A Novel type of *pmoA*: presence and distribution among methanotrophs - expression in *Methylocystis* strain SC2

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Pledge

I certify that the present thesis entitled:

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was carried out without any unlawful devices. I did not use any other than the described literature sources or technical devices.

This work has never been submitted before in this or similar form to any other university and has not been used before any examination.

Marburg, 11.09.2002

Tchawa Yimga Merlin

I dedicate this work to:

- my family,
- my friends,
- and those who will find it valuable.

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2)- Dunfield P. F., M. Tchawa Yimga, S. N. Dedysh; U. Berger, W. Liesack and J. Heyer. 2002. Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. FEMS Microbiol. Ecol. **41:**17-26.

3)- Tchawa Yimga, M.; J. Heyer; P. F. Dunfield, and W. Liesack. 2002. A Novel type of *pmoA*: presence and distribution among methanotrophs – expression in *Methylocystis* strain SC2 (in preparation).

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List of abbreviations

%	percent
µg	Microgram
µl	Microliter
°C	Degree Celsius
b	backward
bp	base pair
BSA	Bovine serumalbumine
cDNA	Complementary DNA
Conv.	Conventional
DEPC	Diethyl pyrocaronate
DGGE	Denaturant gradient gel electrophoresis
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosid triphosphate
EDTA	Ethylenediamin-N, N', N', N' tetra acetic acid
f	forward
FISH	Fluorescence-in-situ hybridisation
g	gram
KAc	Potassium acetate
kb	Kilobase
M	Mole per liter
mg	Milligram
ml	Milliliter
MM	Millimole per liter
MOB	Methane-oxidizing bacteria
MOPS	3-(N-Morpholino)-propanesulfonic acid
NAc	Natrium acetate
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	Potential hydrogen
RNA	Ribonucleic acid

RT-PCR	Reverse transcription-PCR
SDS	Sodium dodecyl sulfate
sp./spec.	species
SSC	Saline sodium citrate
SSCP	Single-stranded conformational polymorphism
S/N Blot	Southern/Northern Blot
TAE	Tris-acetate + Na_2-EDTA
TBE	Tris-borate + Na_2-EDTA
TE	Tris-Cl + Na_2-EDTA
TGGE	Thermal gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
Tris	Tris (hydroxymethyl)-aminomethane
U	Units
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

I. Summary

Methane-oxidizing bacteria (MOB) or methanotrophs are a group of bacteria that have the unique ability to grow on methane as their sole carbon and energy source. They have been isolated from a wide range of terrestrial and aquatic ecosystems and have also attracted a lot of interest biotechnologically.
The aim of the present Ph.D. work was to increase our knowledge on the molecular diversity and genotypic characteristics of methanotrophs. Focus has been made on two important methanotrophic gene markers: - 1) the 16S rRNA gene - and 2) the *pmoA* gene which codes for the α-subunit of the particulate methane monooxygenase.

In the first minor part of this study, some recently published methanotrophic 16S rRNA gene primers were applied in order to assess their target specificity on the ecosystem "flooded rice microcosm". Two clone libraries were generated and analysis of libraries identified broad methanotroph diversity, including new type I MOB and type II MOB sequences. The type I MOB group-specific primers retrieved sequences related to the genera *Methylobacte*r, *Methylomicrobium*, *Methylococcus* and *Methylomonas*. The type MOB II primers detected *Methylocystis*-like sequences. Most of these sequences were related to *Methylocystis* strain SC2, a type II methanotroph recently isolated from a polluted aquifer. Only a very few non-methanotrophic sequences were detected by both type I and type II MOB assays, suggesting that these assays were highly specific on the ecosystem "flooded rice microcosm".

In the second major part of the study, several PCR assays were formulated in order to specifically retrieve additional sequence types belonging to the same phylogenetic group as a recently identified novel *pmoA* (clone M84-P3) that clustered neither with the conventional *pmoA* of type I nor with those of type II methanotrophs. The term "novel" was applied to accommodate the novel type of *pmoA* as distinct to the "conventional" or previously known *pmoA* of type I MOB and type II MOB. Two PCR assays, one of which was a single-round-PCR and the other which was a nested-PCR were successful. Phylogenetic analysis of the derived amino acid sequences of *pmoA* retrieved from the ecosystem "rice microcosm" revealed a cluster of environmental sequences that were related to clone M84-P3. The novel cluster had intracluster amino acid dissimilarities of up to 19%. Amino acid sequence identities were ca. 70% with the conventional *pmoA* of type II methanotrophs, 60% to 65% with that of type I methanotrophs and 44% to 65% with the *amoA* of nitrifiers. Diversity

assessment via T-RFLP analysis showed that the novel environmental *pmoA* sequences could easily be classified based on their T-RFs. The novel environmental *pmoA* sequences grouped with a novel *pmoA* sequence identified in strain SC2. This indicated that the novel *pmoA* sequences retrieved in our study do not originate from a hitherto uncharacterized group of methanotrophs, but rather are an indication of divergent, multiple *pmoA* copies in known methanotrophs. The presence of the novel *pmoA* in strain SC2 was confirmed by Southern hybridization.

The question of whether the novel *pmoA* is expressed or not was addressed by applying total RNA extracts of strain SC2 in Northern hybridisation. An unclear and faint signal was observed. To investigate this by a second independent method RT–PCR was applied. The result confirmed expression of the novel *pmoA*, although at lower level than that of the conventional *pmoA*. This suggested that the gene product of the novel *pmoA* may be a functionally active enzyme. In order to get further evidence for the putative function of the novel *pmoA* and to confirm that methane would be the substrate of the enzyme, we conducted a search for amino acid residues, that indicate whether an enzyme is adapted to methane or to ammonia as a substrate. The novel *pmoA* cluster of sequences contained 93.3% (42 of 45) of the universally conserved monoooxygenase (pMMO and AMO) signatures, 78.8% (11 of 14) of the putative pMMO signatures, and none of the putative AMO signatures, indicating that the gene probably codes for the active subunit of a particulate methane monooxygenase.

By using one of the specific PCR assays developed in our study we identified the novel *pmoA* in a wide range of methanotrophs, most of which were type II MOB and a few type I MOB. Confirmation of the presence of the gene in selected strains was possible via Southern hybridization. Phylogenetic affiliation of novel *pmoA* sequences showed that sequences of type I MOB and type II MOB did not separate into monophyletic groups but rather were intermixed, indicating that the novel gene cannot be used as a marker for inferring MOB phylogeny.

II. Introduction

Since the last major glaciation (about 18,000 years ago) the concentration of methane in the atmosphere has increased from 0.35 to 1.7 ppm. During the last 300 years atmospheric methane concentration has been increasing from 0.8 to 1.0% per year until recently when slight decreases in the rate of increase has been reported (Blake *et al*, 1988; Craig *et al*, 1982; Khalil *et al*., 1989; Rowland *et al*. 1990). It is estimated that the concentration of methane will reach 2.1 to 4.0 ppm by the year 2050 (Ramanathan *et al*, 1985). This accumulation makes methane one of the most abundant greenhouse gases in the atmosphere. The release of methane to the atmosphere results in an increased rate of global warming and causes changes in the chemical composition of the atmosphere (Lelieveld *et al*., 1993). As other greenhouse gases, methane absorbs terrestrial radiation in the 4- to 100-nm region (infrared radiation) and while reemitting the absorbed radiation the environment becomes warmer (Lelieveld *et al*., 1993). Evidence suggests that an increase in the atmospheric concentrations of CH_4, CO_2, NO_2 and H_2O is the major cause of global warming. Although the concentration of methane in the atmosphere is lower than that of carbon dioxide, it has been estimated that one mole of methane contributes 26 times more than one mole of carbon dioxide to climate change (Lelieveld *et al*., 1993). In the past century methane has accounted for 15 to 25% of the thermal trapping while carbon dioxide has contributed 60% (Hogan *et al.*, 1991; Ramanathan *et al*., 1985; Rodhe *et al*., 1990).

It has been predicted that increases in methane production in the atmosphere will decrease OH radical concentrations and thus increases the lifetime of methane in the atmosphere (Lelieveld *et al*., 1993). On the other hand, decreases in the rate of methane emissions would have a positive feedback on the decrease of methane in the atmosphere, because of the increase in the atmospheric concentrations of OH radicals that would result. By this process atmospheric methane oxidation would result in an increase in the tropospheric ozone (Tie *et al*., 1992) and stratospheric water concentrations (Cicerone *et al*., 1983; Ehalt, 1974; Vaghjiani *et al*., 1991).

1. Balancing the global methane budget

Global balances in the CH_4 budget have been updated repeatedly. Total sources of atmospheric methane have been estimated at 520 Tg year^{-1} (Cicerone *et al*., 1983, Fung *et al.*, 1991, Lelieveld *et al*., 1993). The lifetime of this gas in the atmosphere is approximately 8 to 12 years (Whalen, 1993). An amount equal to approximately 90% of the annual emissions (450 Tg year^{-1}) is oxidized through photochemical reactions initiated by OH radicals in the troposphere, and a smaller but significant amount (approximately 10 Tg year^{-1}) is lost by

microbial oxidation in soils (Cicerone *et al*., 1983, Fung *et al.*, 1991, U.S. Environmental Protection Agency, 1990). The net annual increase in atmospheric methane is estimated to be 40 Tg $year^{-1}$.

The increase in atmospheric methane is believed to result primarily from human activities (agricultural and industrial emissions), whereas pre-industrial sources of methane were primarily methanogenic activity in natural wetlands with minor contributions from ruminants, fires, oceans, and insects (Hogan *et al.*, 1991; Lelieveld *et al*., 1993, Oremland, 1998; Reeburgh *et al*., 1977; Reeburgh *et al*., 1991; Reeburgh *et al.*, 1993; Whalen, 1993). Estimates of methane budgets, including terms for global production, oxidation, and atmospheric emissions have been reviewed by Cicerone *et al.* (1981; 1988), Lelieveld *et al.* (1993), Reeburgh *et al.* (1993) and Bartlett and Harriss (1993).

2. Methanotrophic bacteria

Methane-oxidizing bacteria (MOB) or methanotrophs are a subset of a larger physiological group of bacteria known as methylotrophs. Methanotrophs are characterized by their ability to utilize methane as their sole source of carbon and energy (Hanson and Hanson, 1996). Methane escapes from anaerobic environments to the atmosphere when it is not oxidized by methanotrophs. Söhngen in 1906 recognized that methane was produced in large amounts in sediments and suggested that the low atmospheric concentrations of this gas were due to its oxidation by microbes. The oxidation of methane is known to occur in both aerobic and anaerobic environments, although little has been published about the microbiology or biochemistry of anaerobic methane oxidation.

Methanotrophs are ubiquitous in environments where methane and oxygen are present. These environments include rivers, lakes, swamps, rice paddies, sediments, soils, seawage sludge etc (Corpe, 1985; Hanson, 1980; Hanson *et al.*, 1991; Hanson and Watenburg, 1991, Heyer *et al.*, 1984; Heyer *et al.*, 1984a; Holmes *et al*., 1995; Seiburg *et al*., 1987, Topp and Hanson, 1991). Methanotrophs alone would oxidize 5-10 % of total methane produced in these environments before it reaches the atmosphere (Conrad, 1995; King, 1997; Neue, 1997).

Methanotrophs are strictly aerobic, gram-negative bacteria that are obligately methylotrophic, and can be classified into two major groups on the basis of their intracytoplasmic membranes, pathways for formaldehyde assimilation and 16S rRNA sequence. The genera *Methylomonas, Methylobacter, Methylococcus, Methylocystis and Methylosinus* proposed by Whittenbury *et al.* (1970) largely have remained unaltered (Bowman *et al.,* 1993) except for the addition of new genera of methanotrophs including *Methylomicrobium* (Bowman *et a.l*, 1993; Bowman *et*

al., 1995), *Methylosarcina* (Wise *et al.*, 2001), *Methylosphaera* (Bowman *et al.*, 1997), *Methylothermus* (Brodossy *et al*., 2000), *Methylocella* (Dedysh *et al.*, 2000) and *Methylocapsa* (Dedysh *et al.*, 2001). Type I methanotrophs, such as *Methylobacter* and *Methylomonas*, are γ-*Proteobacteria* that possess bundles of intracytoplasmic membranes throughout the cell and fix carbon into cell biomass using the ribulose monophosphate cycle (Figure 1). Type II methanotrophs including *Methylosinus* and *Methylocystis* are α-*Proteobacteria* that have their membranes arranged around the periphery of the cell and fix carbon at the level of formaldehyde via the serine cycle. A new group, type X, was added to accommodate methanotrophs similar to *Methylococcus capsulatus* that, like type I methanotrophs utilize the ribulose monophosphate pathway (RuMP) for formaldehyde assimilation. Type X methanotrophs were distinguished from type I methanotrophs because they also possessed low levels of enzymes of the serine pathway, and ribulose biphosphate carboxylase, an enzyme present in the Calvin cycle (Whittenbury, 1981; Whittenbury and Dalton, 1981; Whittenbury and Krieg, 1984). Type X methanotrophs grew at higher temperatures than type I and type II methanotrophs and possessed DNA with a higher mol% G + C content than that of most type I methanotrophs (Gal'chenko *et al.*, 1984; Green, 1992; Hanson *et al.*, 1991).

The interest in methanotrophs over the last 30 years has largely been due to their biotechnological potential for the production of single cell protein, propylene oxide and other biotechnological products (Leak, 1992). sMMO- (soluble methane monooxygenase) containing methanotrophs are particularly useful in biotransformation reactions since they are able to degrade the groundwater pollutant trichloroethylene (TCE) and other halogenated hydrocarbons (Oldenhuis and Jansen, 1993; Brusseau *et al*, 1990; Bowman *et al.,* 1994; Baker *et al.,* 2001).

3. Physiology, biochemistry and molecular biology of methane oxidation

Methane is oxidized by methanotrophs to CO_2 via the intermediates methanol, formaldehyde, and formate. Approximately 50% of the formaldehyde produced is assimilated into cell carbon and the remainder is oxidized to CO_2 and lost from the cell (Anthony, 1982).

The dissimilatory reactions, converting formaldehyde to CO_2, generate reducing power for biosynthesis and the oxygenation step (Figure 1). The first enzyme in the methane oxidation pathway is methane monooxygenase (MMO). There are two distinct types of MMO enzymes: a soluble, cytoplasmic enzyme complex (sMMO) and a membrane-bound, particulate enzyme system (pMMO).

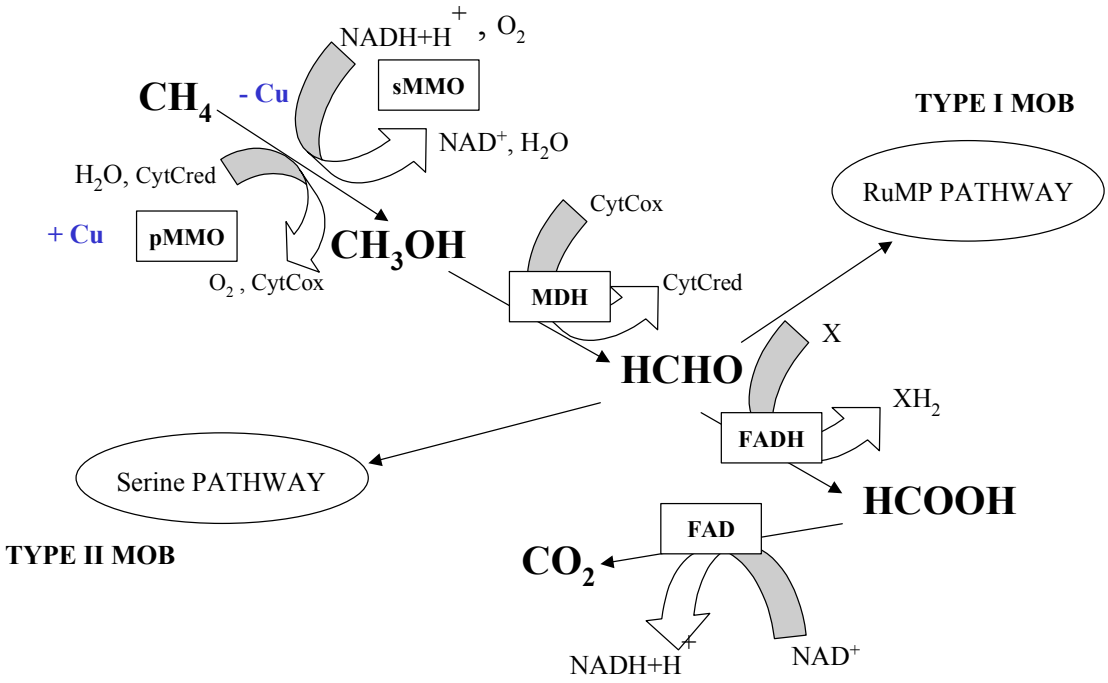

Fig. 1. Pathways for methane oxidation and formaldehyde assimilation in type I and type II methanotrophs.

Until recently it was thought that sMMO was found only in the genera *Methylosinus*, *Methylocystis* and *Methylococcus*. Although it has subsequently been observed in some *Methylomonas* (Shigematsu *et al*., 1999) and *Methylomicrobium* (*Fuse et al.*, 1998) species, not all methanotrophs contain this enzyme. sMMO is only expressed when the copper-to-biomass ratio of the culture is low, that is under low-copper growth conditions. There is also evidence that copper can inhibit sMMO activity (Green *et al.*, 1985). sMMO has an extremely broad substrate specificity, co-oxidizing a wide range of alkanes, substituted aliphatics and even aromatic compounds, making it an extremely attractive enzyme for biotransformation processes and bioremediation (Brusseau *et al.*, 1990). The most well-characterized sMMO enzymes are those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Lipscomb, 1994; Gassner *et al.*, 1999; Lee *et al.*, 1999). sMMO is a non-heme, iron-containing enzyme complex consisting of three components: hydroxylase, protein B and protein C. The hydroxylase has three subunits, α, β and γ, of 60, 45 and 20 kDa respectively, which are arranged in an $\alpha_2\beta_2\gamma_2$ configuration. The α subunit contains a non-heme bis-μ-hydroxo-bridged binuclear iron centre at the active site of the enzyme, where methanol is formed from methane and oxygen. The genes encoding sMMO have been cloned and

sequenced from several methanotrophs, including *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. In methanotrophs these genes are clustered on the chromosome. *mmoX, mmoY* and *mmoZ* encode the α, β and γ subunits of the hydroxylase, respectively; *mmoB* and *mmoC* code for protein B and protein C , respectively. An open reading frame (ORF) of unknown function, *orfY*, separates *mmoY* and *mmoZ* in all methanotrophs examined to date (McDonald *et al.,* 1997). sMMO genes are highly conserved in all methanotrophs examined with sequence identities of 55-95 % and 46-96 % being observed between the corresponding DNA and amino acid sequences, respectively.

