccoid cells	-
KS8a	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00	Coccoid cells and bent rods, sometimes motile	-
KS9, KS12	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00		-

Table 1. (Cont.)

Strain	Species	Location and source for enrichment culture	Sampling date	Unusual cultural and morphological traits	<i>mmoX</i>
KS30, KS31	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00	Viscous colonies with coccoid cells and thick (> 1 µm) rods in aggregates	–
KS33	Mc	Lake Kinneret (Israel), oxic surface sediment†§	11/00	Coccoid cells of different sizes	–
F10V2a	Mc	Neuglobsow, Fuchskuhle (Germany), surface sediment of dystrophic peat bog lake*	07/00	Viscous colonies with coccoid cells and thick (> 1 µm) rods in aggregates	+
2¶	Mc		77	<i>Methylocystis echinoides</i>	–
45¶	Mc		77	<i>Methylocystis echinoides</i>	–
82¶	Mc		77	' <i>Methylocystis minimus</i> '	+
41¶	Mc		77	' <i>Methylocystis minimus</i> '	+
48¶	Mc		77	' <i>Methylocystis minimus</i> '	–
56¶	Mc		77	' <i>Methylocystis minimus</i> '	–
10¶	Mc		77	' <i>Methylocystis methanolicus</i> '	–
39¶	Mc		77	' <i>Methylocystis methanolicus</i> '	–
81¶	Mc		77	<i>Methylocystis parvus</i>	–
54¶	Mc		77	<i>Methylocystis parvus</i>	–
57¶	Mc		77	<i>Methylocystis parvus</i>	–

* Sampling by Eckmann dredge.

† Sampling by gravity corer from a ship.

‡ Sampling by multi corer from a ship.

§ Enrichment with rotary shaking (120 r.p.m.).

|| Enrichment medium contained vitamin solution.

¶ Strains isolated and described by Galchenko *et al.* (1980).

Table 2. Oligonucleotide primers used in this study for the PCR amplification of genes from DNA extracts of MOB cultures

Name*	Target	Reference	Sequence (5'-3')
A189f	<i>pmoA</i>	Holmes <i>et al.</i> (1995)	GGNGACTGGGACTTCTGG
A682r	<i>pmoA</i>	Holmes <i>et al.</i> (1995)	GAASGCNGAGAAGAASGC
1003f	<i>mxoF</i>	McDonald <i>et al.</i> (1997b)	GCGGCACCAACTGGGGCTGGT
1561r	<i>mxoF</i>	McDonald <i>et al.</i> (1997b)	GGGCAGCATGAAGGGCTCCC
9f	16S rDNA	Weisberg <i>et al.</i> (1991)	GAGTTTGATCMTGGCTCAG
1492r	16S rDNA	Weisberg <i>et al.</i> (1991)	ACGGYTACCTTGTTACGACTT
A166f	<i>mmoX</i>	Auman <i>et al.</i> (2000)	ACCAAGGARCARTTCAAG
B1401r	<i>mmoX</i>	Auman <i>et al.</i> (2000)	TGGCACTCRTARCGCTC

* Primers with names ending in 'f' target the antisense strand, those ending in 'r' target the sense strand.

and onto plates of mineral salts medium incubated in an atmosphere containing no CH₄. Isolated cultures were maintained by periodic transfer (6–12 month intervals).

Morphological identification. Morphological characterization was performed at the time of isolation, and again immediately before molecular analyses to ensure that contamination of cultures did not occur. Cell form, size, motility, exospore formation, lipid cyst formation, and cell arrangement (especially rosette formation) were determined by phase-contrast microscopy of 1-week-old (exponential phase) and 2- to 3-week-old (stationary phase) liquid cultures and agar plate cultures. Agar plate cultures were also characterized based on colony form, size, surface appearance, edge, consistency and pigmentation.

Type II MOB can be distinguished on the basis of visible morphological characteristics (Whittenbury *et al.*, 1970; Bowman *et al.*, 1993; Bowman, 2000). The type species are *Methylosinus trichosporium* strain OB3b, *Methylosinus sporium* strain 5, *Methylocystis parvus* strain OBBp, and *Methylocystis echinoides* strain IMET 10491. *Methylocystis* are coccoid (0.6–1.5 µm diameter) or short, slightly bent rods (1.2–3.5 × 0.8–1.5 µm), occasionally reniform, and often contain reserve material (polyhydroxybutyrate) at the poles. They form lipid cysts (enlarged, irregularly shaped, refractive cells) in older cultures. They are non-motile (with few exceptions noted in our collection, Table 1) and do not form rosettes. *Methylosinus* are motile, rod-shaped cells which form exospores and often cluster in rosettes. *Methylosinus sporium* differs from *Methylosinus trichosporium* in the form and size of cells. *M. trichosporium* cells are generally long (2–5 µm), thick (0.8–1.3 µm) rods which are only slightly bent and are slightly thicker at the basal pole (holdfast site during rosette formation) than at the apical pole (site of exospore formation). *Methylosinus sporium* cells are shorter (1.5–3 µm), thick (0.8–1.2 µm) cells which are strongly bent, often crescent-shaped and vibrioid. They are clearly thicker at the basal pole than the apical pole.

Methylocystis parvus and *Methylocystis echinoides* are difficult to distinguish without electron micrographs to visualize the spinae which project from the cell envelope of *M. echinoides*. Therefore, the identification of new *Methylocystis* isolates was made to the genus level only. However, molecular analyses were also performed on the type strain *Methylocystis echinoides* strain IMET 10491 (Table 1) which was isolated by Meyer *et al.* (1986) (originally designated strain IC 493s/5),

and phenotypically characterized by Bowman *et al.* (1993). In addition, molecular analyses were performed on 11 *Methylocystis* strains that were isolated from various sites in Russia and divided based on phenotypic traits into four morphotypes: *parvus*, *echinoides*, 'minimus' and 'methanolicus'. Detailed phenotypic descriptions of these strains, including morphological observations from electron microscopy, physiological tolerances (pH, temperature, salt), phospholipid fatty acid (PLFA) profiles, G + C content and various enzymic tests, have been published elsewhere (Galchenko *et al.*, 1980) and are also available online at <http://inmi.da.ru/>.