Virtually all methanotrophs examined possess a membrane-bound, particulate methane monooxygenase, which is expressed only when the copper-to-biomass ratio of the culture is high. The only exception is *Methylocella palustris* that harbours only sMMO. Unlike sMMO, pMMO has a relatively narrow substrate specificity, oxidizing alkanes and alkenes of up to five carbons in length. However it can be useful for biotransformation (DiSpirito, 1992). The pMMO from *Methylococcus capsulatus* (Bath) consists of three subunits of 45, 27 and 23 kDa. The 45- and 27-kDa subunits probably constitute the active site because they can be labelled by the suicide substrate acetylene (Zahn and DiSpirito, 1996). Particulate methane monooxygenase has proven difficult to purify to homogeneity with high activity. However this enzyme has now been purified from membranes of *Methylococcus capsulatus* (Bath) (Nguyen, 1998; Zahn and DiSpirito, 1996). There are two nearly identical copies of the genes encoding pMMO (*pmoCAB*) in the chromosome of *Methylococcus capsulatus* (Bath) (Semrau *et al.*, 1995; Stolyar *et al.*, 1999) and a third, separate copy of *pmoC* has also been identified (Stolyar *et al.,* 1999). Mutagenesis experiments have suggested that the two sets of genes are functionally equivalent (Stolyar *et al.*, 1999). This gene duplication has also been observed with the analogous enzyme ammonia monooxygenase (*amoCAB*) in ammonia-oxidizing bacteria (Sayavedra *et al.*, 1996); comparison of *pmo* and *amo* gene sequences suggests that pMMO and AMO could be evolutionarily related (Holmes *et al.,* 1995; Klotz and Norton, 1998). The *pmo* gene clusters have also been sequenced from *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Gilbert *et al.,* 2000). These species also have two copies of *pmoCAB* and there is a high degree of similarity (80-94 %) at the derived amino acid sequence level between the pMMO polypeptides from different methanotrophs. The *pmoA,* which encodes the α subunit (PmoA) of the pMMO, has been shown to be evolutionary highly conserved among methanotrophs (Holmes *et al.,* 1995).

In methanotrophs such as *Methylosinus trichosporium* OB3b, which possess both pMMO and sMMO, there is a metabolic switch mediated by copper ions. When cells are starved for

copper, and the copper-to-biomass ratio of the cell is low, sMMO is expressed. Cells grown under conditions of excess copper express pMMO and there is no detectable sMMO expression (Murrell *et al.*, 2000). No other metal ion effects this metabolic switch. However, the exact mechanism for reciprocal regulation of the sMMO and pMMO gene clusters by copper ions is not clear at present.

4. Molecular ecology of methanotrophs

Culture-based techniques have successfully been used to isolate methanotrophs from environmental samples. However, culture-based methods are limited because many methanotrophs do not grow on conventional media and pure cultures isolated constitute only a small fraction of the viable species diversity and the fraction of cells recovered from environmental samples is also believed to be a small fraction of those present (Bone *et al.*, 1986; Hanson, 1980; Hanson and Wattenburg, 1991). The physiological types of methanotrophs isolated from environmental samples may reflect the conditions used for enrichments and isolation attempts and thus may not be the dominant organisms in the original population (Amaral *et al.*, 1995; Hanson, 1992; Hanson and Wattenburg, 1991; Whittenbury and Dalton; 1981).

Another method for identification of methanotrophs in natural environmental samples is phospholipids and fatty acid analysis. Phospholipids extracted from environmental samples are useful for measuring changes in community structure and physiological stress within a microbial community (Nichols *et al*. 1985; Nichols *et al.*, 1987). This approach has been particularly useful for detecting populations of type I and type II methanotrophs, because each contains characteristic fatty acids. However the technique is relatively expensive and requires an extensive database for methylotrophic organisms.

The use of fluorescent antibodies prepared against killed cells from pure cultures is a potentially effective tool for identifying and enumerating methanotrophs without culturing them (Abramochkina *et al.*, 1987; Bohlool and Schmidt, 1980; Broxrukova *et al.*, 1983; Gal'chenko *et al.*, 1988). However these techniques require that the organisms used to prepare the antisera belong to all the serotypes present in the habitats under study and that the cells being used are permeable to antibodies.

Non-cultured methanotrophs can be detected with nucleic acid probes or by sequencing genes amplified by PCR directly from environmental samples (Amann *et al.*, 1990; Fox *et al.*, 1980; Giovannoni *et al.*, 1988; Miyata *et al.*, 1993; Olsen *et al.*, 1986). These methods are useful for identification of taxa and for determination of the phylogenetic positions of microbes. Current

classification schemes (Green, 1992; Hanson *et al.*, 1991) have been strengthened as a result of the comparative sequence analysis of both the 5S and the 16S ribosomal RNA (rRNA) from a large number of methanotrophs and methylotrophs (Tsuji *et al.*, 1990; Bratina *et al.*, 1992; Bowman, 1990). Type II methanotrophs, such as *Methylocystis parvus* and *Methylosinus trichosporium*, appear to cluster in the α-2 sub-class of the class *Proteobacteria* and form a separate cluster from other serine pathway methylotrophs. Type I methanotrophs, such as *Methylomonas methanica*, *Methylomicrobium album* and *Methylobacter luteus* are found in the γ-sub-class of *Proteobacteria* (reviewed by Hanson and Hanson, 1996). Hanson and colleagues designed oligonucleotide probes from 16S rRNA data, which can be end-labelled with ^{32}phosphorous or dyes, such as fluoresceine and rhodamine, for fluorescence microscopy work. The first probe, designated 9α, was complementary to target sequences in 16S rRNA of serine-pathway methylotrophs and is specific for these organisms. The second, 10γ, was specific for RuMP-pathway methylotrophs. These two probes, when labelled with two different dyes, have successfully been used to differentiate two different groups of methanotrophs (Tsien *et al.*, 1990; Hanson *et al.*, 1993). Several other group-specific probes have been designed for type I and type II methanotrophs and have been successful in a wide range of applications. A partial list of these probes is given in table 1.

5. Functional gene probes for methanotrophs

The high degree of identity between sMMO genes has enabled the design of PCR primers which specifically amplify each of the five sMMO structural genes (McDonald *et al.*, 1995 Mini review) such that amplification of the five sMMO structural genes is now possible from cultured methanotrophs containing sMMO and from total DNA from a variety of different freshwater, estuarine, soil, wetlands and rice root samples. Subsequent cloning and DNA sequencing of a number of these PCR products has uncovered sMMO-encoding DNA genes very similar to, but not identical to known sMMO gene sequences (McDonald *et al.*, 1995; Horz, 2001). Although sMMO-based approaches are useful for studying methanotroph diversity in copper-depleted environments such as wetlands or contaminated aquifers, these genes, however, are not present in all known methanotrophs. A better approach would be based on the pMMO, present in all known methanotrophs except for *Methylocella palustris*. Sequence data on *pmoA* and *amoA* genes have allowed the design of degenerate primers,

Table 1. Group-specific probes targeting type I and type II methanotrophs

Probe name	Sequence 5'-3'	Description of specificity	Reference
1034-Ser	CCATACCGGACATGTCAAAAGC	Serine pathway methanotrophs	Brusseau *et al.*, 1994
1038-Ser-M	GGTAACATGCCATGTCCAG	==//==	===//==
1041-5	CTCCGCTATCTCTAACAGATT	RuMP pathway methanotrophs	===//==
1035-RuMP	GATTCTCTGGATGTCAAGGG	==//==	===//==
Mb1007	CACTCTACGATCTCTCACAG	*Methylobacter*	Holmes *et al.*, 1995
Mc1005	CCGCATCTCTGCAGGAT	*Methylococcus*	===//==
Mm1007	CACTCCGCTATCTCTAACAG	*Methylomonas*	===//==
Ms1020	CCCTTGCGGAAGGAAGTC	*Methylosinus*	===//==
Mm850	TACGTTAGCTCCACCACTAA	*Methylomonas*	===//==
MG-64	CCGAAGGCCTRTTACCGTTC	*Methylococcus, Methylomonas, Methylomicrobium*	Bourne *et al.,* 2000
Mc1029	CCTGTGTCTTGGCTCCCGAA	*Methylococcus*	===//==
MA-221	GGACGCGGGCCGATCTTTCG	Type II Methanotrophs	===//==
MA-621	TCAAAGGCAGTTCCGAGGTT	===//==	===//==
Mcell-1026	GTTCTCGCCACCCGAAGT	*Methylocella*	Dedysh *et al*., 2001
Mcell-181	TCTTTCTCCTTGCGGACG	*Methylocella*	===//==
Mα450	CTATTACTGCCATGGACCTA	Type II Methanotrophs	Eller *et al*., 2001
Mα464	ATTACTGCCATGGACCTATT	Type II Methanotrophs	===//==
Mγ84	AGCCCGCGACTGCTCACC	Type I Methanotrophs	===//==
Mγ705	CTAGACTTCCTTGTGGTC	Type I Methanotrophs	===//==
Mγ983	TGGATGGGAACTGTAGGT	Type I Methanotrophs	===//==
Mγ993	CTGTAGGTCTCTTTAGACA	Type I Methanotrophs	===//==

that specifically amplify a 525-bp internal DNA fragment of these genes from a variety of methanotrophs and nitrifiers (Holmes *et al.*, 1995). Use of these allowed amplification of *pmoA* or *amoA* fragments from diverse cultures, and the results suggested that the pMMO and AMO may be evolutionary related enzymes, despite their different physiological roles (Holmes *et al.*, 1995).

The degenerate oligonucleotide PCR primers described above have now been successfully used to specifically amplify *pmoA/amoA* from DNA isolated from a variety of environments (Murrell *et al.*, 1998; Costello *et al.*, 1999; Horz *et al.*, 2001; Auman *et al.*, 2000). At present an extensive database of sequences from extant methanotrophs and nitrifiers is being established and will aid further molecular ecological studies. Moreover, PCR primers have been designed, that specifically amplify either *amoA* (Rotthauwe *et al.*, 1997) or *pmoA* (Costello *et al.*, 1999). These new primers may help to assess the relative roles of methanotrophs and nitrifiers in the cycling of methane in a number of interesting environments.

Another potentially useful marker is the *mxaF* gene. PCR primers that specifically amplify a 550-bp fragment of *mxaF* sequences from methanotrophs have been used to extend the database of *mxaF* genes of methanotrophs and methylotrophs and to identify *mxaF* sequences in marine, soil and wetland samples (McDonald *et al.*, 1997, Holmes *et al.,* 1995, McDonald *et al.*, 1995). The *mxaF* gene is not specific for methanotrophs, however.

Finally degenerate *nifH* primers have been applied to amplify *nifH* sequences of methanotrophs based on their nitrogen fixation capabilities. Although these primers could discriminate among type I and type II methanotrophs, not all type I organisms seemed to harbor the *nifH* gene (Auman *et al.*, 2001). Moreover, as with the *mxaF,* the *nifH* gene is not specific for methanotrophs.

6. Anaerobic methane oxidation

Recently, consortia have been found, that are responsible for anaerobic methane oxidation. This process probably contributes to a significant proportion of global methane oxidation. The consortia consist of microorganisms affiliated to lineages of *Archaea* and bacterial sulfate-reducers (Boetius *et al.,* 2000). They occur in anaerobic environments in the deep sea and consume methane coming out off gas hydrates (Orphan *et al.,* 2001). This process does not occur in terrestrial ecosystems. Obviously, these anaerobic consortia are important for controlling emission of methane out off gas hydrates, and play a role in global methane budget (DeLong, 2000). But for oxidation of atmospheric methane only terrestrial ecosystems seem to be relevant.

7. Aim of work

PCR-mediated preferential amplification of MOB 16S rDNA and *pmoA*, and subsequent cloning and sequencing has extensively been applied to create phylogenetic inventories of MOB in numerous environments (Costello *et al.*, 1999; Henckel *et al.*, 2000; Horz *et al.*, 2001, Wise *et al.*, 1999).

The *pmoA* encodes the α subunit (PmoA) of pMMO and it has been shown to be evolutionarily highly conserved among methanotrophs (Holmes *et. al*, 1995). Consequently, the *pmoA* has been used as a functional gene marker to detect MOB in a wide range of environments (Costello *et al.*, 1999; Auman *et al.*, 2000; Henckel *et al.*, 2000; Horz *et al.*, 2001; Pacheco-Olivier *et al.*, 2002). In numerous studies, phylogenetic trees constructed

based on *pmoA* sequences closely mirrored 16S rDNA-based phylogenies for the same organisms, and therefore it has been suggested that *pmoA* is a useful phylogenetic marker for MOB in molecular ecology studies (Murrell *et al.*, 1998; Costello *et al.*, 1999; Horz *et al.*, 2001; Auman *et al*., 2000). This view is based on the assumption that multipe *pmoA* copies present in a single MOB species are nearly identical, as has been shown for the duplicate *pmoA* gene copies present in the type I MOB *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8 (Semrau *et al.*, 1995; Stolyar *et al*., 1999), as well as the type II species *Methylosinus trichosporium* OB3b and *Methylocystis* sp. M (Gilbert *et al.*, 2000). Both RFLP patterns and comparative sequence analysis suggested that the one to three *pmoA* copies present in five type I and six type II MOBs isolated from lake sediment are almost identical (Auman *et al.*, 2000). However, if divergent *pmoA* copies were present in the same organism, the interpretation of environmental data would become rather more complicated. Moreover, incomplete coverage of cultured MOB in the current 16S rRNA and *pmoA* data sets renders it impossible to decide whether a novel environmentally retrieved 16S rRNA or *pmoA* sequence represents a previously uncultured MOB, or is identical to an already isolated MOB, that is not yet included in the respective database.

The objectives in the present study were as follows:

1. 16S rDNA-based detection of methanotrophs on rice roots

In a previous study, Wise *et al.* (1999) exploited the limited database of obligate methane oxidizers to design degenerate methanotroph-specific 16S rRNA PCR primers, and used these primers to construct clones libraries from DNA extracted directly from landfill soil, in an effort to describe methanotrophic community structure in a cultivation-independent manner. Primer sets were specific for the retrieval of either type I or type II sequences on the environment tested. However, a decision about the target specificity requires that these assays be tested on a wide range of environments. One of the goals in our study was to test the reliability of the newly developed methanotroph specific 16S rRNA assays on DNA extracted from rice roots.

2. Development of specific PCR assays for the detection of novel *pmoA*-like sequences on rice roots

In an effort to generate a larger methanotrophic *pmoA* database we identified a novel *pmoA*-like sequence that clustered neither with the conventional *pmoA* sequences of type I, nor with those of type II methanotrophs. Analyses of *pmoA* signatures showed that the novel sequence

exhibited amino acid residues normally found at conserved positions within the *pmoA* gene and therefore could be assumed to code for *pmoA* than *amoA*. We therefore assumed that the novel sequence was indicative of a novel group of methanotrophs whose members were characterized not yet. In order to further investigate this problem and to identify novel sequences belonging to the same phylogenetic group, we designed PCR primers that were specific to the novel *pmoA*-like sequence cluster, then evaluated the target specificity of the primers for further application to environmental studies.

3. Screening of pure cultures isolates for the presence of the novel *pmoA* gene

In parallel to our study mentioned in section 2, colleagues (Dr. Peter F. Dunfield and Dr. Jürgen Heyer) in our department were developing a culture collection and a molecular database for MOB isolated from a number of environments. During their study they identified a *Methylocystis* strain (termed strain SC2) with two very different *pmoA*-like genes (Dunfield *et al.*, 2002). The first gene (*pmoA*1 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 the first gene at the nucleotide level and 68.5% identity at the amino acid level. The *PmoA2* of strain SC2 was closely related to the above-mentioned *pmoA*-like sequence retrieved in our previous study by cultivation-independent methods from rice field soil (86.3% identity on amino acid level), indicating that the presence of multiple, diverse *pmoA* copies might not be unique to strain SC2. This finding was strengthened by detection of a novel *pmoA*2-like copy in *Methylosinus trichosporium* strain KS21 (Dunfield *et al.*, 2002). This is intriguing because the *pmoA* has been used instead of 16S rDNA as a phylogenetic marker (Auman *et al.,* 2000; Horz *et al.,* 2001). However, the presence of diverse copies in single strains may change this view and the interpretation of environmental *pmoA* data may become more complicated. It also poses the questions of how widely distributed these novel *pmo* genes are among methanotrophs and of what function they may have. To address the first question, we used our newly designed PCR assays (as mentioned in section 2) to screen for the presence of the novel type of *pmoA* gene among members of type I and type II methanotrophs obtained from the culture collection established by Peter F. Dunfield and Jürgen Heyer. Then, selected strains, which tested positive by PCR were subjected to Southern hybridization, a PCR-independent approach. Finally, the expression of the novel *pmoA* gene was investigated by Northern hybridization and the results confirmed via RT-PCR.

III. Materials and Methods

1. Materials

1.1 Environmental samples and DNA extracts

DNA extracts were either obtained from extraction performed during the present study (see Methods section) or they were made available from previous Ph.D. studies. The list of extracts is given in table 2.

Table 2. Environmental DNA extracts applied in the course of this study

Designation	Provider	Origin
Rr90a	Dirk Rosencrantz	Roots of 90-day-old rice plants (1)
RsVb, RsVc, RsVd	Heiner Lüdemann	Rice soil (2)
Rz90e, Rz90f	Regine Großkopf	Root mat of 90-day-old rice plants
Rr90g	Hans-Peter Horz	Roots of 90-day-old rice plants (1)
Rr90h	Present study	Roots of 90-day-old rice plants (1)

The microcosms were cultivated using soils sampled from drained rice fields of the Italian Rice Research Institute in Vercelli, Italy. (1) Soils were obtained in 1995. (2) Soils were obtained for samples RsVb, RsVc and RsVd in 1993, 1995 and 1997, respectively.

1.2 Microorganisms

A number of methanotrophic pure cultures, including isolates and type strains, were applied in the course of this study in either PCR or hybridization assays. Taxonomic designation and sources of microorganisms are given in table 2. *E. coli* strain INVαF' (Invitrogen, de Schelp, The Netherlands) was applied in cloning experiments (not shown in table 2).

1.3 Cloning vectors

pCR™ II and pCR™ 2.1 (TA Cloning® Kit, Invitrogen, de Schelp, Netherlands).

Table 3: List of methanotrophic pure cultures

Strain designation	Taxonomic name	Origin
SM16	*Methylocystis* spec.	High polluted River Saale, Maua, Germany
LRI	*Methylocystis* spec.	Agricultural soil, Ottawa, Canada
B2/7	*Methylocystis* spec.	Meadow soil, Gießen, Germany
62/12	*Methylocystis* spec.	Oligotrophic Lake Stechlinsee, Neuglobsow, Germany
KS9	*Methylocystis* spec.	Mesotrophic Lake Kinneret, surface sediment, Israel
014c	*Methylocystis* spec.	Baltic Sea, Bornholm-Becken, surface sediment, Germany
51	*Methylocystis* spec.	Meadow soil, Gießen, Germany
IMET10484	*Methylocystis* spec.	Soil near oil extraction plant, Mannhäger Moor, Germany
21/1	*Methylocystis* spec.	Soil near oil extraction plant, Wittenhagen, Germany
IMET10499	*Methylocystis* spec.	Water, Insel Rügen, Germany
F10v12a	*Methylocystis* spec.	Peatsludge, Neuglobsow, Germany
Ks7	*Methylocystis* spec.	Mesotrophic Lake Kinneret, surface sediment, Israel
IMET10486	*Methylocystis* spec.	Water, Wittenhagen, Mecklenburg-Vorpommern, Germany
SC2	*Methylocystis* spec.	Highly polluted River Saale, Weißen, Germany
IMET 10491	*Methylocystis echinoides*	Clearing station, sludge, Jena-Lobeda, Germany
Pi6/2	*Methylocystis* spec.	Rice field, paddy soil, Los Banos, Philippines
NCIMB 11132	*Methylocsystis parvus*	National collection of industrial and marine bacteria, Aberdeen , Scotland, United Kingdom
SC8	*Methylosinus sporium*	Highly polluted River Saale, Weißen, Germany
SK13	*Methylosinus sporium*	Highly polluted River, Saale, Weißen, Germany
20/3	*Methylosinus sporium*	Soil near oil extraction plant, Wittenhagen, Germany
H1b	*Methylosinus sporium*	Eutrophic Lake Haussee, surface sediment, Feldberg, Germany
SC6	*Methylosinus trichosporium*	Highly polluted River, Saale, Wichmar, Germany
H3	*Methylosinus trichosporium*	Sea sediment, Feldberg, Germany
M23	*Methylosinus trichosporium*	Mangrove roots, Hisal, India
39/3	*Methylosinus trichosporium*	Water, Rügen Island, Mecklenburg-Vorpommern, Germany
NCIMB 11131	*Methylosinus trichosporium*	National collection of industrial and marine bacteria, Aberdeen , Scotland, United Kingdom
SC10	*Methylosinus trichosporium*	Water, Saale, Wichmar, Thürigen, Germany
IMET 10556	*Methylomonas* spec.	Clearing station sludge, Jena-Lobeda, Germany
D1a	*Methylomonas* spec.	Eutrophic Lake Dagowsee, surface sediment, Neuglobsow, Germany
E10a	*Methylocaldum* spec.	Field soil, Eiterfeld, Germany
08a	*Methylocaldum* spec.	Ostsee, Gotland-Tief, Germany, surface sediment
NCIMB 11853	*Methylococcus capsulatus*	National collection of industrial and marine bacteria, Aberdeen , Scotland, United Kingdom
NCIMB 11123	*Methylomicrobium album*	National collection of industrial and marine bacteria, Aberdeen , Scotland, United Kingdom

1.4 Enzymes and Kits

Item	Source
AmpliTaq®-DNA-Polymerase ABI PRISM™ Dye Terminator Cycle Sequencing Ready reaction Kit ABI PRISM™ BigDye Terminator Cycle Sequencing Ready reaction Kit	Perkin Elmer Applied Biosystems, Weiterstadt, Germany
AmpliTaq-DNA-Polymerase Restriction enzymes: *Msp*I, *BamH*II, *Bgl*II, *Pst*I, *Hind*III, *EcoR*I, *Sma*I, *Xho*I	Promega, Mannheim, Germany
Lysozyme from chicken egg Proteinase K	Sigma Aldrich, Deisenhofen, Germany
RNase A, RNase T1	Boehringer Mannheim, Mannheim, Germany
Qiagen Omniscript^{TM} Kit	Qiagen, Hilden, Germany
TOPO TA Cloning® Kit	Invitrogen, Groningen, The Netherlands

1.5 Nucleic acid standards

DNA Smart Ladder (Eurogentec, Searing, Belgium)
RNA Ladder (New England Biolabs, Frankfurt)
Genescan-standard Rox 1000, with Rox (6-carboxy-X-Rhodamin) labelled (Perkin Elmer Applied Biosystems).

1.6 Oligonucleotide primers and probes

Primer and probe sequences are summarized in table 3. Primers for T-RFLP analyses had a 5(6)-carboxyfluorescein-N-hydroxysuccinimidester (fluorescein) labeling whereas probes for hybridization had a Digoxygenin labeling.

1.7 Chemicals and reagents

Chemicals and reagents used in this study were purchased from the following companies:
Bio-Rad, Munich; Biozym, Hess. Oldendorf; Boehringer Mannheim, Mannheim; New England Biolabs Frankfurt; Fluka, Buchs, Switzerland; Gibco, Eggenstein; Merck, Darmstadt; MWG-Biotech, Ebersberg; Metabion, Martinsried; Perkin Elmer Applied Biosystems, Weiterstadt; Amersham Pharmacia, Freiburg; Biometra Goettingen; Qiagen, Hilden; Stratagene, Heidelberg; Sigma, Deisenhofen.

1.8 Gases

The following gases were used for cultivation of methanotrophic bacteria: CH_4, CO_2, and air. Liquid nitrogen was used in nucleic acid extraction protocols. All gases were purchased from Messer-Griescheim (Düsseldorf).

1.9 Buffers and solutions

Buffers and solutions were prepared with distilled water and sterilized 30 min at 121°C. Solutions which did not require autoclaving such as 10% SDS were prepared with sterile distilled water and filtered (0.2 μm diameter Whatman paper, Schleicher and Schuell, Dassel) under sterile conditions.

Table 4: Oligonucleotide primers and probes used in this study

Name	Target	Strategy	Gene positions	Sequence 5'→3'	Reference
A189f	*pmoA*	PCR	171-189	GGNGACTGGGACTTCTGG	Holmes *et al.*, 1995
A682b	*pmoA*	PCR	703-685	GAASGCNGAGAAGAASGC	Holmes *et al.*, 1995
PmoA206f	*pmoA*	PCR	171-206	GGNGACTGGGACTTCTGGATCGACTTCAAGGATCG	This study
PmoA703b	*pmoA*	PCR	703-668	GAASGCNGAGAAGAASGCGGCGACCGGAACGACGT	This study
PmoA190f	*pmoA*	PCR	190-206	TCGACTTCAAGGATCG	This study
PmoA668b	*pmoA*	PCR	686- 668	ACCGGAACGACGTCCTTA	This study
PmoA671b	*pmoA*	PCR	689-671	ATCATGCGGATGTATTCMGGSGTGCC	This study
PomA636b	*pmoA*	S/N Blot	610-636	CATCGACGTGCGGACGAAGTGGA	This study
PmoA593b	*pmoA*	S/N Blot	615-593	CATCGACGTGCGGACGAAGTGGA	Dunfield *et al.*, 2002
MethT1dF	16S rDNA	PCR	988-1006	CCTTCGGMGCYGACGAGT	Wise *et al*., 1999
MethT1bR	16S rDNA	PCR	84-102	GATTCYMTGSATGTCAAGG	----------
MethT2R	16S rDNA	PCR	997-1017	CATCTCTGRCSAYCATACCGG	----------
Eub9f	16S rDNA	PCR	9-27	GAGTTTGATCMTGGCTCAG	Lane, 1991
Eub1492b	16S rDNA	PCR	1512-1492	ACGGYTACCTTGTTACGACTT	Weisburg *et al.*, 1991
536f	16S rDNA	16S rDNA sequencing	519-536	CAGCMGCCGCGGTAATWC	Lane, 1991
536r	16S rDNA	16S rDNA sequencing	519-536	GWATTACCGCGGCKGCTG	Lane, 1991
907f	16S rDNA	16S rDNA sequencing	907-926f	AAACTRAAAMGAATTGACGG	Lane; 1991
907r	16S rDNA	16S rDNA sequencing	907-926f	CCGTCAATTCMTTTRAGTTT	Lane, 1991
Puc/M13f (-20)	PCR™II	Clone sequencing	433-448	GTAAAACGACGGCCAG	TA Cloning Kit®, Invitrogen
Puc/M13r	PCR™II	Clone sequencing	205-221	CAGGAAACAGCTATGAC	TA Cloning Kit®, Invitrogen

N=(A,T,C,G), M=(C,A), W=(T,A), K=(G,T), R=(A,G), Y=(C,T)

Numbering for *pmoA* is referred to the *pmo* gene sequence of *Methylococcus capsulatus* (Bath) (Semrau *et al.,* 1995). Numbering for 16S rDNA is referred to the 16S rDNA gene sequence of *E. coli* (Brosius *et al.,* 1978). Numbering for PCR™II is referred to the vector map (see TA Cloning Kit® booklet).

A) Buffers and solutions for DNA extraction:

- Extraction buffer for environmental DNA

100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.0 mM Dithiothreitol (DTT)

- TE-Buffer (Sambrook *et al*., 1989)

10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA, pH 8.0

- Lysozyme buffer: 1 mg/ml Tris, pH 8.0

- Other solutions

10% SDS, 5 mM KAc, pH 7.5; chloroform/isoamylalcohol (24:1, v/v); isopropanol, ethanol 70%.

B) Buffers and solutions for separation of nucleic acids:

- TBE-buffer (Sambrook *et al*., 1989)

90 mM Tris-boric acid, pH 8.0; 2 mM EDTA, pH 8.0

- TAE-Buffer (Sambrook *et al*., 1989)

40 mM Tris-HCl, pH 7.5; 20 mM NaAc, 1 mM EDTA, pH 7.5

- 10 x loading buffer for agarose gel electrophoresis

40% (w/v) saccharose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylenecyanol, 0.25% (w/v) Orange G2

- Ethidium bromide solution

0.0001% (w/v)

C) Buffers and solutions for cloning:

- X-Gal solution 5 % (w/v)

50 mg 5-bromo-4-chloro-3-indolyl-ß-galactopyranoside (X-Gal) in 1.0 ml dimethylformamid (storage at –20°C).

- Glycerin buffer

65 % glycerol, 0.1 M $MgSO_4$, 25 mM Tris-HCl, pH 8.0

D) Buffers and solutions for Southern hybridization: (see the DIG Application manual for filter hybridization)

- Sterile double distilled water for rinsing gel and eventually dilution of DNA
- Depurination solution: 250 mM HCl
- Denaturation solution: 0.5 M NaOH, 1.5 M NaCl
- Neutralization solution: 0.5 Tris-HCl, pH 7.5; 1.5 M NaCl
- 20 x SSC buffer, 3 M NaCl, 300 mM sodium citrate, pH 7.0
- Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5
- 10 x blocking solution: 1:10 (w/v) in maleic acid buffer
- Hybridization buffer: 5 X SSC, 0.1% N-Lauroylsarcosin, 0.02% SDS, 1% blocking solution
- Washing buffer: 0.1 M maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20
- Low stringency buffer: 2 x SSC containing 0.1% SDS
- High stringency buffer: 0.5 x SSC containing 0.1% SDS, pH 7.5; 0.3 % (v/v) Tween 20
- Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5
- Stripping buffer: 0.2 M NaOH, 0.1% SDS

E) Buffers and solutions for Northern hybridization: (see the DIG Application manual for filter hybridization)

- 10 x MOPS buffer: 200 mM MOPS, 50 mM NaAc, 20 mM EDTA, pH 7.0
- Loading buffer:

MOPS/deionized formamide/formaldehyde/glycerol/bromophenol blue

- Others: Hybridization buffer, maleic acid buffer, 10 x blocking solution, washing buffer, low stringency buffer, high stringency buffer, detection buffer buffer.
- Stripping buffer: 50 % deionized formamide; 5 % SDS; 50 mM Tris-HCl, pH 7.5
- DEPC-treated deionised water: 1 ml DEPC (diethyl pyrocarbonate) in 1000 ml deionised H_2O

1.10 Culture media

A) Media for growth of *Escherichia coli*

- LB-Medium (Luria-Bertani-Medium, Sambrook *et al.*, 1989)

Liquid medium: 1.0 % Bacto-Trypton; 0.5 % Beef extract; 1.0 % NaCl, pH 7.0

Solid medium: 1.0 % Bacto-Trypton; 0.5 % Beef extract; 1.0 % NaCl, 1.5 % Agar, pH 7.0

Antibiotic selection required addition of Kanamycin at final concentration of 50 µg/ml.

2. Methods

2.1 DNA extraction from environmental samples

Construction of microcosms, growth of rice plants, handling and harvesting of rice roots were performed in previous studies (Großkopf *et al*., 1998; Horz *et al.*, 2001)

DNA was extracted from dried root material following a procedure adapted from Großkopf *et al.* (1998). Ca. 100 mg of lyophilised roots, were pulverized with a mortar under liquid nitrogen. The pulverized root material was resuspended in 1 ml of extraction buffer. The homogenisate was transferred in a 2-ml tube. The tube was placed into liquid nitrogen for 2 minutes, then for another 2 minutes in a 65°C water bath. The operation was repeated twice, followed by addition of 2 mg lysozyme (40 µl of a 50 mg/ml solution) and 1 h incubation at 37°C. Subsequently, 0.1 mg of proteinase K (5 µl of 20 mg/ml solution) and 50 µl of a 10% SDS solution were added and the mixture was incubated for another hour at 37°C. Finally, SDS was added to a final concentration of 2%, followed by incubation at 65°C for 10 minutes and addition of 5 M potassium acetate and further incubation for 20 minutes on ice. The mixture was centrifuged at 13 000 x g for 15 minutes and the supernatant was transferred into a new tube. The supernatant was extracted three times with chloroform/isoamylalcohol (24:1) followed by precipitation of total nucleic acids with isopropanol and then with ethanol (70%). The pellet was dried and suspended in 150 µl of Tris-EDTA buffer. Further purification was carried out with the "Prep-A-Gene Purification Kit" (Bio-Rad, Hercules, USA) following the recommendations of the supplier.

2.2 Nucleic acid extraction from pure cultures

DNA extracts intended for PCR studies were provided by Dr Peter F. Dunfield. Extraction protocols are described in Dunfield *et al*. (2002).

For hybridization studies pure cultures grown under standard cultivation conditions (5% CO_2, 20% CH_4, 75% air) were provided by Dr. Peter F. Dunfield and Dr. Jürgen Heyer. The protocols that were used for extraction of DNA or RNA are almost identical except that solutions for extracting RNA were prepared using DEPC-treated deionised water.

Cells were first pelleted at 6 000 x g for 20 min at 4°C and washed once with TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). The pellet was then suspended in 10 ml TE buffer to which 25 mg of lysozyme and 20 µl of proteinase K (25 mg/ml) were added. The solution was placed in a 37°C water bath for two hours after which a 10% (w/v) SDS solution was added to a final concentration of 1%, followed by a 1.5-h incubation at 37°C. After centrifugation at 5 000 x g for 15 min at 4°C the supernatant was transferred to a new tube, and to this 1 ml of 5 M potassium acetate [pH 7.5] was added, followed by another 5 000 x g centrifugation step (15 min at 4°C). Total nucleic acids were extracted from the supernatant twice with one volume of chloroform/isoamylalcohol (24:1 v/v) and then precipitated with isopropanol and resuspended in 5 ml TE buffer. The resulting extract was used either for DNA extraction or for RNA extraction. For DNA extraction, 30 µl of 100 mg/ml RNase A and 20 µl of 100 000 U/ml RNAse T1 were added, followed by incubation for two hours at 37°C. Finally, DNA was extracted three times with chloroform/isoamylacohol and then precipitated with isopropanol and resuspended in TE buffer. For RNA extraction, 20 µl of RNAse-free DNase was added, followed by incubation for two hours at 37°C. RNA was extracted three times with chloroform/isoamylacohol and then precipitated with isopropanol and resuspended in DEPC-treated TE buffer. The quality of DNA or RNA was checked on a 1% agarose gel.

2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was applied to check the completeness of restriction enzyme digestions, and to determine the yield and purity of a nucleic acid extraction or PCR reaction. Prior to gel casting, dried agarose was dissolved and heated in 1% TAE-buffer. Then the warm gel solution was poured into a mold, which is fitted with a well-forming comb. The percentage of agarose in the gel varied depending on the purpose of experiment. Agarose gels were submerged in electrophoresis buffer (1% TAE-buffer) in a horizontal electrophoresis

apparatus. DNA samples were mixed with gel tracking dye and loaded into the sample wells. Electrophoresis was usually carried out at 150 to 200 mA for 0.5 to 1 hour, depending on the desired separation. Size markers (in our case λ-DNA cleaved with restriction endonuclease *Pst*I) were co-electrophoresed with DNA samples. After electrophoresis, the gel was stained with ethidium bromide and placed on a UV light box and a photo of the fluorescent ethidium bromide-stained DNA separation pattern was taken with a video camera (Gel Jet Imager, INTAS, Goettingen, Germany).

2.4 Quantification of nucleic acids

Extracted nucleic acids and PCR products were quantified by measuring the optical density (OD) at 260 nm. At this wavelength an OD of 1.0 would correspond to 50 μg/ml for double-stranded DNA or 40 μg/ml for single-stranded DNA or RNA, or 20 μg/ml for oligonucleotides.
The purity of nucleic acid solutions is given by the ratio of OD_{260}/OD_{280}. For a good purity this ratio should vary between 1.8 and 2.0 (Sambrook *et al.*, 1989).

2.5 PCR amplification

2.5.1 Amplification of 16S rDNA of type I and type II methanotrophs

Specific PCR assays (Wise *et al*., 1999) were used to amplify the 16S rDNA of type I and type II methanotrophs inhabiting the rice roots. The different primer sets applied are listed in Table 4.
All reactions were carried out in a volume of 100 μl, containing 1 μl of target DNA, 2.5 U Taq Polymerase (Applied Biosystems, Weiterstadt, Germany), 0.3 μM each primer, 1.5 mM $Mg^{2+,}$ 0.2 mM of each dNTP (USB, Cleveland, Ohio, USA) and 1 x PCR *Taq* buffer (10 μl in 100 μl final volume). The reactions were performed in a thermal cycler (model 2400, PE Applied Biosystems) and the PCR profile consisted of an initial denaturation at 94°C for 3 min, and 33 cycles consisting of denaturation at 94°C for 60s, annealing at 55°C for 60s, elongation at 72°C for 60s, and final extension step at 72°C for 7 min. The size and purity of PCR products were checked by agarose gel electrophoresis (1% agarose) and PCR products were cloned into pCR®II-TOPO® of the TA Cloning kit.

2.5.2 Development of specific PCR assays for the retrieval of sequence types belonging to a novel *pmoA* lineage

Different PCR assays were designed in an attempt to specifically detect sequences of a novel *pmoA* gene cluster in extracts of environmental DNA. Primer sequences and descriptions are given in table 4.