Colorimetric detection of sMMO activity. To test for sMMO activity, the naphthalene oxidation assay was performed on 1- to 2-week-old streaked plate cultures of selected isolates (Graham *et al.*, 1992). Medium was made with and without added copper. Some isolates grew poorly on the medium of Graham *et al.*, (1992); in these cases the test was made on medium 10 instead. A positive assay was scored by the development of a deep purple colour, indicating the oxidation of naphthalene to α -naphthol by sMMO.

Molecular analyses. DNA was extracted from cultures grown on agar slants using a procedure based on mechanical agitation in a FastPrep FP120 cell disrupter (Savant Instruments) of 2 ml screw-cap reaction vessels filled with a mixture of culture, 0.1 mm diameter silica-zirconium beads and a phosphate buffer (pH 8) containing 2% SDS (Henckel *et al.*, 1999). Oligonucleotide primer sets listed in Table 2 were used to amplify partial gene products of 16S rDNA, *pmoA*, *mmoX* and *mxoF* on a PE GeneAmp PCR System 9700 temperature cyclor (Perkin-Elmer Applied Biosystems). Temperature programs were as described previously (Henckel *et al.*, 1999), except for *mmoX*, which was as described by Auman *et al.* (2000). PCR mixtures (50 µl) contained 0.5 µM each primer, 1 × Premix F (Epicenter Technologies), 1 µl template DNA and 1 U *Taq* DNA polymerase (Q Biogene). PCR products were sequenced on an ABI 373 automated sequencer (Perkin-Elmer Applied Biosystems) as described by Henckel *et al.* (1999) or on an ABI Prism 377 DNA sequencer using BigDye terminator chemistry as specified by the manufacturer (Perkin-Elmer Applied Biosystems).

It has been shown that at least some type II MOB contain a second *pmoA*-like gene (*pmoA2*) which possesses only about 30% nucleotide sequence identity to the *pmoA* that has been well-studied in pure cultures of type II MOB (Dunfield *et al.*, 2002). However, the 'normal' *pmoA* gene is favoured by the

A189/A682 oligonucleotide primer set and ambiguous nucleotide assignments were rarely evident in our sequencing analyses. In the few cases where many ambiguities in nucleotide assignment of *pmoA* sequences were evident, the PCR was repeated with a higher annealing temperature (67 °C) and most ambiguities disappeared.

Phylogenetic analyses. Comparative sequence analyses of the nearly complete 16S rRNA genes and of the partial sequences of the various functional genes, were performed using the ARB software (Strunk & Ludwig, 1996). Sequencing errors were minimized by comparing each gene sequence obtained to a cumulative consensus sequence for type II MOB and double-checking all mismatches against the original ABI sequencer output. Identical sequences from different strains were input only once into each analysis.

The 16S rDNA treeing analysis included all complete sequences from type II MOB isolates that are available in public-domain databases, with a few exceptions. When performing phylogenetic analyses on closely related species such as type II MOB, tree topology can be greatly affected by only a few erroneous nucleotide position assignments. Therefore the earliest published sequences (GenBank accession numbers L20848, L20803, L20844, M29026, M29024, M95665) were not used, since subsequent analysis of two strains has demonstrated that the original sequences contained many errors (Dedysh *et al.*, 2000). Two sequences from the present study should also be used to replace older sequences in GenBank: that of *Methylocystis echinoides* strain 2 (new accession number AJ458502; old accession number L20848) and that of *Methylosinus trichosporium* strain SM6, identified previously by its classification number in the Ukrainian Collection of Microorganisms, IMV B-3060 (new accession number AJ458477; old accession number L20845). Based on the new data, the earlier sequences contained 6.5% and 1.5% erroneous nucleotide assignments, respectively. Finally, the 16S rDNA sequence of '*Methylosinus pucelana*' (AF107461) was nearly identical (99.5%) to *Methylocystis parvus* strain 81; we suspect that this strain has been falsely identified as *Methylosinus*.

For phylogenetic analyses of *pmoA*, all sequences from type II MOB isolates in the public-domain database were included, along with representative sequences that were obtained from cultivation-independent studies and fall into the type II MOB cluster of *pmoA* sequences.