First-round-PCR strategies were designed as follows:

- PCR strategy A: A189f/A682b
- PCR strategy A°: pmoA190f/pmoA668b or pmoA190f/pmoA671b
- PCR strategy A': pmoA189f/pmoA668b
- PCR strategy B1: pmoA206f/pmoA703b

Second-round- (NESTED or semi-nested) PCR strategies were applied using the amplification product of PCR strategy A as follows:

- PCR strategy A1: pmoA189f/pmoA668b or pmoA189f/pmoA671b
- PCR strategy A2: pmoA190f/pmoA668b or pmoA190f/pmoA671b

PCR was carried out in a volume of 75 µl containing 1 µl of target DNA, 2.5 U Taq Polymerase (Promega, Mannheim, Germany), 0.125 µM each primer, 1.5 mM Mg^{2+}, and 0.15 mM of each dNTP. The PCR profiles are given in Table 5.
The sizes of PCR products were checked on a 1% agarose gel, and PCR products were cloned into pCR®2.1.

2.5.3 PCR amplification of *pmoA* from methanotrophic pure cultures

Depending on the experimental strategy (T-RFLP analysis or sequencing), different PCR strategies were designed for amplifying either the conventional or the novel *pmoA* genes from methanotrophic isolates or type strains.
pmoA genes were amplified in a 75 µl reaction mixture containing 1 µl of target DNA, 2.5 U Taq Polymerase (Promega, Mannheim, Germany), 0.125 µM each primer, 1.5 mM Mg^{2+} and 0.15 mM of each dNTP. Conventional *pmoA* genes were amplified with the primer combination A189f/A682b at an annealing temperature of 55°C whereas the novel type of *pmoA* was amplified with the primer combination pmoA206f/pmoA703b at annealing temperature 60°C or 66°C. The reactions were carried out in a Perkin-Elmer thermocycler and

Table 5: PCR profiles for amplification of *pmoA* in environmental DNA extracts

Strategy	Fragment length (bp)	PCR profile	Cycle number
A	531	94°C, 60s; 55°C, 60s; 72°C, 60s	32
A°	Ca. 492	94°C, 60s; 57.8°C, 60s; 72°C, 60s	35
A1	Ca. 510	94°C, 60s; 51°C, 60s; 72°C, 60s	35
A2	Ca. 492	94°C, 60s; 58°C, 60s; 72°C, 60s	23
A'	Ca. 508	94°C, 60s; 62.8°C, 60s; 72°C, 60s	35
B1	531	94°C, 60s; 59°C, 60s; 72°C, 60s	34

PCR profiles consisted of an initial denaturation at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 60s, annealing at 60°C or 66°C for 60s, elongation at 72°C for 60s and final extension step at 72°C for 7 min.

2.6 Amplification of 16S rDNA from methanotrophic pure cultures

In order to verify the identity of cultures, specific PCR assays (Wise *et al.*, 1989) were used to amplify the 16S rDNA of either type I or type II methanotrophs. The primer combination MethT1dF/Metht1bR was attempted for amplifying the 16S rDNA of type I methanotrophs whereas the primer combination 27f/MethT2R was applied for amplifying the 16S rDNA of type II methanotrophs.

2.7 Cloning of PCR products

The plasmid vector (pCR®II-TOPO® or pCR®2.1-TOPO®) of the TOPO TA Cloning kit (Invitrogen, de Schelp, Netherlands) is supplied linearised with single 3'-thymidine (T) overhangs for TA Cloning® and topoisomerase I covalently bound to the vector (referred to as "activated" vector). Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in the TOPO TA Cloning kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. Vectors of the pUC series such as those of the TA cloning kit carry the coding information for the first 146 amino acids of the β-galactosidase gene. Embedded in this coding region is the polycloning site into which the DNA insert is cloned. When expressed, this 146 amino acid fragment of β-

galactosidase protein is incapable of acting on the chromogenic substrate (X-gal). But when expressed in appropriate host cells, which express the carboxyl terminal fragment of the β-galactosidase protein, these two protein fragments can associate to form an enzymatically active protein. This is called the α-complementation and such cells turn blue when plated on plates containing X-gal. But if inserts DNA were cloned in the polycloning site, it almost invariably results in production of an amino-terminal fragment that is not capable of α-complementation and hence those colonies remain white.

PCR products were cloned into pCR®II or pCR®2.1 and transformed in *E. coli* strain INVαF' following the instructions of the manufacturer. Positive clones were selected based on transformation of X-gal on kanamycin-containing LB-agar plates. 25 µl of 50 mg/ml X-gal were spread onto LB-agar plates that were placed 15 min at dark. Subsequently, variable amounts of the transformation reaction were spread onto the plates, which then were incubated at 37°C overnight. Positive clones were picked by means of a sterile toothpick and replated onto new plates for growth. After the second growth, the plates were stored at 4°C and the clones could either be screened by means of PCR or they could be stored in liquid LB-medium containing kanamycin.

2.8 Screening of clone libraries

Individual colonies containing inserts were suspended in 60 µl of water and boiled for 6 min. The cells debris were spun down, and 1 µl portions of the supernatants were used in PCR mixtures to reamplify the insert from the vector with the primer combination M13f and M13r at an annealing temperature of 55 °C. The size of the PCR product was checked on a 1% agarose gel.

2.9 Long-term storage of clones

Single colonies were inoculated into 1-2 ml of liquid LB-medium containing 50 µg /ml kanamycin. After overnight incubation at 37°C, 0.85 ml of cultures were mixed with 0.15 ml of sterile glycerol buffer and stored at –80°C.

2.10 T-RFLP analysis

The T-RFLP (Terminal restriction fragment length polymorphism) analysis is a recent molecular approach (Liu *et al.,* 1997) that can assess subtle genetic differences between strains as well as provide insight into the structure and function of microbial communities (Reviewed in Marsh, 1999; Osborn *et al.*, 2000). The method is based on the restriction endonuclease digestion (normally with 4 bp cutters) of fluorescently end-labelled PCR products. Either one or both primers used in the PCR can be fluorescently labelled with a distinct fluorescent dye. The digested product is mixed with a DNA size standard, itself labelled with a distinct fluorescent dye, and the fragments are then separated by electrophoresis using either gel- or capillary-based systems, with laser detection of the labelled fragments using an automated analyser (in our case, Applied Biosystems). Upon analysis, only the terminal end-labelled restriction fragments are detected. The output from such an analysis is in two forms: first an electropherogram is produced which shows the profile of a microbial community as a series of coloured peaks of varying heights and the second output consists of a table, which includes most importantly the size (in base pairs) and the height of each peak.

The presence of divergent *pmoA* gene copies in methanotrophs (Dunfield *et al.*, 2002) suggests that both copies are different enough to be screened based on differences in their restriction patterns. T-RFLP analysis was applied in this study for the following aims:

- Assessing the community profiles of environmental DNA extracts.
- Screening the intragenomic *pmoA*-based diversity of methanotrophic isolates.
- Optimising PCR conditions for a rapid retrieval of novel *pmoA* sequence types (if present) without cloning the PCR product prior to sequence analysis.

PCR amplifications

Single-round-PCR amplifications were carried out using a combination of either 5'-Fam-A189f/A682b or 5'-Fam-pmoA206f/pmoA703b. Nested- or semi-nested-PCR were carried out using the combination of primers A189f/A682b in a first-round and then using its product in a second-round with primers 5'-Fam-pmoA190f/pmoA668b.

All reactions were carried out in a volume of 75 µl reaction containing 1 µl of target DNA, 2.5 U Taq Polymerase (Promega, Mannheim, Germany), 0.125 µM each primer, 1.5 mM Mg^{2+} and 0.15 mM of each dNTP. PCR profiles consisted of an initial denaturation at 94°C for 3 min and 28 cycles of 94°C for 60s, annealing at 50°C (5'-Fam-A189f/A682b) or 60°C or

66°C (5'-Fam-pmoA206f/pmoA703b) for 60s and elongation at 72°C for 60s and final extension step at 72°C for 7 min. The sizes of PCR products were checked on a 1% agarose gel.

Restriction digestion

An aliquot (ca. 200 ng) of the PCR product was digested in reaction tubes containing 10 U *Msp*I (Promega, Mannheim), 1 µl buffer B (Promega Mannheim), 0.1 µl BSA and H_2O for a total volume of 10 µl. All reactions were incubated at 37°C for 3 h and stored at 4°C thereafter.

Analyses of the restricted products

2.5 µl of the restricted products were mixed with 2 µl of formamide and 0.5 µl of an internal size standard (Gene-Scan-1000 ROX, PE Applied Biosystems, Weiterstadt). Mixtures were denatured at 94°C for 4 min and electrophoresed on a 6 % polyacrylamide gel containing 8.3 M urea for 6 h at 2, 500 V, 40 mA and 27 W on an ABI sequencer 373. T-RFLP profiles were analysed using the GENESCAN software (version 2.1) (Applied Biosystems). The size, in base pairs, of terminal restriction fragments (T-RFs) was estimated by reference to the internal lane standard. T-RFs with a peak height of less than 100 fluorescence units were excluded from further analysis.

2.11 Southern hybridization

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). In this method, genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured in situ and transferred from the gel onto a solid support (usually nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to a probe and the positions of bands of interest can be located via a suitable detection method. In this study, Southern hybridization was applied to a number of methanotrophic isolates and type strains. The aim was to confirm the results of PCR-based strategies applied for the retrieval of sequence types belonging to the novel *pmoA* lineage.

Restriction digestion and hybridization

Aliquots of genomic DNA (10 μg) were digested overnight at 37°C using 50 U restriction enzyme, by following the protocols as recommended by the supplier (Promega, Wisconsin, MA, USA). The restricted DNA was precipitated with ethanol, dried in a vacuum dessicator (Savant, Holbrook, NY, USA), 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 outlined in the DIG Application manual for Filter hybridization (Roche Molecular Diagnostics GmbH, Gemany, 2000). This consisted of denaturation and neutralization of the electrophoresed genomic DNA and blotting overnight on a Hybond-N-membrane (Amersham, Piscataway, NJ, USA). The DNA was UV cross-linked using a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) at 266 nm for 3 min.

Based on the alignment of *pmoA* sequences obtained from environmental samples and pure cultures, a 26-bp oligonucleotide probe (pmoA636b) was designed to specifically detect the novel *pmoA* gene. DNA fragment probes were generated as a mixture of DIG-labelled amplification products of the different strains tested in a PCR using the primer combination A189f/A682b. Hybridizations were carried out overnight at 40°C with a standard hybridization solution (5 x SSC, 0.1% N-lauroylsarcosin, 0.02% SDS, 1% blocking solution). Membranes were washed in a 0.5 x SSC at 60°C for 30 min and positive bands were detected on a Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) after reaction of ECF substrate for Western blotting (Amersham).

2.12 Northern hybridization

Northern hybridization or RNA blotting allows the determination of the size and the amount of specific RNA molecules in preparations of total RNA (Alwine *et al.*, 1977, 1979). The RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to activated cellulose (Alwine *et al.*, 1977; Seed 1982), nitrocellulose (Goldberg, 1980; Thomas, 1980), or nylon membranes (Bresser and Gillespie, 1983). The RNA of interest is then located by hybridization with a radiolabelled or non-radiolabelled DNA or RNA probe followed by detection via a suitable method. Using *Methylocystis* sp. strain SC2 as model organism, Northern hybridization was applied to assess the expression of the novel *pmoA* gene type under standard cultivation conditions (see “Methods).

Total RNA separation and hybridization

Ca. 60 μg of total RNA from strain SC2 were first separated on a 1% formaldehyde agarose gel at 60 V for 3 h in the presence of 1 x MOPS buffer (10 x 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 GmbH, Gemany, 2000). Blotting was carried out overnight on a Hybond-N-membrane (Amersham, Piscataway, NJ, USA). The DNA was UV cross-linked using a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) at 266 nm for 3 min. Hybridizations were carried out overnight at 40°C with a standard hybridization solution (5 x SSC, 0.1% N-lauroylsarcosin, 0.02% SDS, 1% blocking solution), by using probes pmoA636b or A593b. Membranes were washed in 0.5 x SSC at 60°C for 30 min and positive bands were detected on a Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) after reaction of ECF substrate for Western blotting (Amersham).

2.13 Reverse transcription-PCR (RT-PCR)

Reverse transcription-PCR is a common method used in the analysis of gene expression. In this method, the RT step is used to produce a cDNA that serves as template in subsequent PCR. This method is highly sensitive and, in contrast to Northern hybridization, only very little amounts of RNA are applied. RT-PCR was applied in this work as a complementary method to confirm the results obtained by Northern hybridization.

Reverse transcription

Reverse transcription was performed using a Qiagen Omniscript^{TM} Kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. The reaction was carried out in a 20-μl (total volume) mixture containing 2 μg of strain SC2 total RNA, 0.5 mM each dNTP, reverse transcriptase buffer, 10 U of RNase inhibitor, 1.0 μM of primer A682b and 4 U of Omniscript Reverse Transcriptase. The reaction mixture was incubated at 37°C for 30 min.

PCR amplification

PCR amplification was carried out in a volume of 100 μl containing 1 μl RT product, 2.5 U of Taq polymerase, 0.25 μM each primer, 1.5 mM Mg^{2+} and 0.2 mM of each dNTP. Primer combinations were as follow: A189f/pmoA593b to amplify the cDNA of the conventional *pmoA* gene and PmoA206f/PmoA636b to amplify the cDNA corresponding to the novel

pmoA gene. The reactions were performed in a thermal cycler (model 2400, PE Applied Biosystems), and the PCR profile consisted of an initial denaturation at 94°C for 3 min, and 32 cycles of 94°C for 40s, 62°C for 40s and 72°C for 45s and final extension step of 72°C for 7 min.

PCR products were checked by agarose gel electrophoresis and comparative sequence analysis.

2.14 Dideoxynucleotide DNA sequencing (Sanger *et al.*, 1977)

Prior to sequencing, PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) by following the instructions of the manufacturer. Reactions for sequencing were performed using the "ABI PRISM™ Dye (or BigDye) Terminator Cycle Sequencing Ready Reaction kit" (PE Applied Biosystems, Weiterstadt). All reactions were set in 20 µl (or 10 µl) (total volume) and contained 6 µl (or 3 µl) master mix, ca. 5 pmol primer, ca. 100 ng DNA and H_2O. Sequencing reactions were carried out on a GeneAmp PCR System 9600 (PE Applied Biosystems, Weiterstadt) and the PCR profile consisted of 25 cycles of denaturation at 96°C for 10s, annealing at 50°C for 5s, extension at 60°C for 4 min and cooling at 4°C. Reaction samples were purified using Microspin G-50 chromatography columns (Pharmacia, Upsala, Sweden) according to the recommendations of the supplier, and purified samples were dried on a vaccum dessicator (Savant, Holbrook, NY, USA). Dried pellets were mixed with 3 µl of formamide/EDTA (25 mM, pH 8.0) in a ratio of 4:1 (v/v), denatured at 100°C for 3 min and separated on a 5 % polyacrylamide gel containing 8 M urea. The gel was run on an ABI sequencer (model 373 or 377) for 16 h at 2500 V, 40 mA and 27 W.

2.15 Blast searches and phylogenetic analyses

BLAST searches

In order to determine whether the sequence data obtained exhibited homologies with 16S rDNA or *pmoA* sequences available in public domain databases, searches were performed using the BLAST programs of the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) and the EMBL (European Molecular Biological Laboratory, http://www2.ebi.ac.uk/). BLAST® (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of

whether the query is protein or DNA. BLAST is able to detect relationships among sequences which share only isolated regions of similarity (Altschul *et al.*, 1990). The scores assigned in BLAST create a list of most similar sequences to the query sequence, with homology values given in percent.

16S rDNA-based phylogenetic inference

The new 16S rRNA sequences were added to a 16S rDNA alignment of about 14 000 homologous primary structures from members of the domain *Bacteria* using the alignment tool of the ARB program package (O. Strunk and W. Ludwig, http://www.biol.chemie.tu-muenchen.de/pub/ARB). Sequences were first aligned with most similar sequences by using methods for pairwise or multiple sequences comparison (Maidak *et al.*, 1996; Van de Peer *et al*., 1996; Ludwig, 1995). By these methods it was possible to compare conserved and variable regions in the 16S rRNA sequences. Alignments were refined by visual inspection and manual correction. Variabilities in the individual alignment positions were determined using the ARB package and were used as criteria for removing or including variable positions for phylogenetic analyses. An alignment was performed for every single sequence introduced to the database and new sequences were inserted into a phylogenetic tree constructed on the basis of all 16S rRNA sequences available in the ARB database.

Phylogenetic analyses were performed for selected sequences by applying the distance matrix methods using the respective tools in the ARB and PHYLIP (Phylogeny Inference Package; J. Felsenstein, Depatment of Genetics, University of Washington, Seattle) program packages. Evolutionary distances between pairs of sequences were calculated using the "Jukes-Cantor" correction (Jukes and Cantor, 1969). Trees were constructed by applying "Neighbor-Joining method" (Saitou and Nei, 1987). The stastistical significance of interior node, were determined by performing bootstrap analyses by the "neighbor-joining" method. For each calculation, 500 bootstrap resamplings were analysed.

PmoA-based phylogenetic inference

The novel *pmoA*-like sequences were added to an ARB sequence database, which was created based on *pmoA* sequences downloaded from public databases. Deduced amino acid sequences were aligned using the editor GDE 2.2 (S. W. Smith, C. Wang, P. M. Gillevet, and W. Gilbert, Genetic Data Environment and the Havard Genome Database, Genome Mapping and sequencing, Cold Spring Harbor Laboratory) implemented in the ARB software package. To construct phylogenetic trees based on an alignment of amino acid residues, distances were inferred by using the maximum likelihood method implemented in the PROTDIST program,

with the Kimura (1983) matrixes as the amino acid replacement models. Trees were inferred from the distances by using the "Neighbor-Joining" method with global arrangements and randomised input order of species. Boostrap values for deduced amino acid sequences were calculated for 500 data resamplings.

IV. Results

1. 16S rDNA-based diversity of type I and type II methanotrophs on rice roots

16S rDNA PCR products obtained by using target DNA extracted from rice roots were used to construct two clone libraries. The primers used to construct both libraries were group-specific primers for type I or type II methanotrophs as described and published by Wise *et al.* (1999).

1.1 Type I MOB clone library

The Type I group-specific primer MethT1dF was used in combination with MethT1bR to generate a PCR product of approximately 920 bp. The PCR product was used to construct a clone library from which 24 clones were randomly selected for further analysis. Database searches indicated that the great majority of the clones were related to *Methylobacter* sp. BB5.1 whereas a few clones were related to known type I MOB of the genera *Methylomicrobium*, *Methylocaldum* and *Methylococcus.* Three clones could not be assigned to any known MOB.