RESULTS AND DISCUSSION

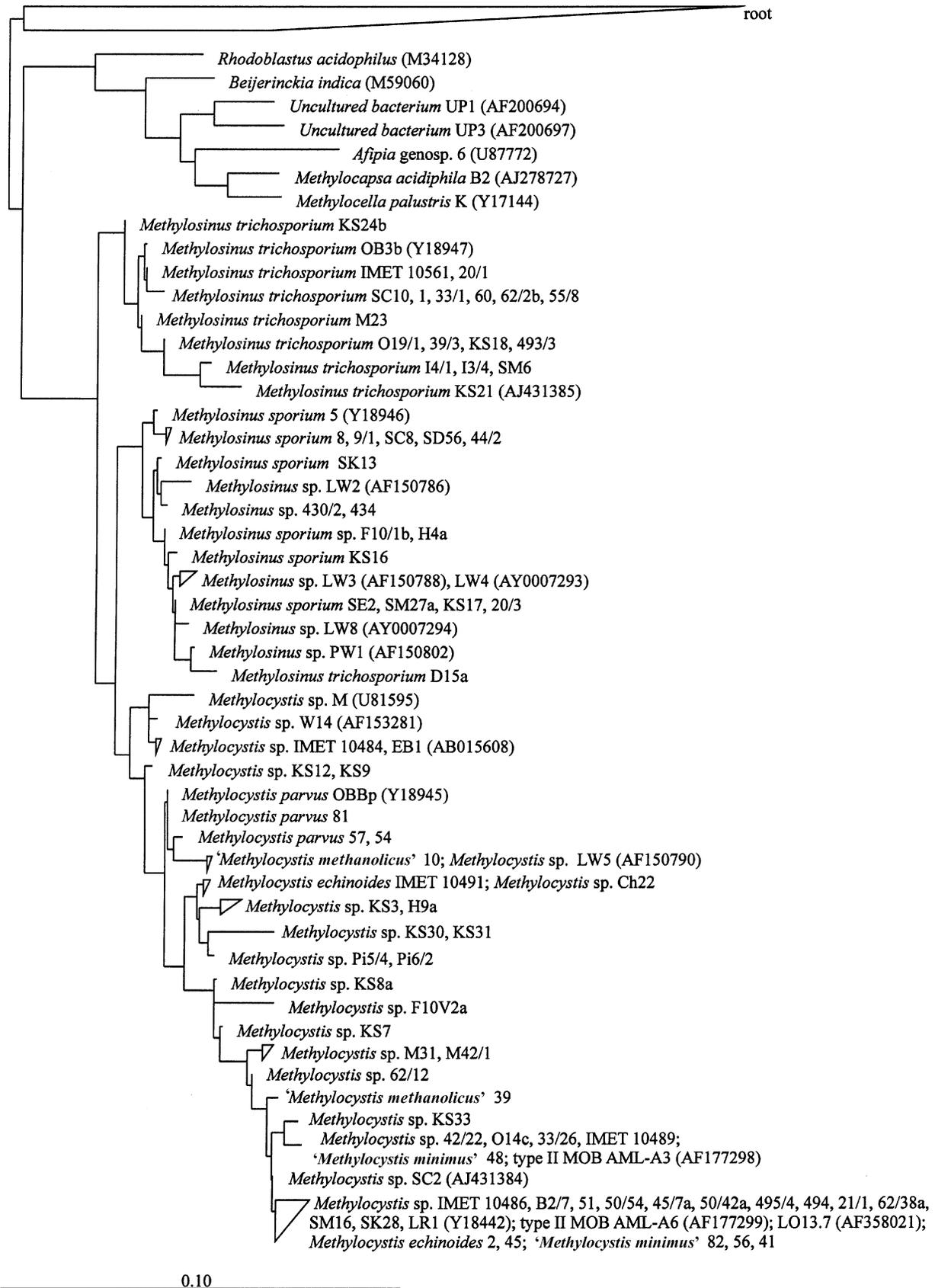
Phylogeny and taxonomy of type II MOB isolates

With few exceptions, the type II MOB isolates could be identified based on visible morphological and cultural traits as *Methylosinus sporium*, *Methylosinus trichosporium* or *Methylocystis* sp. Some isolates deviated in one or more characteristics from the standard species morphologies outlined in Methods. These exceptions are noted in Table 1. For example, *Methylocystis* strains KS7 and KS8a were motile, a characteristic not previously observed in any *Methylocystis* strain. However, other characteristics of KS7 and KS8a (e.g. shape, cyst formation) were typical for *Methylocystis*. Other atypical strains included two unusually long and slender *Methylosinus* strains (KS21, D15a), some *Methylocystis* strains which were large, aggregated, rod-shaped cells

and formed viscous colonies on agar plates (F10V2a, KS30, KS31) and several red-pigmented or yellow-pigmented *Methylosinus trichosporium* strains (SM6, KS24b, I4/1, I3/4). The red pigment of strain SM6 has been characterized chemically (Strauss & Berger, 1983). No strains of *Methylocella*- or *Methylocapsa*-like bacteria were isolated, probably due to enrichment biases caused by the medium used (Dedysh *et al.*, 2000, 2002). Therefore, further discussion of type II MOB will primarily be limited to the *Methylocystis*/*Methylosinus* group.

A phylogenetic tree based on 16S rDNA sequences and constructed using a maximum-likelihood algorithm suggests that the three morphologically identified groups of type II MOB are also each monophyletic (Fig. 1). The only exception was strain D15a, which was identified as *Methylosinus trichosporium* based on morphology but which groups within the *Methylosinus sporium* cluster based on 16S rDNA phylogeny. Possibly this strain represents an intermediate between the two species (a hypothesis supported by *pmoA* phylogeny, see below). Otherwise the phylogenetic analysis identified three distinct clusters which corresponded to morphologically recognizable species of type II MOB. This monophyletic nature of different type II MOB species has not always been evident in previously constructed phylogenies (e.g. Hanson & Hanson, 1996; Bowman, 2000; Murrell & Radajewski, 2000). A maximum-parsimony phylogenetic tree of 16S rDNA sequences (100 bootstraps) preserved the monophyletic nature of the three groups (*Methylocystis* spp., *Methylosinus trichosporium* and *Methylosinus sporium*). Bootstrap values for the species cluster nodes were not high (25%, 32% and 84%, respectively); however, bootstrap values are not especially useful when evaluating a 'bush'-like structure with a gradient of many closely related sequences. Neighbour-joining trees varied depending on the filter and distance correction used (data not shown). The neighbour-joining tree which best preserved the topology of the maximum-likelihood and maximum-parsimony trees used a Jukes-Cantor or Felsenstein correction and incorporated no filter, which is reasonable considering that the most variable nucleotide positions are the most useful in discerning differences among closely related strains and should therefore not be filtered out (Swofford *et al.*, 1996). In this tree the *Methylosinus sporium* remained monophyletic, but some *Methylosinus trichosporium* strains (KS21, I4/1, I3/4, SM6) formed a separate cluster from the other *Methylosinus trichosporium*, branching nearest to *Methylocystis* F10V2a and thereby separating the *Methylocystis* into two clusters.

Some of the *Methylocystis* strains studied have been classified based on intense morphological characterization as either *Methylocystis parvus* or *Methylocystis echinoides*, or as the morphotypes '*Methylocystis minimus*' and '*Methylocystis methanolicus*' (Table 1) (Galchenko *et al.*, 1980). All *Methylocystis parvus* 16S rDNA sequences were very similar (Fig. 1). However, *Methylocystis echinoides* strains were more phylo-



genetically diverse, and strains classified as 'minimus', which do not show the spinae formation typical of *Methylocystis echinoides*, had nearly identical 16S rDNA sequences to some strains of *Methylocystis echinoides*. Molecular phylogeny of 16S rDNA therefore does not clearly resolve the species *Methylocystis echinoides* from other morphotypes of *Methylocystis*.

pmoA

For each pairwise permutation of type II strains, the percentage 16S rDNA sequence difference was compared to the percentage partial *pmoA* gene sequence difference. The nucleotide substitution rate of the partial *pmoA* gene was 3.5 times the nucleotide substitution rate of 16S rDNA (i.e. percentage difference was on average 3.5 times greater). There was a strong linear relationship between pairwise *pmoA* and 16S rDNA differences ($r^2 = 0.90$), and therefore comparative analysis of partial *pmoA* gene sequences yielded a tree topology largely in agreement with that produced from 16S rDNA sequences (Fig. 2). The three main groups (*Methylocystis* spp., *Methylosinus trichosporium* and *Methylosinus sporium*) were again monophyletic, with the possible exception of *Methylosinus trichosporium* strains D15a and KS21. The monophyletic nature of the three groups in the neighbour-joining analysis (Fig. 2) was preserved in a maximum-parsimony tree with 100 bootstraps. Maximum-likelihood analysis also preserved the three groups, except that again the morphologically unusual *Methylosinus* strains KS21, KS24b and D15a often were not clearly assigned to either the *Methylosinus sporium* or *Methylosinus trichosporium* clusters in separate runs (bootstraps).

Phylogenetic analysis of genes other than 16S rDNA are commonly based on derived amino acid sequences rather than on nucleotide sequences. However, because much of the information content is lost by translating nucleotide sequences, pairwise differences in 16S rDNA sequences were actually better correlated to pairwise *pmoA* nucleotide sequence differences ($r^2 = 0.90$) than to pairwise *pmoA* amino acid sequence differences ($r^2 = 0.83$). When dealing with closely related species, *pmoA*-based phylogeny is therefore more reliable when constructed based on nucleotide than on derived amino acid sequences. However, the topology of the tree remains nearly the same (data not shown).

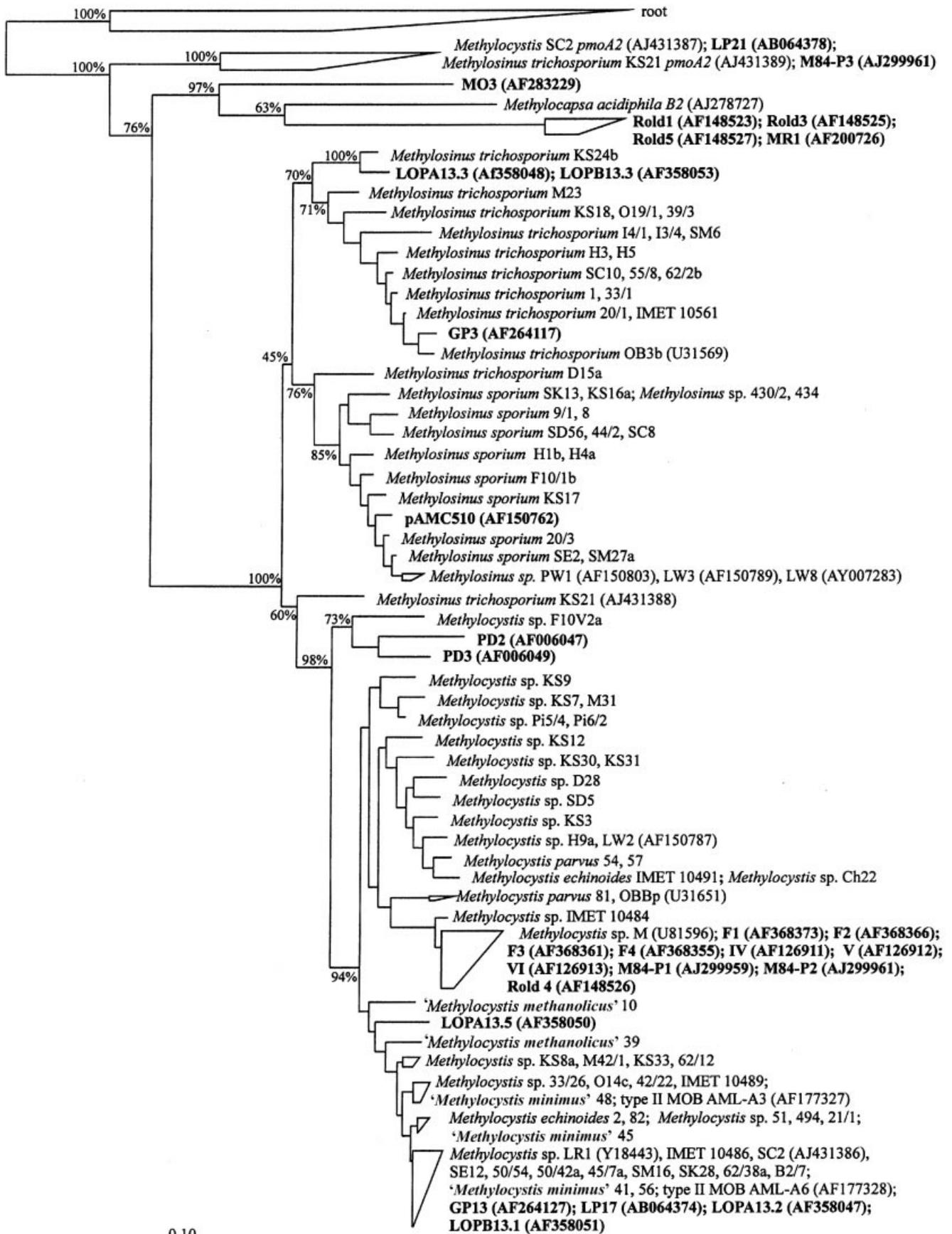
The *pmoA* gene has been extensively used in cultivation-independent studies of MOB (e.g. McDonald & Murrell 1997a; Costello *et al.*, 1999; Henckel *et al.*, 1999;