Figure 2 shows the results of the phylogenetic analysis of all type I MOB clones and their relationship to representative members of the genera *Methylobacter*, *Methylocaldum*, *Methylomonas*, *Methylococcus* and other characterized *Proteobacteria*. Seventeen clones formed a distinct cluster most closely related (sequence identities of 96% to 97%) to members of the genus *Methylobacter*. The high boostrap value separating these clone sequences from any other known methanotrophs suggests that these clones form a monophyletic group. One clone was related (identity 98%) to *Methylomicrobium album* whereas two clones were related (identity 93%) to members of the genus *Methylocaldum* and one clone was related to *Methylococcus capsulatus* (92% identity). Three clones were not related to known MOB and were related to the genus *Coxiella* (two clones) or to *Proteobacterium* strain DSM 1181 (one clone).

1.2 Type II MOB clone library

The type II MOB specific primer-(probe) MethT2R was used in combination with the *Bacteria*-specific primer 27F, which targets most members of the domain *Bacteria*. The expected product had a size of approximately 950 bp. Thirty clones were randomly selected

from the clone library for further analysis. Database searches indicated that sequences were related to *Methylosinus* or *Methylocystis*.

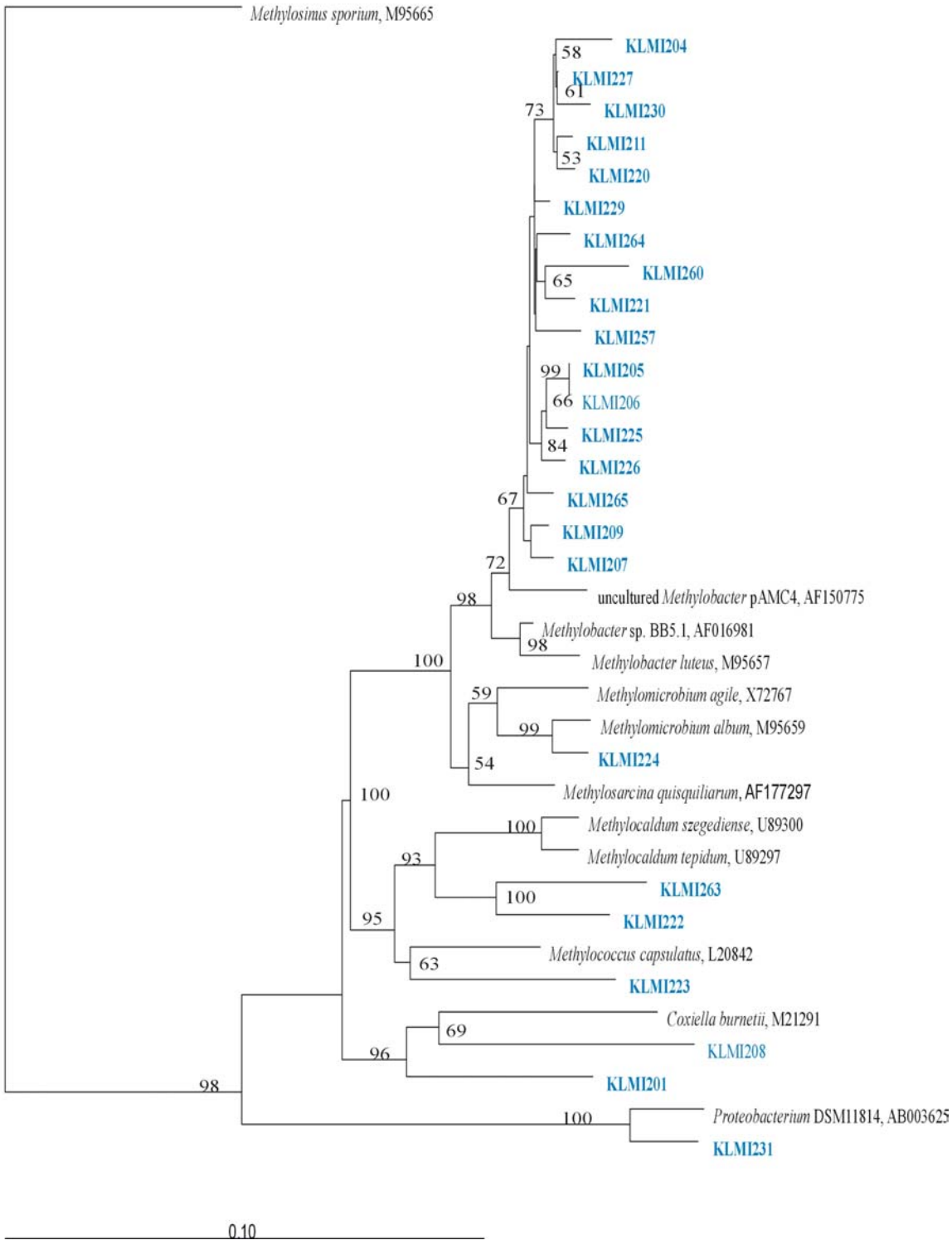

Fig 2. Phylogenetic tree showing the relationship of rice root type I methanotroph 16S rDNA clone sequences to some characterized type I methanotrophs and γ-*Proteobacteria*. The tree was constructed using the Neighbour-Joining method with a Jukes-Cantor correction. The 16S rDNA of *Methylosinus sporium* served as outgroup. The scale bar represents 0.10 substitutions per base position. The numbers at nodes of the tree indicate bootstrap values (data resamplings values below 50 are not shown).

Figure 3 shows the results of the phylogenetic analysis of the type II MOB clone sequences in relation to representative type II MOB. Seventeen clones were closely related to *Methylocystis* strain SC2 with sequence identities ranging from 98.1% to 98.4%. Five clones (KLMII236, KLMII260, KLMII210 KLMII281 and KLMII233) exhibited the highest percentages of sequence identity to *Methylocystis* sp. strain M (98.0% to 98.5%), an sMMO-containing isolate that can degrade high levels of trichloroethylene (McDonald *et al.*, 1997). Three clones (KLMII227, KLMII228 and KLMII209) were found most closely related to *Methylocystis* strain Lw5, a type II methanotroph recently isolated from lake sediment (Costello *et al.,* 1999). Sequence identities varied from 97.1% to 97.9%. The clones KLMII2114, KLMII2200 and KLMII2108 were more closely related to *Methylocystis* sp. M and *Methylocystis* sp. Lw5 (97.6% to 97.8% identity) than to other type II MOB. Clone KLMII2122 was related to *Afipia* genospecies 7 with sequence identity of 99%.

2. Development of PCR assays for the specific retrieval of novel *pmoA*-like sequences

2.1 Specific primers for novel *pmoA*

In our effort to generate a *pmoA* sequence database by using target DNA extracted from rice roots, we identified a novel *pmoA*-like sequence. A more detailed analysis of the derived amino acid sequence revealed that, although this sequence clusters closer to type II MOB than to type I MOB, it does form a distinct branch (Horz *et al.*, 2001). Many of the conserved amino acid residues of known *pmoA*/*amoA* sequences are present within the novel sequence, suggesting that the sequence is indeed *pmoA*-like. We assumed that the novel sequence was indicative of a novel group of methanotrophs whose members were characterized not yet. In order to specifically retrieve further sequences of this novel *pmoA*-like cluster, a set of PCR primers was designed by aligning the novel sequence with conventional *pmoA* sequence types from representatives of different MOB subgroups. Overall, we manually developed four oligonucleotide primers termed PmoA190f, PmoA206f, PmoA668b and PmoA671b (see table 4). These primers were highly specific to the novel *pmoA*-like gene and exhibited enough mismatches to enable differentiation of known methanotrophs. However, a few mismatches were observed with conventional *pmoA* sequences of *Methylocystis*-group members (two to three mismatches). According to database searches, none of the primers perfectly matched to conventional *pmoA* or *amoA* sequences, suggesting that our assays should be specific.

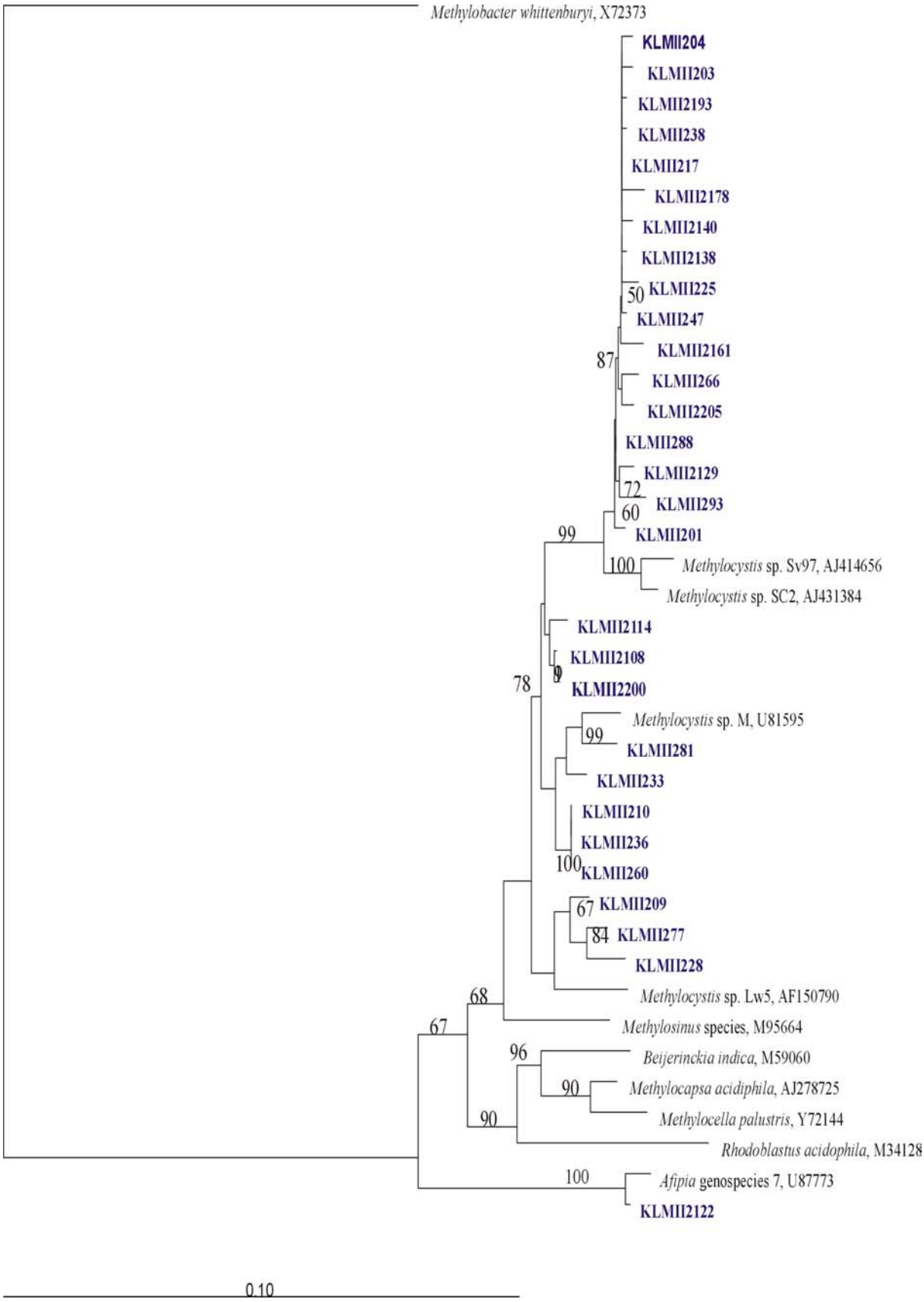

Fig 3. Phylogenetic tree showing the relationship of rice roots type II methanotroph 16S rDNA clone sequences to some characterized type II methanotrophs and other members of α-*Proteobacteria*. The tree was constructed using the Neighbour-Joining method with a Jukes-Cantor correction. *Methylobacter whittenburyi* served as outgroup. The scale bar represents 0.10 substitutions per base position. The numbers at nodes of the tree indicate bootstrap values, from 100 bootstrap resamplings (values below 50 are not shown).

2.2 Specific PCR assays and *pmoA* sequences of the novel lineage

In order to investigate the intracluster diversity of the novel *pmoA* in environmental samples, DNA extracts of various compartments of rice microcosms were applied in molecular studies. Using a combination of the newly designed primers, various PCR assays were formulated and applied to environmental DNA extracts (see table 4).

Initially primer pairs PmoA190f/PmoA668b and PmoA190f/PmoA671b were used. A band of approximately 492 bp was observed and this product was cloned. Ten clones were randomly selected for further analysis. Database searches indicated that the sequences amplified by these assays were not *pmoA*.

In order to increase the specificity and to detect target sequences, new primer combinations were formulated. Single-round-PCR was carried out by using the primer combinations PmoA206f/PmoA703b and PmoA190f/A682b. Nested-PCR was also formulated by amplifying *pmoA* sequences in a first-round-PCR with the primers A189f/A682b and using the amplified product in a second-round-PCR with primer pairs PmoA190f/PmoA668b or pmoA190f/PmoA671b. Products of the correct size (492 bp) were observed in both cases and these were used to generate clone libraries. A total of 7 clone libraries were produced from rice roots (samples Rr90h and Rr90a, one and two libraries, respectively), or from root mats (samples Rz90e and Rz90f, one library each), or from rice soil (sample RsVc, two libraries). Twelve to fifteen clones were randomly selected from every single library for further analysis. Database searches indicated that all the environmental sequences exhibited a higher level of identity with *pmoA* than with *amoA*.

Environmental *pmoA* sequences were added to the existing *pmoA* database and the alignments were used to generate a phylogenetic tree (Fig. 4). PCR assay PmoA190f/A682b failed to retrieve MOB sequences belonging to the novel lineage, and sequences belonged mostly to a novel sublineage within the type I MOB. A few other sequences were conventional *Methylocystis*-like *pmoA* sequences. The novel cluster of type I MOB sequences had low sequence identities to known type I methanotrophs. Identities at the amino acid level were 86% to *Methylococcus capsulatus* (Bath), 77% to *Methylobacter* sp. LW1, 71% to *Methylomonas methanica*, ca. 84% to *Methylocaldum zegediense* and 76% to *Methylosarcina quisquiliarum*. Single-round-PCR assay PmoA206f/PmoA703b and nested-PCR were specific for detecting sequence types belonging to the novel *pmoA* lineage. The large majority of clone sequences retrieved clustered with the novel *pmoA*-like sequence of clone M84-P3 previously retrieved in a cultivation-independent approach (Horz *et al,* 2001). However, a few clones could also be identified as type II-like conventional *pmoA* sequences belonging to the *Methylocystis* group. Sequences of the novel *pmoA* cluster had amino acid identities of ca.

70% with known type II methanotrophs and ca. 60%-65% with type I MOB. The intracluster diversity was much greater. Sequences formed three distinct clusters with identities varying from 81% to 98%.

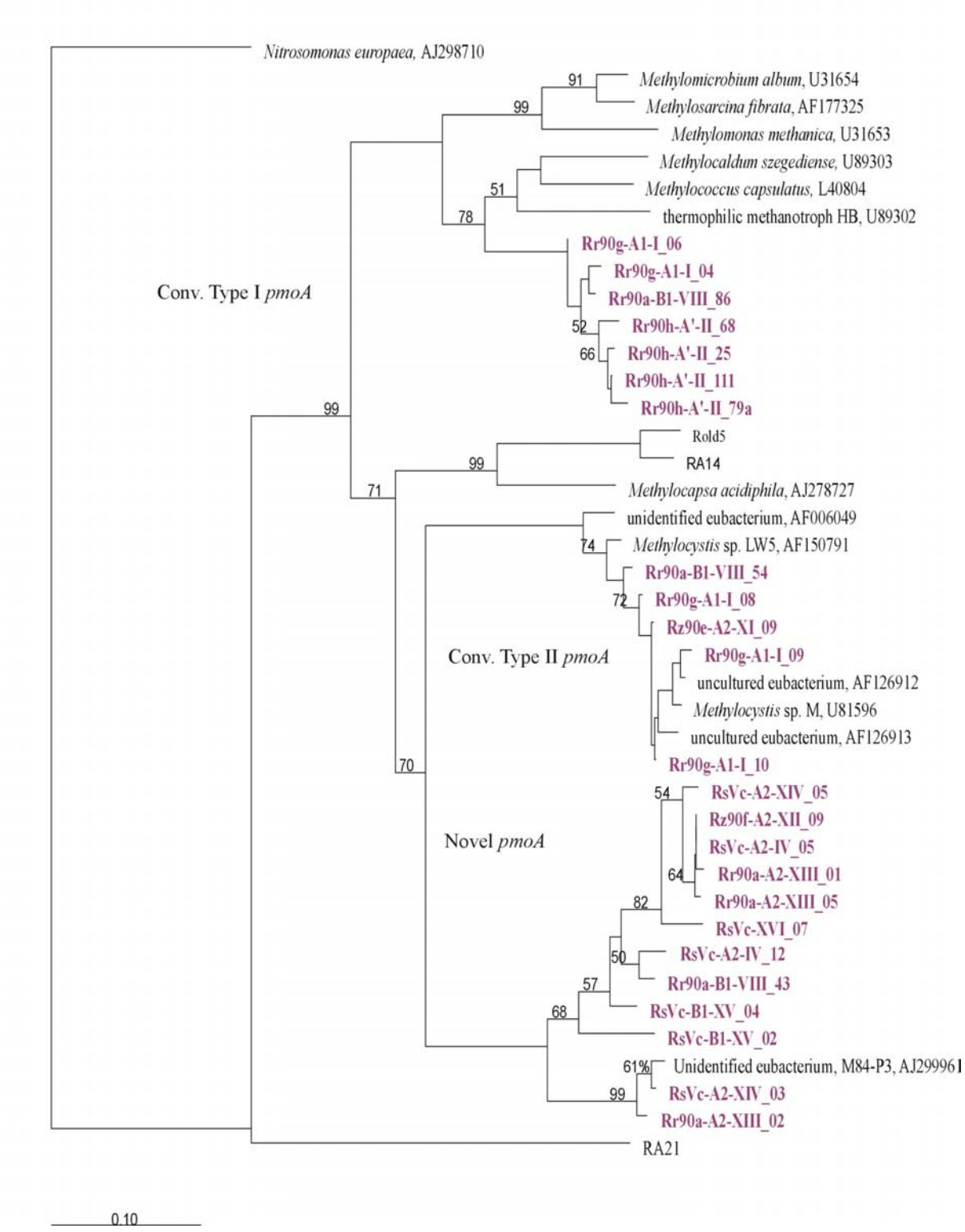

Fig 4. Phylogenetic tree based on *pmoA* showing the relationship of the derived amino acid sequences of environmental clones to some cultured and uncultured methanotrophs. The tree was constructed using a Neighbour-Joining method with a Kimura correction. The sequences RA14, RA21 and Rold5 were retrieved by cultivation-independent methods from forest soils and the sequence M84-P3 was retrieved from rice paddy soil.Bootstrap values >50% are indicated. The scale bar represents 0.10 substitutions per base position.

2.3 T-RFLP for assessing *pmoA* diversity in selected environmental DNA extracts

Horz *et al.* (2001), showed recently that *Msp*I is the most appropriate restriction enzyme for analysing *pmoA* diversity of methanotrophic communities by T-RFLP analysis. This conclusion was based *on silico* analysis of *Msp*I cutting sites of *pmoA* sequences derived from cultured methanotrophs, and was later confirmed with experimental findings. The comparison of T-RFLP profiles obtained from rice roots allowed group-based and even genus-based differentiation among methanotrophs and nitrifiers (Horz, 2001; Horz *et al.,* 2001). In order to assess *pmoA* diversity in environmental DNA extracts via T-RFLP analysis and to confirm the extent of diversity based on comparative sequence analysis, we manually searched for *Msp*I restriction sites in all environmental *pmoA* sequences retrieved from rice roots. We found that environmental sequences could be differentiated on the basis of their T-RFs.

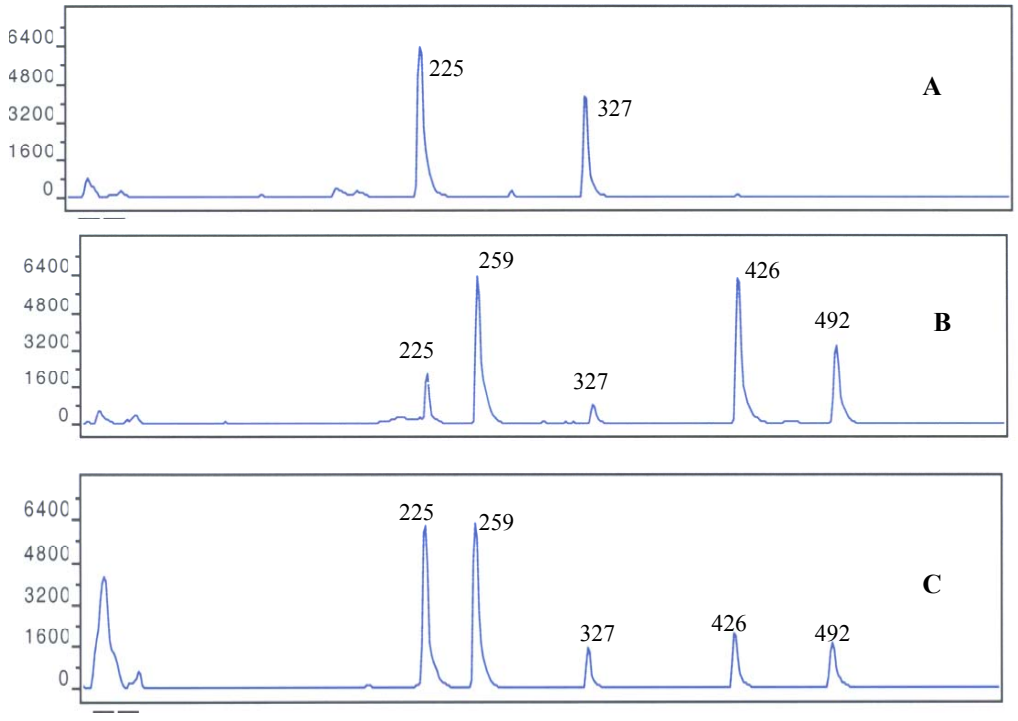

Fig. 5. *pmoA*-based T-RFLP profiles of three environmental samples: A (Rz90f, root mate of 90-day-old rice plants), B (Rr90a, roots of 90-day-old rice pants) and C (RsVc, roots of 90-day-old rice plants). The x-axis shows the lengths (in base pairs) of the T-RFs, and the y-axis shows the signal intensities of the fragments in arbitrary units. The numbers are T-RFs of individual *pmoA* sequences.

DNA extracts from samples Rr90a, Rz90f and RsVc were used for nested-PCR of *pmoA* (see "Methods") and the products were subjected to *Msp*I-based T-RFLP analysis. These samples exhibited a considerable diversity of sequence types belonging to the novel *pmoA*. The T-RFLP patterns of individual samples reflected the diversity provided by sequence analysis. Comparison of T-RFLP profiles (Fig. 6) allowed the identification of T-RFs with a size of 225 bp, 259 bp, 327 bp, 426 bp and 492 bp in samples Rr90a and RsVc, whereas T-RFs 225 bp and 327 bp were identified in sample Rz90f. Except for 492 bp T-RF, all the T-RFs identified by T-RFLP analysis could be assigned to specific clusters of environmental sequences, confirming that T-RFLP analysis would be suitable as a rapid tool for assessing the diversity of the novel *pmoA* gene cluster in environmental samples.

3. *Methylocystis* strain SC2 harbours a novel *pmoA*-like gene

In parallel to our study on novel environmental *pmoA* sequences, colleagues (Dr. Peter F. Dunfield and Dr. Jürgen Heyer) in our department created a culture collection and a molecular database for type II MOB. In the course of their study they identified a *Methylocystis* strain (termed strain SC2) that harboured two very different *pmoA*-like genes (Dunfield *et al*., 2002). The first gene 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 possessed only 73% identity with *pmoA1* at the nucleotide level and 68.5% identity at the amino acid level. *pmoA2* of strain SC2 was closely related to the above-mentioned *pmoA*-like clone sequence M84-P3 (Fig. 5) retrieved in our previous study by cultivation-independent methods from rice field soil (86.3% sequence identity), indicating that the presence of multiple, diverse *pmoA* gene copies might not be unique to strain SC2. This finding was strengthened by detection of a novel *pmoA*2-like gene copy in *Methylosinus trichosporium* strain KS21 (Dunfield *et al.*, 2002). The translated amino acid sequences of strain SC2 were aligned with those corresponding to some of our environmental sequences. The alignments were used to construct a phylogenetic tree (Fig. 6). *pmoA*1 clustered with conventional *pmoA* sequences of the *Methylocystis* group whereas *pmoA*2 clustered with environmental sequences belonging to the novel cluster, indicating that the novel *pmoA* gene is not indicative of a novel uncharacterized group of methanotrophs. Instead, it is present in some currently known methanotrophs.

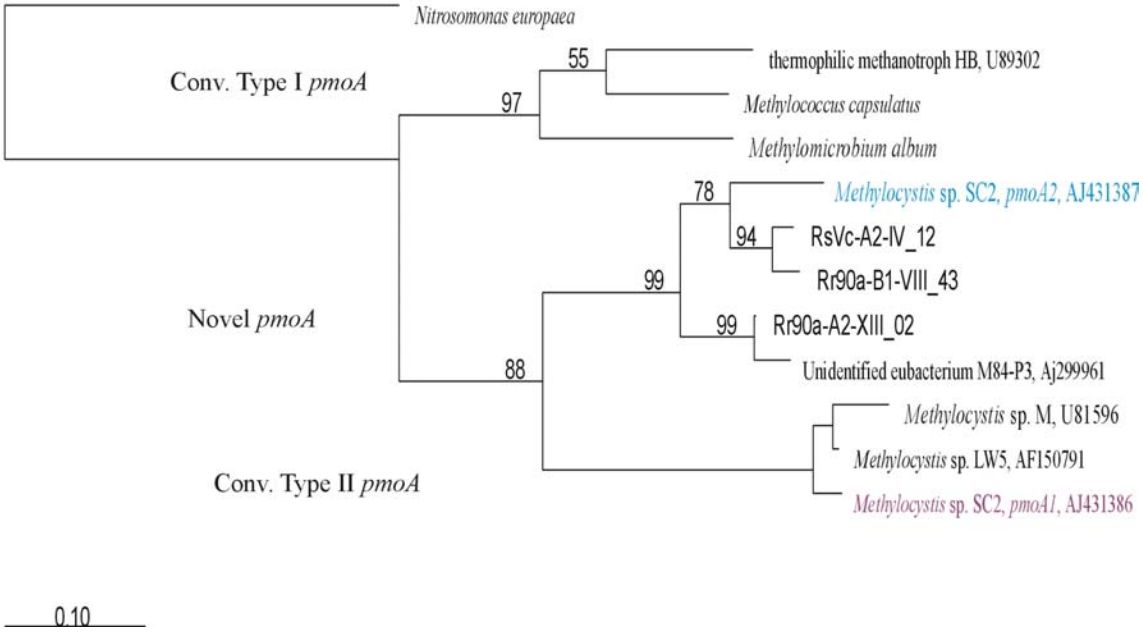

Fig. 6. Phylogenetic tree showing the relationship of the derived amino acid sequences of the two partial *pmoA* fragments of *Methylocystis* sp. SC2 to those of some environmental clones, as well as selected type II and type I MOB. The tree was constructed using a Neighbour-Joining method with Kimura correction. Sequence M84-P3 was retrieved by cultivation-independent methods from rice paddy soil.
Bootstrap values>50% are indicated. The scale bar represents 0.1 change per amino acid position.

In order to confirm the above results by a non-PCR-based method, genomic DNA was extracted from cell biomass of strain SC2 and used in Southern hybridization in conjunction with oligonucleotide probes specific either for *pmoA*1 or for *pmoA*2 (Dunfield *et al.*, 2002).
The probe A539b specific for the *pmoA*1 gene (containing eight mismatches to the *pmoA*2 gene) hybridized to two DNA size fragments (Fig. 7). The *pmoA*2 probe, specific for the novel gene type (12 mismatches to the *pmoA*1 gene), hybridized to a single, distinct size fragment. The results were the same regardless of the restriction enzyme used. The Southern hybridization data therefore confirmed that both *pmoA*-like genes detected by PCR-based methods were present in the genome of *Methylocystis* strain SC2. The probe A593b was employed instead of the probe pmoA1b to detect the *pmoA*1 gene. The rationale behind the use of probe A593b was that this probe produced three hybridization signals, two strong signals identical to those produced by probe A593b, and a very weak, third hybridization signal corresponding to the same size fragment to which the pmoA2b probe hybridized strongly.

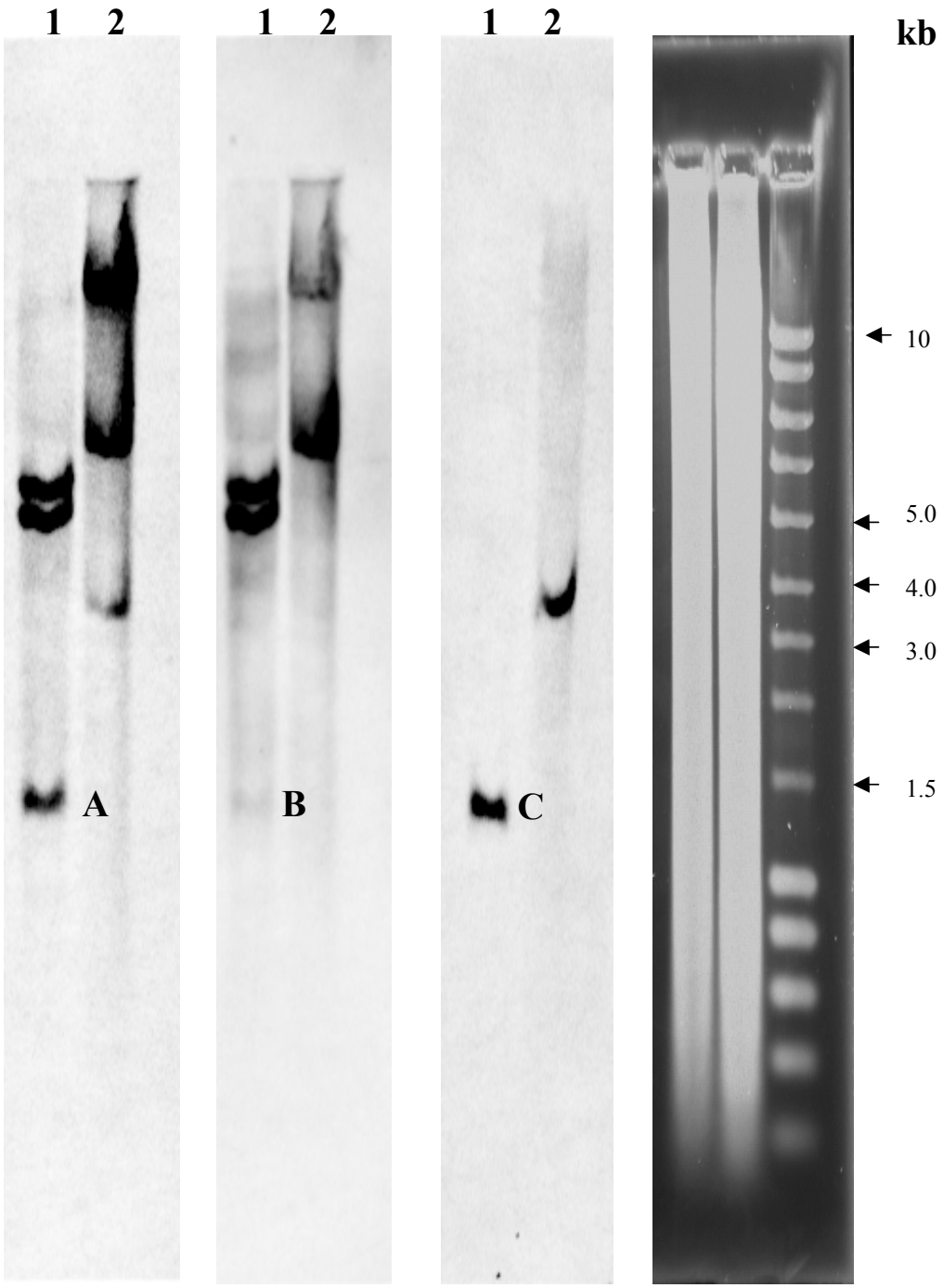

Fig. 7. Southern hybridization of genomic DNA extracted from *Methylocystis* strain SC2. DNA was digested with either *Xho*I (1) or *Eco*RI (2). A, hybridization with probes A593b and pmoA2b. B, hybridization with probe A593b (*pmoA1*-specific). C, hybridization with probe pmoA2b (*pmoA2*-specific). The rightmost panel shows digested genomic DNA and a DNA ladder stained with ethidium bromide.

4. Screening of pure cultures for the presence of the novel *pmoA* gene type

The presence of multiple conventional *pmoA* gene copies within a single strain has been demonstrated for laboratory strains (Semrau *et al*., 1995; Stolyar *et al*., 1999), and for both *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, the two copies of the *pmoCAB* operon are almost identical (Gilbert *et al.*, 2000, Stolyar *et al.*, 1999). This propenty has facilitated the rapid identification of methanotrophic strains (Holmes *et al.*, 1995; Wise *et al.*, 1999) by direct sequencing of *pmoA* PCR products i.e a single sequence corresponds to a particular species. However, if divergent multiple copies of *pmoA* are present within a single strain, amplification of *pmoA* genes with universal *pmoA* primers would result in mixtures of amplicons in PCR reactions, thereby affecting further characterization by direct sequencing of PCR products. Therefore, there was a need to find an appropriate method to isolate the divergent *pmoA* gene copies present in a single organism.

4.1 Establishment of a T-RFLP-based screening method

The ability of T-RFLP analysis to distinguish divergent *pmoA* sequence types within a single strain was assessed by computer simulation of the T-RF size distribution of the two *pmoA* sequence types present in *Methylocystis* strain SC2. Based on the PCR primers pmoA206f and pmoA703b, 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 a *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 with a fluorescently labelled forward primer pmoA206f. 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 two different annealing temperatures (60°C and 66°C). The PCR products were digested with *Msp*I and separated on an ABI 373 automated sequencer. No discrepancy was observed between the results expected by *in silico* analyses and the empirical data obtained. Two different T-RFs with a size of 245 bp 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 8). This finding led us to conclude that there were at least two distinct *pmoA* sequence types within the genome of strain SC2. To verify that only *pmoA2* was amplified at 66°C, another PCR was carried using the annealing temperature of 66°C (with no fluorescently labelled primers) and the resulting

PCR product was sequenced. Comparative sequence analysis confirmed the product as derived from *pmoA2*. This finding allowed us to conclude that T-RFLP analysis in combination with comparative sequence analysis may be a rapid and reliable method for screening methanotrophic pure cultures for the presence of multiple, divergent *pmoA* gene copies.

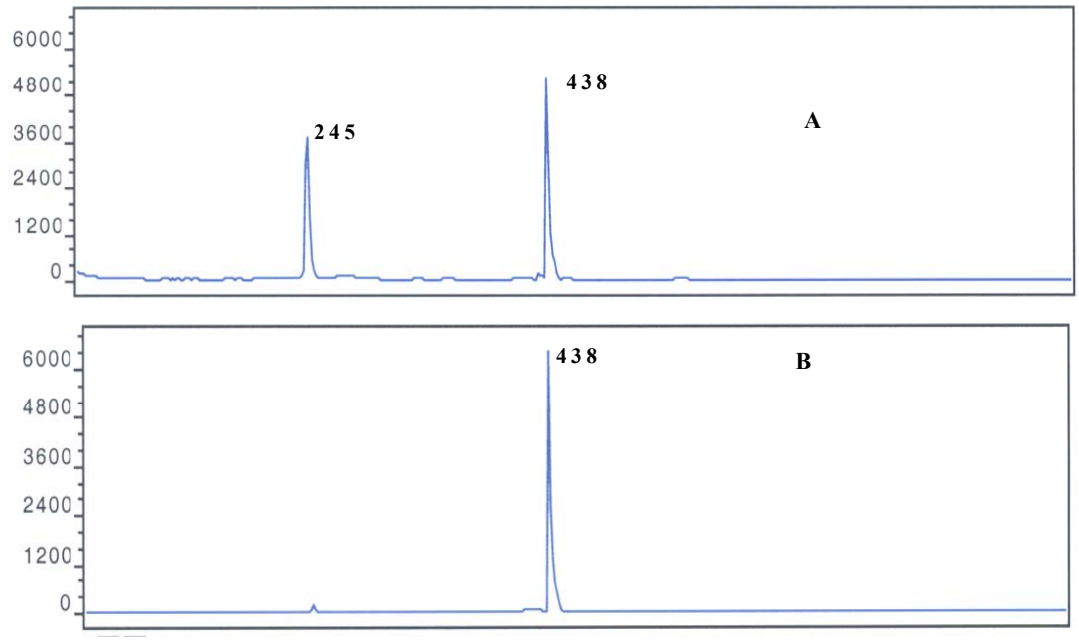

Fig. 8. *pmoA*-based T-RFLP profiles of *Methylocystis* strain SC2. PCRs were carried out with primers pmoA206f and pmoA703b at two different annealing temperatures: A (60°C) and B (66°C). On the y-axis are represented the intensities of fragments in arbitrary units. Numbers are the lengths of T-RFs corresponding to individual *pmoA* sequences.

4.2 Screening of pure cultures

Thirty-one pure cultures, including 9 type strains and 22 environmental isolates, were screened for the presence of a novel *pmoA* gene copy.

Prior to *pmoA*-based analysis, the identity of each strain was confirmed by comparative sequence analysis of its 16S rRNA gene and the conventional *pmoA* gene copy. The 16S rRNA and the *pmoA* genes had been amplified using group-specific 16S rRNA gene primers described by Wise *et al.*, (1999) and the *pmoA* primers A189f/A682b, respectively.