2000a, b; Holmes *et al.*, 1999; Bourne *et al.*, 2001; Fjellbirkeland *et al.*, 2001; Horz *et al.*, 2001; Reay *et al.*, 2001; Morris *et al.*, 2002). Sequences of *pmoA* which were directly retrieved from environmental samples and which group within the type II MOB cluster were included in phylogenetic analyses to demonstrate the usefulness of the database for species identification. Some of these environmental sequences (Rold1–Rold4 and MR1) do not fall within the *Methylosinus/Methylocystis* cluster and may represent novel type II MOB species most closely related to *Methylocapsa* (Fig. 2). Others (LP21, M84-P3) fall within a cluster that has been shown to represent a second *pmoA*-like gene present in some type II methanotrophs (Dunfield *et al.*, 2002). Finally, some fall clearly within one of the *Methylosinus trichosporium*, *Methylosinus sporium* or *Methylocystis* clusters, and can be assumed to indicate the presence of the respective species in that particular environment. For example, two *pmoA* sequences retrieved from *Sphagnum* peat, PD2 and PD3 (McDonald & Murrell, 1997a), are most closely related to *Methylocystis* strain F10V2a, which was also isolated from a *Sphagnum* bog environment. Interestingly, despite the diversity of type II sequences from the present culture collection, most *pmoA* sequences recovered in cultivation-independent studies fall into two very closely related groups within the *Methylocystis* cluster. This suggests either that these strains are the numerically dominant MOB in several environments, or that the oligonucleotide primer systems that are widely used are biased towards the recovery of certain sequences.

mxoF

A comparative phylogenetic analysis of *mxoF* sequences from selected isolates is shown in Fig. 3. The phylogenetic tree does not quite show the same division into three main clusters as did the 16S rDNA and *pmoA* phylogenies. The unique feature of the *mxoF* phylogeny is that some *Methylosinus trichosporium mxoF* sequences group closely with some *Methylocystis mxoF* sequences, while others are more closely related to *Methylosinus sporium mxoF* sequences. The two clusters of *Methylosinus trichosporium* separate some species with nearly identical *pmoA* and 16S rDNA sequences (i.e. the bifurcation is not caused only by unusual species such as KS21 and D15a) and it therefore appears likely that horizontal transfer of this gene has occurred across type II MOB. The *mxoF* gene may still be useful to identify different type II MOB, but it is probably less useful than *pmoA* as a phylogenetic marker of evolutionary relationships.

Fig. 1. (see facing page) Maximum-likelihood phylogenetic tree showing the relative positions of 16S rDNA sequences of type II MOB isolates, along with the non-MOB *Beijerinckia indica*, *Afipia* genospecies 6, *Rhodoblastus acidophilus*, and three sequences (UP1, UP3 and LO13.7) retrieved from soil by cultivation-independent methods (Radajewski *et al.*, 2000; Reay *et al.*, 2001). All sequences are from this study except where a GenBank accession number is given in parentheses after the name. Some sequences with <0.1% sequence difference are grouped for simplicity. The root was composed of 24 sequences from (non-methanotrophic) α -Proteobacteria. The distance bar represents 0.1 substitution per nucleotide position.



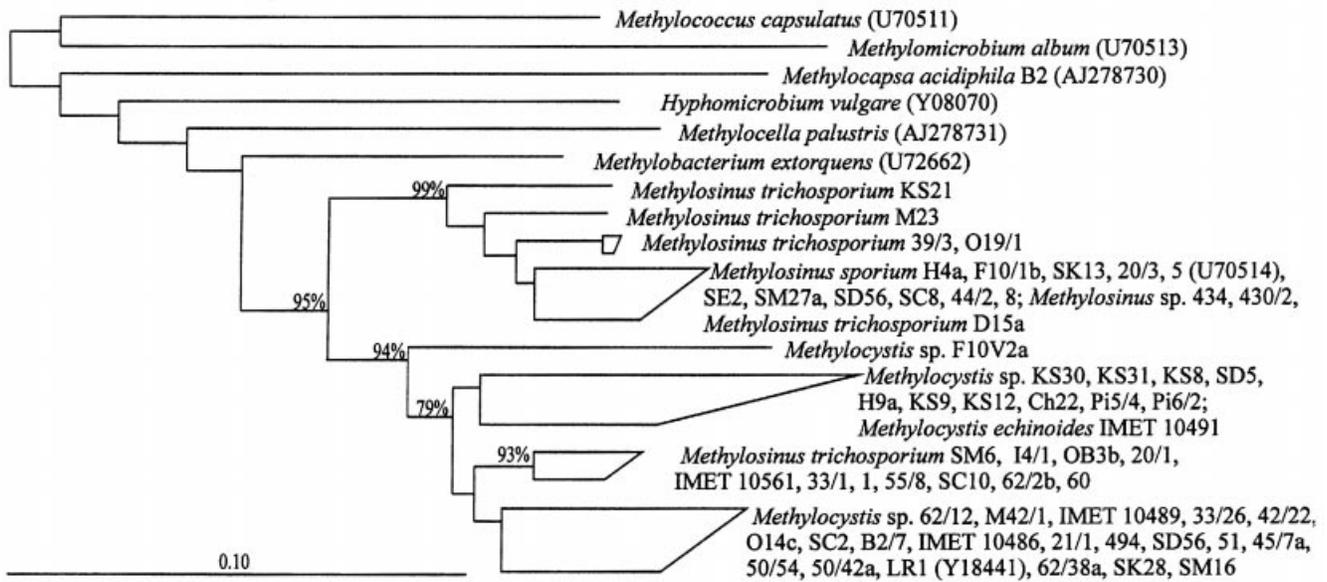


Fig. 3. Neighbour-joining tree (with Jukes–Cantor distance correction) showing the relationship of partial *mxAF* gene sequences of selected type II MOB isolates to those of other methylotrophic bacteria. All sequences are from this study except those where a GenBank accession number is given in parentheses after the species name. Some sequences are grouped for simplicity. Bootstrap values >70% (100 data resamplings) are shown. The scale bar represents 0.1 substitution per nucleotide position.

sMMO

sMMO has previously been detected in only some type II MOB. *Methylocystis parvus* OBBp, for example, appears not to contain this enzyme (Hanson & Hanson, 1996). We used both an enzymic test for sMMO activity (Graham *et al.*, 1992), and PCR-based detection of the *mmoX* gene to indicate whether sMMO was present in selected isolates. Several available *mmoX* primer sets (McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000; Horz *et al.*, 2001), were first tested on a subset of 15 isolates. The primer set published by Auman *et al.* (2000) was the most universal of these. Other primer sets sometimes failed to amplify a product from isolates for which a detectable product was obtained using this primer set, but the opposite was never the case.