The *pmoA*-based diversity present within a single strain (including 6 type I MOB and 25 type II MOB) was assessed by determining the number and the size of fragments (T-RFs) observed in restriction digests of *pmoA* genes amplified at two different annealing temperatures (60°C and 66°C). PCR products were obtained for nearly all type II MOB and the type I *Methylomicrobium album*. Three type strains and two environmental isolates failed to give a

positive PCR with this *pmoA2*-specific primer set, including *Methylococcus capsulatus*, *Methylosinus trichosporium* OB3b, *Methylocaldum* E10A, *Methylomonas* spec. D1a, *Methylosinus* spec. H3 and *Methylocaldum* spec. O8a. The T-RFLP patterns obtained (referred to as "strain fingerprints") are composites of the number of fragments with unique lengths and the relative abundance of each fragment as reflected by the size of each peak in the electropherogram. Based on an analysis of electropherograms from the sequencing gel images, the 5' T-RFLP patterns revealed various distinct T-RFs among the strains, suggesting that there occurred a high degree of "*pmoA* sequence diversity" (i.e. multiple T-RFs, examplarily shown for *Methylocystis parvus*). For most of the strains that tested positively for the novel gene, the *pmoA* sequence diversity decreased with increasing annealing temperature and a single T-RF was observed at 66°C. This allowed the identification of T-RFs with sizes of 130 bp, 159 bp, 209 bp, 245 bp, 350 bp, and 438 bp. It was possible to classify the strains that were tested positively by PCR in groups of T-RFs identified, and on the basis of this classification representatives of 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 amino acid sequences of environmental clones. It was confirmed that the 245 bp T-RF was indeed characteristic of conventional *pmoA* sequences of the *Methylocystis/Methylosinus* group that could still be amplified with the presumed *pmoA2*-specific primer set at 66°C. Phylogenetic analyses (Fig. 10) showed that novel *pmoA* sequences of methanotrophic isolates were related to environmental clone sequences. Sequences of isolates grouped in two larger clusters. Amino acid sequence identities between these clusters were ca. 85% to 89%. Novel *pmoA* sequences of *Methylocystis parvus* and *Methylomicrobium album* were almost identical and grouped in a separate cluster.

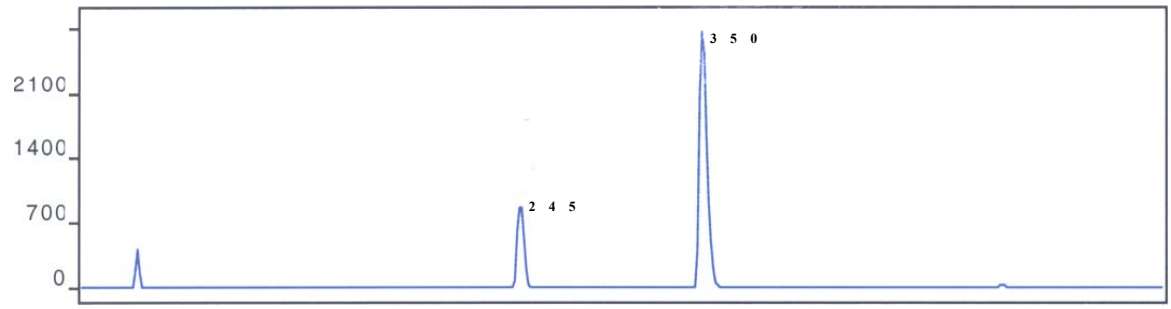

Fig. 9. *PmoA*-based T-RFLP profiles of *Methylocystis parvus*. PCR were carried out with primers pmoA206f and pmoA703b at annealing temperature 60°C. On the y-axis are represented the intensities of fragments in arbitrary units. Numbers are T-RFs corresponding to individual *pmoA* sequences.

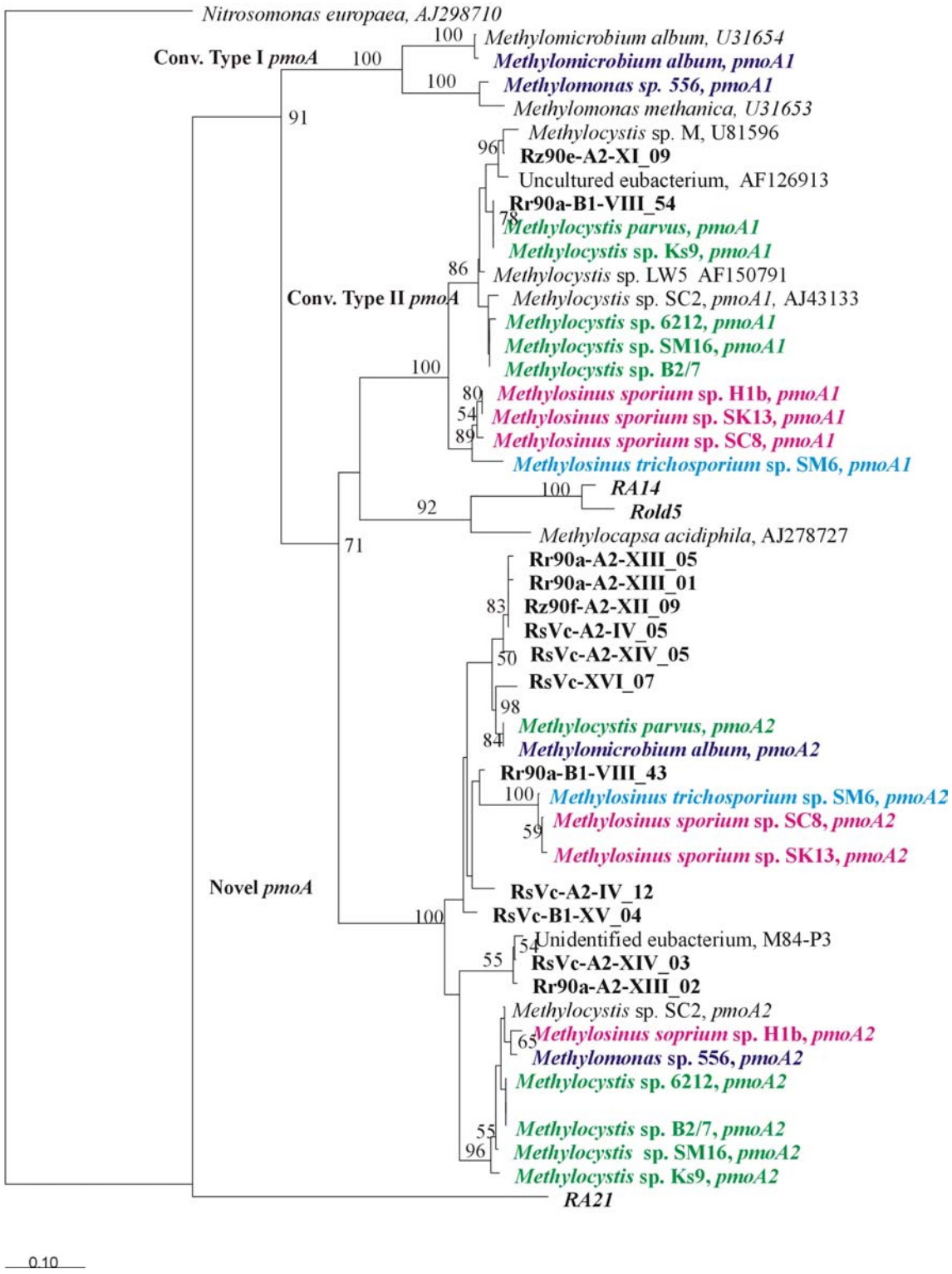

Fig. 10. Phylogenetic tree based on *pmoA* sequences showing the relationship of the derived amino acid sequences of two partial *pmoA1* and *pmoA2* of methanotrophic isolates to environmental clones as well as to selected type I and type II MOB. The sequences RA14, RA21 and Rold5 were retrieved by cultivation-independent methods from forest soils, and the sequence M84-P3 was retrieved from a rice-paddy soil. The tree was constructed using a neighbour-joining method with a Kimura correction. Bootstrap values >50% are indicated. The scale bar represents 0.1 change per amino acid position.

In order to confirm the PCR-based screening results for a representative set of MOB by Southern hybridization, a 26-bp oligonucleotide probe was developed for the detection of the