The results of the PCR assay of Auman *et al.* (2000) on all isolates are indicated in Table 1. An *mmoX* gene was detected in all *Methylosinus* strains, but only in about half of the *Methylocystis* strains. Generally, the results

of the naphthalene plate assay for sMMO enzyme activity agreed with the PCR-based detection of the *mmoX* gene, but there were exceptions (Table 3). Several strains which possessed *mmoX* displayed no naphthalene oxidation activity. Two strains (*Methylocystis* Pi6/2 and KS30) displayed naphthalene oxidation activity but an *mmoX* gene could not be amplified with any of three primer sets (Table 3). Interestingly, nearly identical strains often gave different results. For example the *Methylocystis* strain KS30 displayed sMMO activity, but the strain KS31 (identical 16S rDNA sequence) did not. The *Methylocystis* strains 62/38a, 50/42a, SK28 and 51 were all positive for *mmoX* and for naphthalene oxidation activity, but the very similar strains SC2 and 21/1 (99.9% 16S rDNA sequence identity to the above strains) were negative on both tests. The sMMO results are therefore something of a mystery. Possibly, nearly identical strains indeed differ in either possessing or lacking this enzyme, but it is also likely that neither the enzyme test nor the PCR assay are universal for this enzyme.

Fig. 2. (see facing page) Neighbour-joining phylogenetic tree (with Jukes–Cantor distance correction) constructed from partial *pmoA* sequences of type II MOB. Some sequences are grouped for simplicity. The root was composed of 10 *pmoA* sequences from type I MOB. The distance bar represents 0.1 substitution per nucleotide position. Bootstrap values (100 data resamplings) for the initial six nodes are given. All sequences were obtained during this study except where a GenBank accession number is given in parentheses after the name. The sequences in bold were recovered by cultivation-independent methods in various studies. There are: pAMC510 (lake water; Costello & Lidstrom, 1999); PD2, PD3 (peat bog; McDonald & Murrell, 1997a); M84-P1, M84-P2, M84-P3 (ricefield soil; Horz *et al.*, 2001); GP3, GP13 (various upland soils; Reay *et al.*, 2001); LP17, LP21 (trichloroethylene-contaminated aquifer; Baker *et al.*, 2001); Rold1, Rold3, Rold4, Rold5 (various forest soils; Holmes *et al.*, 1999); MR2 (forest cambisol; Henckel *et al.*, 2000a); IV, V, VI (ricefield soil; Henckel *et al.*, 1999); F1, F2, F3, F4 (various upland soils; Bourne *et al.*, 2001); LOPA13.2, LOPA13.3, LOPA13.5, LOPB13.1, LOPB13.3 (peat soil; Morris *et al.*, 2002); and MO3 (ricefield soil; Henckel *et al.*, 2000b).

Table 3. Detection of sMMO in selected isolates by a colorimetric plate assay, and PCR-based detection of the *mmoX* gene encoding a subunit of the sMMO enzyme

Species are *Methylosinus sporium* (Mss), *Methylosinus trichosporium* (Mst) and *Methylocystis* spp. (Mc).

Strain	Species	PCR detection of <i>mmoX</i> *	sMMO activity by naphthalene oxidation assay†			
			Medium‡...	G – Cu	G + Cu	10 – Cu
IMET 10484	Mc	+	++	?		
IMET 10491	Mc	–	–	–		
21/1	Mc	–	–	–		
33/26	Mc	–	–	–		
51	Mc	+	++	+		
50/42a	Mc	+	++	+	++	?
62/38a	Mc	+	++	–		
Pi 6/2	Mc	–			++	++
SC2	Mc	–			–	–
SK28	Mc	+			++	–
F10V2a	Mc	+			++	–
KS8a	Mc	–			–	–
KS30	Mc	–			+	–
KS31	Mc	–			–	–
20/3	Mss	+	?	?		
SC8	Mss	+	–	–		
KS15	Mss	+			++	+
OB3b	Mst	+	++	+		
60	Mst	+	++	+		
KS21	Mst	+			–	–
KS24b	Mst	+			++	+
D15a	Mst	+			–	–

* Based on the primer set published by Auman *et al.* (2000). Negative results were verified with the primer sets of Miguez *et al.* (1997) and Horz *et al.* (2001).

† Tests were scored as very positive (++) positive (+), negative (–) or uncertain (?). Results were uncertain when a weak colour development other than purple was observed (orange or pink).

‡ G, medium of Graham *et al.* (1992); 10, medium 10 described in Methods.

The phylogenetic tree of *mmoX* sequences is shown in Fig. 4. Unlike 16S rDNA and *pmoA* phylogenies, this does not show a clear division into three main clusters corresponding to *Methylosinus trichosporium*, *Methylosinus sporium* and *Methylocystis* spp. Nevertheless, a plot of pairwise *mmoX* sequence difference versus pairwise 16S rDNA sequence difference is linear (Fig. 5), providing no evidence for horizontal gene transfer. The lack of the expected arrangement in this phylogenetic tree is probably due to the scarcity of sequences available. Many branches are represented by single species or by a few nearly identical species, and the relationships among these branches therefore cannot be clearly elucidated.

Oligonucleotide probes

Another use of these databases is the design of specific oligonucleotide probes. The maximum pairwise 16S rDNA sequence difference within all type II MOB

sequenced in the present study was 4.2%, within the genus *Methylocystis* 3.6% and within the genus *Methylosinus* 2.8% (*Methylosinus trichosporium* 2.5%, *Methylosinus sporium* 1.7%; when considering strain D15a as a *Methylosinus sporium*). Several oligonucleotides have already been developed with intended target specificity to 16S rRNA (or its encoding gene), and to functional genes of MOB. Some of these, when tested *in silico* against the databases created in this study, lacked the intended target specificity, but others were universal to the isolates (Table 4). The region of the 16S rRNA gene from *Escherichia coli* positions 433–497 seems to be especially useful for the specific and universal targeting of type II MOB. This region has been identified in recent studies as the target of: 1) the hybridization probe ‘type2 457’ (Auman *et al.*, 2000), 2) the fluorescently labelled probes ‘M α 450’ and ‘M α 464’ used for *in situ* whole-cell hybridization with type II MOB (Eller *et al.*, 2001) and 3) the PCR primer ‘Am445’ (Gulledge *et al.*, 2001).

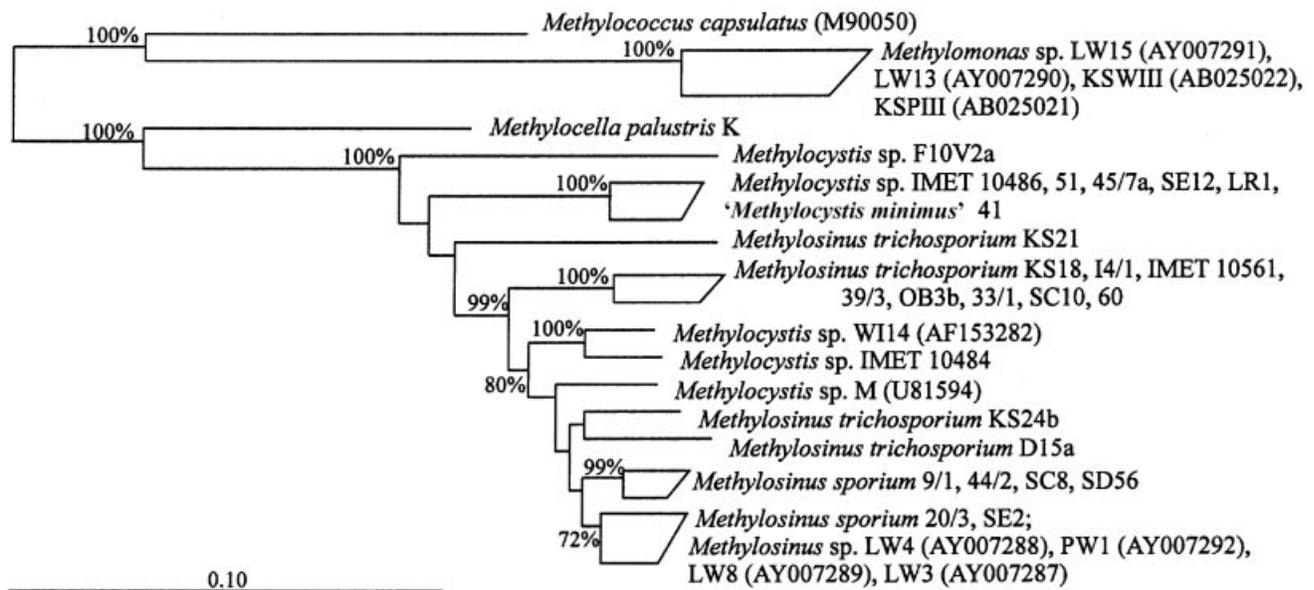


Fig. 4. Neighbour-joining tree (with Jukes–Cantor distance correction) showing the relationship of partial *mmoX* gene sequences of selected type II MOB isolates to those of other methanotrophic bacteria. All sequences are from this study except those where a GenBank accession number is given in parentheses after the species name. Some sequences are grouped for simplicity. Bootstrap values >70% (100 data resamplings) are shown. The scale bar represents 0.1 substitution per nucleotide position.

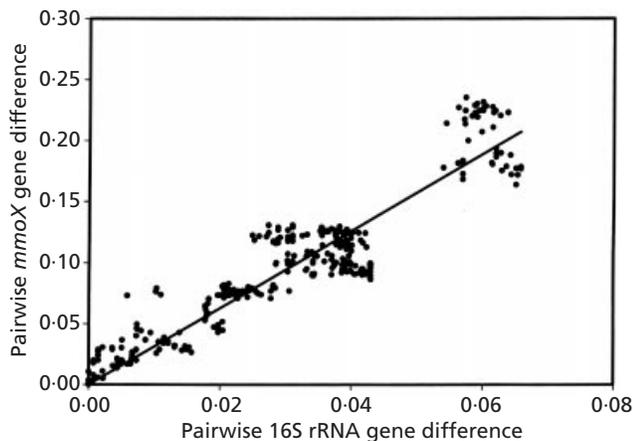


Fig. 5. Percentage difference in *mmoX* sequences versus percentage difference in 16S rDNA sequences for pairwise permutations of type II MOB isolates plus the *mmoX* sequence of *Methylocella palustris* (Dedysh *et al.*, 2000).

Summary

Molecular phylogenies constructed from 16S rDNA (and *pmoA*) sequences identified monophyletic clusters that could be correlated to morphologically recognizable groups of type II MOB: *Methylosinus trichosporium*, *Methylosinus sporium* and *Methylocystis* spp. However, *Methylocystis echinoides* could not be clearly distinguished from *Methylocystis parvus* and other morphotypes of *Methylocystis* based on molecular

phylogeny. Sequence phylogeny based on *pmoA* sequences correlated well to sequence phylogeny based on 16S rDNA sequences, and *pmoA* sequences can therefore be used to identify type II MOB with some certainty to the species level. The *mmoX* and *mxoF* genes were less suitable than *pmoA* as phylogenetic markers at this scale; *mmoX* because it was detectable in few *Methylocystis* strains, and *mxoF* because it showed evidence of horizontal gene transfer.

Certain *Methylosinus* strains (KS21, D15a and possibly I4/1, I3/4, SM6 and KS24b) probably represent evolutionary intermediates to the type species, and were characterized both by unusual morphologies and by phylogenetic relationships which varied depending on the gene sequence (16S rRNA, *pmoA*, *mxoF* or *mmoX*) and on the treeing algorithm used. Gene sequences from these strains frequently branched on the edges of the major species clusters in phylogenetic trees.

Finally, since this culture collection incorporates functionally similar bacterial strains from diverse geographical areas, it might potentially provide some information on the question of bacterial endemism, the ‘Is everything everywhere?’ question (e.g. Cho & Tiedje, 2000). Strains from different environments were often very similar or identical phylogenetically, indicating that these bacteria are not endemic at a level detectable by the gene sequences we determined. However, multiple isolates also coexisted in some environments (e.g. Lake Kinneret contained many diverse strains of both genera). This is a critical point because it is necessary to define a functionally unified group to study endemism. Although

Table 4. Oligonucleotide probes tested *in silico* on the databases created in this study

The maximum number of mismatches with a gene sequence from any isolate of *Methylosinus sporium* (Mss), *Methylosinus trichosporium* (Mst) and *Methylocystis* spp. (Mc) are indicated. Strain D15a is considered in this analysis as Mss (based on phylogeny) rather than Mst (based on morphology).

Name	Target	Intended specificity*	Reference	Sequence (5'–3')	Max. mismatches with type II MOB species		
					Mss	Mst	Mc
9α	16S rDNA	Type II MOB	Tsien <i>et al.</i> (1990)	GTTCGGAATAACTCAGGG	0	0	4
1034-Ser	16S rDNA	Type II MOB	Brusseau <i>et al.</i> (1994)	CCATACCGGACATGTCAAAAAGC	3	1	2
Ms1020r	16S rDNA	<i>Methylosinus</i>	McDonald <i>et al.</i> (1996)	CCCTTGCGGAAGGAAGTC	6	5	6
fm 142f	16S rDNA	Type II MOB	McDonald <i>et al.</i> (1996)	GTTCGGAATAACTCAGGG	0	0	4
Type 2b	16S rDNA	Type II MOB	Costello <i>et al.</i> (1999)	CATACCGGRCATGTCAAAAAGC	2	1	0
MethT2R	16S rDNA	Type II MOB	Wise <i>et al.</i> (1999)	CATCTCTGRCSAYCATACCGG	3	1	1
type2 457	16S rDNA	Type II MOB	Auman <i>et al.</i> (2000)	GCTCTTTCGCTYAGGGACGA	0	0	0
MA-221	16S rRNA	Type II MOB	Bourne <i>et al.</i> (2000)	GGACGCGGGCCGATCTTTTCG	0	1	2
MA-621	16S rRNA	Type II MOB	Bourne <i>et al.</i> (2000)	TCAAAGGCAGTTCGAGGTT	0	0	1
Mα 450	16S rRNA	Type II MOB	Eller <i>et al.</i> (2001)	ATCCAGGTACCGTCATTATC	0	0	0
Mα 464	16S rRNA	Type II MOB	Eller <i>et al.</i> (2001)	TTATCCAGGTACCGTCATTA	0	0	0
Am445	16S rDNA	Type II MOB	Gulledge <i>et al.</i> (2001)	CTTATCCAGGTACCGTCATTATCGTCCC	0	0	0
Am976	16S rDNA	Type II MOB	Gulledge <i>et al.</i> (2001)	GTCAAAAAGCTGGTAAGGTTT	0	0	0
mb661r	<i>pmoA</i>	<i>pmoA</i>	Costello <i>et al.</i> (1999)	CCGGMGCAACGTCYTTACC	3	3	2
LWpmoA	<i>pmoA</i>	<i>pmoA</i>	Auman <i>et al.</i> (2000)	AACTTCTGGGGHTGGAC	0	1	0
325f	<i>pmoA</i>	<i>pmoA</i>	Fjellbirkeland <i>et al.</i> (2001)	TGGGGYTGGACCTAYTTCC	1	1	2
643r	<i>pmoA</i>	<i>pmoA</i>	Fjellbirkeland <i>et al.</i> (2001)	CCGGRCRACGTCCCTTACC	0	2	1
A650	<i>pmoA</i>	<i>pmoA</i>	Bourne <i>et al.</i> (2001)	TGGAAGCCATTTCCTGCA	3	2	2
pmoAfor	<i>pmoA</i>	<i>pmoA</i>	Steinkamp <i>et al.</i> (2001)	TTCTGGGGNTGGACNTAYTTYCC	0	0	0
pmoArev	<i>pmoA</i>	<i>pmoA</i>	Steinkamp <i>et al.</i> (2001)	TCNACCATNCKDATRTAYTCNGG	0	1	1
mmoX1f	<i>mmoX</i>	<i>mmoX</i>	Miguez <i>et al.</i> (1997)	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	3	3	3
mmoX2r	<i>mmoX</i>	<i>mmoX</i>	Miguez <i>et al.</i> (1997)	GGCTCGACCTTGAACCTTGAGCCATACTCG	2	2	2
mmoXr901r	<i>mmoX</i>	<i>mmoX</i>	Shigematsu <i>et al.</i> (1999)	ACCCAGCGGTTCCASGYTTTSACCCA	0	0	1
mmoXD	<i>mmoX</i>	<i>mmoX</i>	Auman <i>et al.</i> (2000)	CCGATCCAGATDCCRCCCCA	0	0	1
534f	<i>mmoX</i>	<i>mmoX</i>	Horz <i>et al.</i> (2001)	CCGCTGTGGAAGGGCATGAA	2	2	2
f882	<i>mmoX</i>	<i>mmoX</i>	McDonald <i>et al.</i> (1996)	GGCTCCAAGTTCAAGGTGCGAGC	1	0	1

*Type II MOB indicates *Methylocystis* and *Methylosinus*, but not *Methylocella* or *Methylocapsa*.

at first glance the obligate MOB is a good candidate group, the coexistence of multiple strains indicates that there is niche separation among them, and that differences cannot therefore be assigned to geographical factors but rather to ecological ones.

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