novel *pmoA* gene type (Fig. 11). The probe exhibited two mismatches to the corresponding target site of the conventional *pmoA* gene copy of *Methylococcus capsulatus* Bath, 4 mismatches to the corresponding target site of *Methylomonas methanica*, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* and 5 mismatches to the corresponding target site of *Methylomicrobium album*. However, the probe [termed pmoA636b (Table 3)], perfectly matched to the target site of all currently available sequence types belonging to the novel *pmoA*-like cluster.

```
Target.....GGCACSCCKGAATACATCCGCATGAT.....
Clone M84-P3..... .....GGCACGCCTGAATACATCCGCATGAT.....
SC2 novel.....GGCACCCCGGAATACATCCGCATGGT.....
RsVc-B1-XV_05.....GGCACGCCGGAATACATCCGCATGAT.....
RsVc-A2-XIV_04.....GGCACACCGGAATACATCCGCATGAT.....
Methylococcus capsulatus.....GGTACGCCCGAGTACATCCGCATGGT.....
Methylomonas methanica.....GGTACACCTGAGTACATCCGTATGGT.....
Methylosinus trichosporium.....TCGATGCCGGAATATATCCGCATGGT.....
Methylocytis parvus conv.....TCGATGCCGGAATATATCCGCATGGT.....
Methylomicrobium album conv.GGTACTCCAGAATATATCCGGATGGT.....
RA21.....AGCATGCCGGAATATCTGCGCATCAT.....
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Fig. 11. Alignment showing the target region of probe pmoA636b among methanotrophic representatives. Bold letters indicate mismatches, underlined letters show differences at degenerated sites.

Southern blots of digested chromosomal DNA were probed respectively with the oligonucleotide probe pmoA636b and a *pmoA* gene probe (generated as a mixture of DIG-labelled amplification products of the different strains tested). All hybridization experiments were carried out using strain SC2 as a positive control (Fig. 12). Probing with oligonucleotide pmoA636b gave one distinct signal for either *Methylomicrobium album* or strain SC2. No signal was observed for *Methylococcus capsulatus*, *Methylomonas sp*. D1a and *Methylosinus trichosporium* OB3b. These results agreed well with those obtained by PCR. Among the strains tested by Southern hybridization, detection and retrieval of sequence information of a novel *pmoA* gene was only the case for *Methylomicrobium album* and for *Methylocystis* strain SC2. Full-length gene probes of *pmoA* detected multiple *pmoA* copies in several of the strains. Two copies were detected in the genomic DNA of *Methylomicrobium album, Methylococcus capsulatus* and *Methylosinus trochosporium* OB3b while only a single copy was detected in M*ethylomonas* sp. strain D1a.

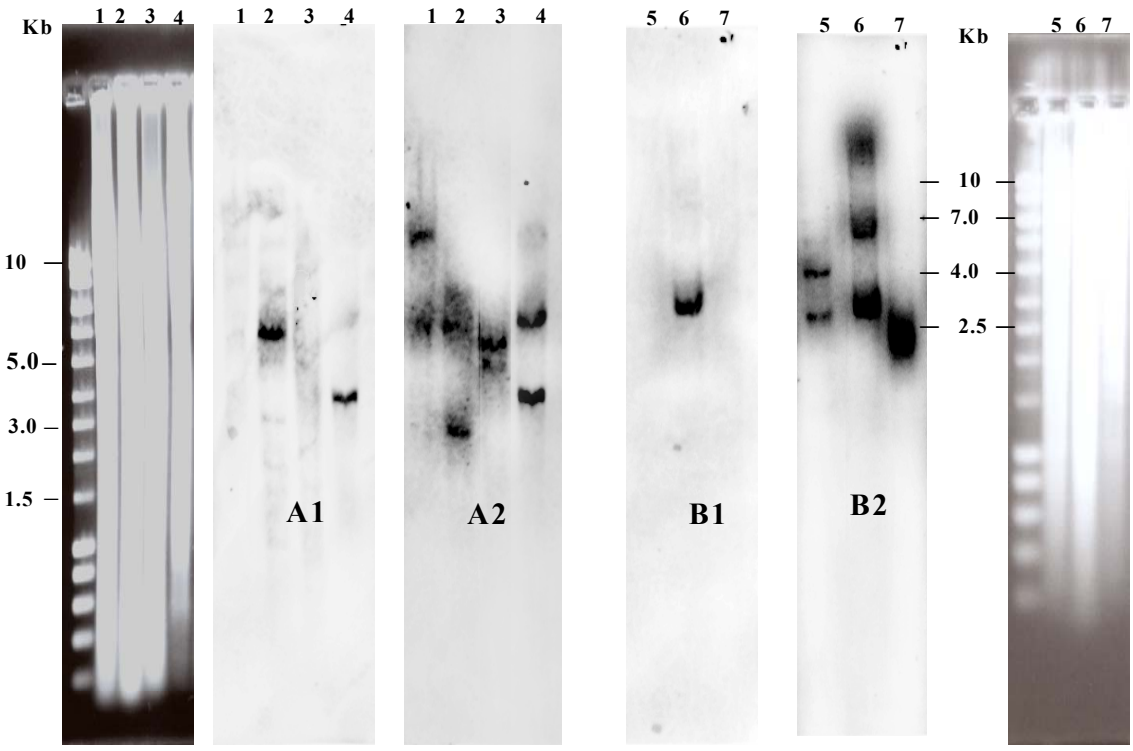

Fig. 12. Hybridization of digests of methanotroph genomic DNA to *pmoA* probes. *Eco*RI digests: 1- *Methylosinus trichosporium* OB3b; 2- *Methylomicrobium album*; *Methylocaldum* sp. E10A; 4- *Methylocystis* strain SC2. *Pst*I digests: 5- *Methylococcus capsulatus* (Bath); 6- *Methylocystis* strain SC2; 7- *Methylomonas* sp. D1a. A1+B1, hybridization with probe pmoA636b. A2+B2, hybridization with DNA fragment probes.

5. Expression of the novel *pmoA*-like gene in strain SC2

Two different experimental strategies were applied to assess whether the novel *pmoA* gene type will be expressed when strain SC2 is grown under standard cultivation conditions (see "Methods").

Detection by Northern hybridization. Attempts were made to check whether or not the novel methanotrophic *pmoA* gene is expressed under standard cultivation conditions. Total RNA of strain SC2 was extracted and separated on a 1% formaldehyde agarose gel and blotted onto a nylon membrane. Probing was carried out with oligonucleotides A593b or PmoA636b specific for either the conventional *pmoA* gene or for the novel *pmoA* gene type. Hybridizations with both oligonucleotides were positive (Fig. 12). However, in comparison to a strong signal observed for the conventional *pmoA* probe, only a very weak signal could be observed for the novel gene type. It was unsure whether the weak signal observed for pmoA636b resulted from experimental biases.

Detection by RT-PCR. An RT-PCR experiment was designed to check by a second, independent approach whether or not the novel *pmoA* gene copy is expressed under standard

laboratory growth conditions. Primers A593b and pmoA636b were initially applied for the reverse transcription. Sequencing of RT-PCR products identified no mRNA product expressed by the novel *pmoA* gene type. However, an unspecific fragment was amplified corresponding to a portion of the 23S rRNA. A strong product could be identified for the conventional *pmoA* gene. It was assumed that an unspecific cDNA was formed when using primer pmoA636b at low binding temperature of the reverse transcription. In an attempt to overcome the problem, the universal *pmoA* primer A682b was used for the generation of cDNA. Specific PCR assays were then carried out either with primers A189f/A593b or pmoA206f/pmoA636b (see tabe 3). In both cases products of the expected size were obtained (Fig. 14) and sequences corresponded either to the conventional *pmoA* gene (primer A593b) or to the novel gene (primer pmoA636b) (Fig. 15).

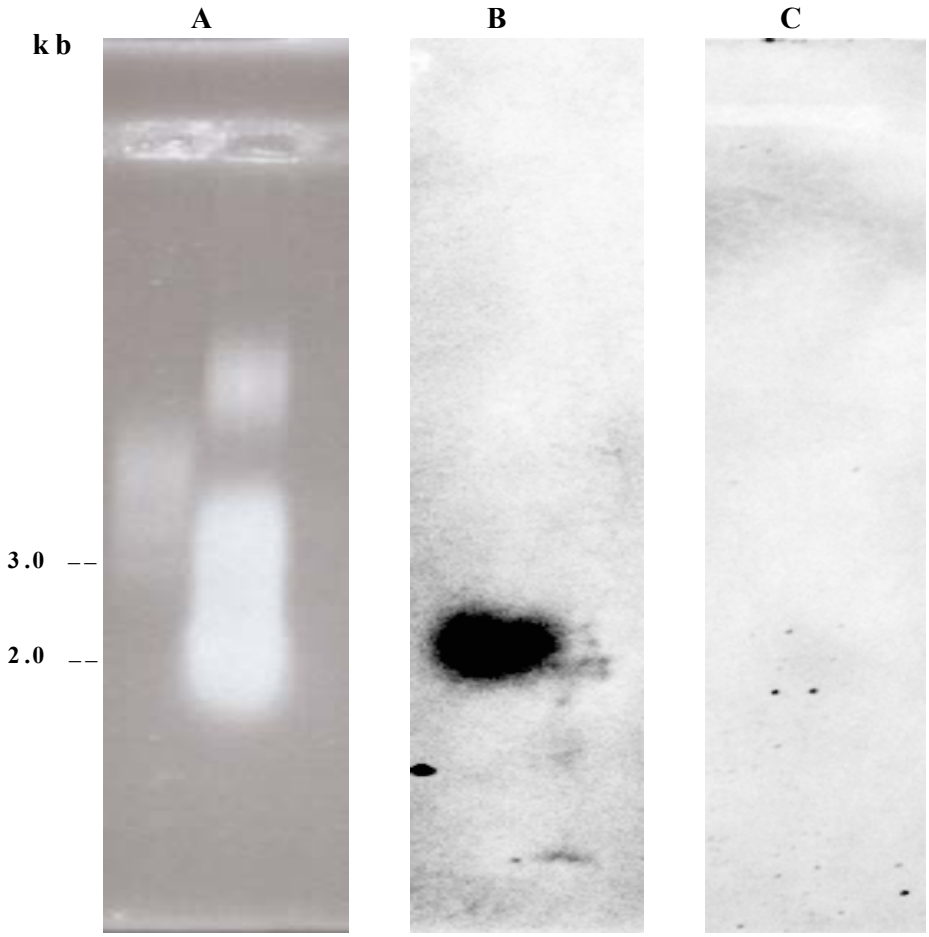

Fig. 13 Hybridization of total RNA from strain SC2 to *pmoA* probes. A) agarose gel electrophoresis of total RNA in presence of an RNA marker (RNA Ladder, New England Biolabs). B) Hybridization with probe A593b specific to the conventional *pmoA* gene. C) Hybridization with probe pmoA636b specific to the novel *pmoA* gene type. Lanes contain ca. 60 µg total RNA of strain SC2.

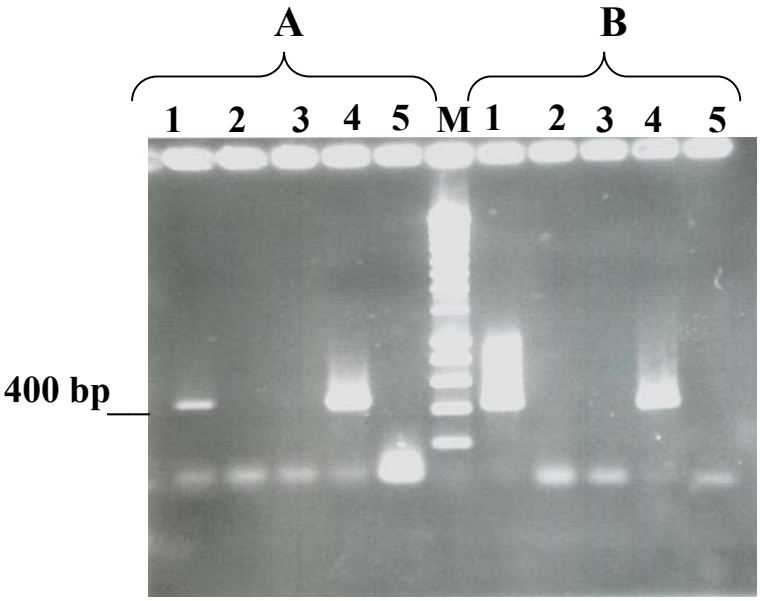

Fig. 14. RT-PCR of total RNA of strain SC2. Reverse transcription was carried out at 37°C with methanotrophic primer A682b. A) Primers specific for the novel methanotrophic *pmoA* gene type. B) Primers specific for the conventional *pmoA* gene. 1-RNA sample + RT-PCR; 2-RNA sample + PCR, without RT; 3-RNAse treated RNA sample + PCR; 4-DNA + PCR; 5-PCR without DNA.

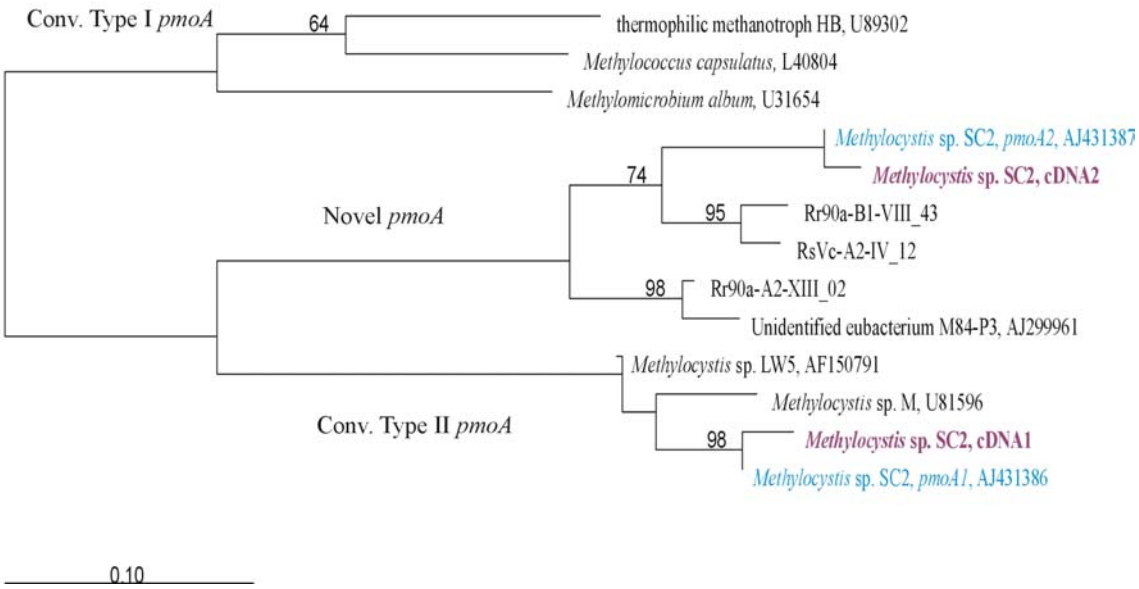

Fig. 15. Phylogenetic tree based on *pmoA* showing the two partial cDNA fragments of *Methylocystis* sp. SC2 in relation to the respective *pmoA* genes as well as representative set of type I and type II MOB and environmental *pmoA* clone sequences. The sequence M84-P3 was retrieved by cultivation-independent methods from a rice-paddy soil.DNA fragments were ca. 525 bp long whereas cDNAs were ca. 400 bp long. The tree was constructed using the Neighbour-Joining method with a Kimura correction. Bootstrap values >50% are indicated. The scale bar represents 0.1 change per amino acid position.

V. Discussion

Methanotrophic bacteria are an environmentally important group due to their role in regulating CH_4 fluxes from ecosystems, as well as their use in bioremediation strategies. However, the cultivation of methanotrophs is laborious and time-consuming and for some species not yet possible using standard techniques. This makes cultivation-based assessments of natural methanotrophic populations problematic. Consequently, the ability to rapidly assess and monitor natural populations of methanotrophs by using molecular techniques holds great promise for understanding the complex role of these bacteria in nature (Hanson and Hanson, 1996). However, in order to develop more reliable molecular tools for monitoring the diversity and dynamics of MOB populations in nature, more information regarding the diversity of *in situ* populations is needed.

Originally, this Ph.D. work aimed at generating methanotroph-specific *pmoA* and 16S rRNA sequence databases with a special focus on rice field soil. These sequence databases were intended to be the basis for the development of population-specific oligonucleotide probes (16S rRNA) and PCR asssays (*pmoA*). However, during the first months of my Ph.D. work, Horz *et al.* (2001) detected a unique *pmoA*-like sequence in rice rizosphere soil. That could not be assigned to any of the known *pmoA* sequences and formed a novel lineage (clone M84-P3). Consequently, the presence of a novel group of methanotrophs was assumed. Due to the detection of this novel *pmoA*-like sequence, the aim of my Ph.D. work shifted towards the retrieval of more information about this novel *pmoA*-like lineage. This included the development of PCR assays and probes for retrieval of additional sequence information from environmental samples as well as pure cultures. Another aspect of the work focused on the expression of the novel gene in *Methylocystis* strain SC2. As a consequence, one of the original aims, the assessment of 16S rRNA will only be briefly mentioned.

1. 16S rRNA-based diversity of type I and type II methanotrophs on rice roots

Several of the 16S rRNA primers and probes published previously for methanotrophs have some disadvantages for studying natural populations of these organisms. Most of the primers currently available were developed on a relatively small sequence database. In addition, some of the previously described sequences on which the primers were based contained errors. This made accurate primer design difficult. Wise *et al.* (1999), designed and published group-specific primers for type I and type II methanotrophs. These primers were successful in

studying methanotroph diversity in a landfill soil. However, strong evidence on target specificity of the assays requires that these assays be tested on a wide range of environments. The aim of this study was twofold: first to test for the specificity and applicability of Wise *et al.* primers on the ecosystem "flooded rice microcosm", and second to increase the database for methanotrophic 16S rDNA sequences that might be useful for the design of population-specific probes for FISH (fluorescent in situ hybridization)-based detection of methanotrophs in rice soil.

The group-specific primers developed by Wise *et al.* (1999) were applied in the course of this study to generate 16S rDNA clone libraries from rice root DNA extracts. Broad methanotroph diversity was identified, including new type I and type II MOB sequences. The type I MOB group-specific primers retrieved sequences related to the genera *Methylobacter*, *Methylomicrobium*, *Methylococcus*, and *Methylocaldum*. We did not detect any 16S rDNA sequences that grouped with *Methylomonas*, *Methylosphaera* or other type I methanotrophs. The type II MOB group specific primers detected *Methylocystis*-like sequences; most of the sequences were related to *Methylocystis* strain SC2, a type II methanotroph recently isolated from a polluted aquifer (Dunfield *et al.,* 2002). We did not detect any 16S rDNA sequences that grouped with the genera *Methylosinus*, *Methylocapsa* or *Methylocella*. Only a very few non-methanotrophic sequences were detected by both type I and type II MOB assays, suggesting that these assays were highly specific on the ecosystem "flooded rice microcosm".

The presence of type I and type II methanotrophs on rice roots has been shown in previous studies (Bodelier et al., 2000; Eller *et al.,* 2001; Horz *et al.,* 2001). The cultivation-independent characterization of type I methanotrophs in unfertilized rhizosphere soil by Bodelier *et al.* (2000) resulted in the detection of one distinct cluster of highly similar 16S rDNA sequence types related to the genus *Methylobacter*. Horz *et al.* (2001) investigated the methanotroph diversity on roots of submerged rice plants by targeting the 16S rDNA, the *pmoA*, *mmoX* and *mxaF* gene and diverse population of both type I and type II methanotrophs. In the study of Horz *et al.* the 16S rDNA primers used were the type I group-specific primers also used in our study. By contrast to our study, the comparative analysis of sequences of cloned RT-PCR products revealed a more complex population, including sequences closely related to the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus* and *Methylocaldum*. The differences observed as compared to our study may be linked to the different retrieval strategies used. RT-PCR targets the 16S rRNA and thus might select for more active populations. One may assume that members of the *Methylomonas* were among the active populations, but that a relatively low cell number did not allow enough

amplification for a greater representation in 16S rDNA-based clone library. By using group-specific 16S rRNA-targeted probes for the detection of type I and type II methanotrophs by FISH, Eller *et al.* (2001) detected both type I and type II methanotrophs in soil and root samples from rice microcosms.

2. The novel *pmoA* gene copy

2.1 Development of molecular tools for the detection of the novel gene copy

In our effort to generate a methanotroph-specific *pmoA* database, we identified in rice paddy soil a novel *pmoA*-like sequence (clone M84-P3), that clustered neither with the conventional *pmoA* sequences of type I MOB, nor with those of type II methanotrophs. Analyses of *pmoA*-specific signatures showed that the novel *pmoA*-like sequence type exhibited the same amino acid residues at conserved positions within the *pmoA* gene as one present in methanotrophs, and therefore could be assigned as one *pmoA*-like than amoA-like. We assumed that the novel sequence was indicative of a new group of methanotrophs whose members were characterized not yet. In order to retrieve additional sequence types belonging to this novel *pmoA* cluster, we used clone M84-P3 as a reference to design oligonucleotide primers and assessed the target specificity of different combinations of these primers on environmental DNA extracts.
In total, five PCR assays were developed (table 4). Three PCR assays (A°, A1 and A') failed to retrieve sequences belonging to the novel lineage. Two PCR assays including a single-round-PCR and a nested-PCR (PCR system B1 and A2, respectively) were successful. Phylogenetic analyses of the derived amino acid sequences of *pmoA* genes obtained from different DNA extracts of the ecosytem "rice microcosm" revealed a novel cluster of environmental *pmoA* sequences with low similarity to known *pmoA*/*amoA* from methanotrophs and nitrifiers. Amino acid sequence identities were ca. 70% with known type II methanotrophs, 60% to 65% with type I MOB and 44% to 65% with nitrifiers. A more detailed analysis of the derived amino acid sequences showed that, although these sequences are more closely related to *pmoA* of known type II methanotrophs, they do form a distinct branch with a close affiliation to clone M84-P3. The intracluster diversity within the novel cluster was great, indicating that members of this novel *pmoA* cluster are numerous in the rice microcosm. Intra-cluster sequence dissimilarity values were up to 19% compared to ca. 30% for type I, and ca. 15% for type II methanotrophs. Moreover, the novel environmental *pmoA* sequence closely matched (85% to 92%, amino acid level) a sequence (LP21), recently detected in a polluted aquifer (Baker *et al.*, 2001). This suggests that this novel type of *pmoA*

is not restricted to the environment rice microcosms, but rather may also be present in other environments. Besides the detection of novel *pmoA* sequence types, we also detected a few type I and type II MOB sequences. Interestingly, the type I MOB sequences formed a novel sublineage with amino acid identities of only 75% to 80% with those of known type I MOB. This finding suggests that a novel subgroup of type I MOB whose presence has not yet been identified may inhabit the rice roots. However, confirmation of this novel subgroup is needed through cultivation approaches.

In addition to the diversity assessment of environmental samples via sequencing, T-RFLP analysis was conducted on three samples. This aimed at correlating the T-RFs observed to novel *pmoA* sequences. All the T-RFs observed could be assigned to environmental *pmoA* sequences. Only one of the T-RFs (492 bp) present in the profiles could not be detected by cloning and sequencing. Figure 5 shows a *Msp*I-based electropherogram of the 5' T-RFLP patterns for samples Rz90f, Rr90a and RsVc. Differences in the numerically dominant T-RFs between two T-RFLP patterns were clearly observed. For example, the dominant T-RFs of samples Rr90a and RsVc were different (Fig. 5B and C). Certain major T-RFs observed in samples Rr90a and RsVc were not observed in sample Rz90f. Above all, the T-RFs corresponded to sequence types of our novel *pmoA,* suggesting that the T-RFLP method is a reliable tool for rapid analysis of environmental samples harbouring the novel *pmoA* sequence types.

2.2 Evidence for the presence of a novel *pmoA* gene in *Methylocystis* strain SC2

Methylocystis strain SC2 was recently isolated from a polluted aquifer, and it has been of interest in molecular-based studies (Dunfield *et al.,* 2002). As shown by DGGE (Denaturant Gradient Gel Electrophoresis) combined with sequencing, strain SC2 has two very different *pmoA*-like genes. The first gene (*pmoA*1 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 the first gene at the nucleotide level and 68.5% identity at the amino acid level. As identified in our study, *pmoA2* of strain SC2 was closely related to the novel *pmoA*-like sequences retrieved from flooded rice microcosms. This indicated that the novel *pmoA*-like sequences in our study do not originate from a hitherto uncharacterized group of methanotrophs, but rather are divergent, extra copies of *pmoA* in known methanotrophs. Interestingly, as confirmed by phylogenetic analyses (Fig. 4) some of our environmental 16S

rRNA gene sequences are affiliated with 16S rDNA of strain SC2, suggesting that SC2-like strains that harbour multiple, divergent *pmoA* gene copies might be present in rice paddy soil. In order to confirm the presence of divergent multiple copies of *pmoA* within a single species, *Methylocystis* strain SC2 was tested for the number of detectable copies of *pmoA* by using oligonucleotide probes specific for either *pmoA1* or *pmoA2*. Our results suggest that these probes detected two copies of *pmoA1* and one copy of *pmoA2* (Fig. 8). The two gene copies of *pmoA1* are the most homologous to *pmoA* genes detected in other type II MOB at two to three nearly identical copies (Auman *et al.,* 2000; Gilbert *et al.*, 2000; Semaru *et al.,* 1995; Stolyar *et al.,* 1999). Therefore, the finding of multiple identical copies of *pmoA* in strain SC2 is consistent with earlier findings.

2.3 Expression of the novel gene in strain SC2

The finding of a novel *pmoA* within the strain SC2 raised the question of whether the gene is functionally active or not. In order to address this question, Northern hybridization and RT-PCR were carried out on total RNA of strain SC2 grown under standard cultivation conditions (see "Methods"). Both methods confirmed the expression of the novel gene, suggesting that the expression product of the novel *pmoA* may be an active enzyme and not a pseudogene. However, hybridization showed that, the expression of the novel *pmoA* gene was low compared to that of the conventional *pmoA* gene. We are unable based on these findings to predict the function that the novel *pmoA* gene has in strain SC2 and in other MOB. Further studies will need to be conducted to elucidate this.

2.4 Screening of methanotrophic pure cultures for the presence of the novel *pmoA*

The specific primers developed for the retrieval of the novel *pmoA* had few mismatches with conventional *pmoA* sequences of the *Methylocystis/Methylosinus* group. At lower annealing temperature, both *pmoA* copies present within a single strain would be amplified and mixtures of amplicons therefore resulted. These could not be characterized by direct sequencing. An alternative to alleviate the problem would to clone the PCR product or to use it in fingerprint methods such as DGGE, TGGE (Muyzer *et al.,* 1993) or SSCP (Lee *et al.,* 1996). Although the current methods of cloning and sequencing have proven suitable for the retrieval of multiple sequences present in environmental samples as well as pure cultures (Auman *et al.,* 2000), these methods would be too laborious if a large number of isolates would have to be

screened. Moreover construction and screening of a clone library for every single isolate would be rather time-consuming and expensive. Consequently, there was a need to establish a reliable method that would circumvent these limitations.

Our data on environmental *pmoA* sequences suggested that T-RFLP analysis would be the easiest way to classify the sequences. Ideally, each single sequence could be assigned to a given T-RF. In contrast to the laborious aspects of cloning and sequencing and other methods such as DGGE or its cousin TGGE or SSCP, T-RFLP has the advantage that on-line analysis of data is immediate and the output is digital. Moreover, direct reference to the sequence database can be made, i.e. all T-RFs sizes observed in a *pmoA*-based T-RFLP pattern can be compared with T-RFs derived from the growing sequence database. Another advantage is that several isolates are analyzed simultaneously on the same gel. Moreover, DGGE, TGGE and SSCP have other advantages: bands observed on gels can be blotted on membranes and characterized by probing or they can be exised and sequenced.

At an annealing temperature of 66°C, the combined T-RFLP and sequencing analysis seemed ideal for the retrieval of the novel *pmoA*. One important aspect of our investigation was the finding that the novel *pmoA* is distributed among most type II MOB and a few type I MOB examined in this study. However, some MOB including type I as well as type II strains failed to give a positive PCR with our specific assays. The observation that the novel *pmoA* can be found only in a restricted number of species is not surprising. Other functional genes such as sMMO (Auman *et al.*, 200; Bowman *et al.,* 1993; Fuse *et al.,* 1998) or *nifH* (Auman *et al.,* 2001, Oakley and Murrell, 1988) are found only in a limited number of methanotrophs. We also identified false positive sequence types in this study. *pmoA* sequences belonging to the *Methylocystis*/*Methylosinus* group were still amplified at an annealing temperature of 66°C, suggesting that these sequences had only a very few mismatches to our specific primers.

The phylogenetic analysis of novel *pmoA*-like sequences led to some unexpected results. We observed a close affiliation of *pmoA2* of *Methylomicrobium album* (type I MOB) and *pmoA2* of *Methylocystis parvus* (type II MOB). However, based on comparative analysis of 16S rDNA, those are only distantly related MOB. Thus, the finding that their novel *pmoA* are closely related suggests that the *pmoA2* cannot be considered a reliable phylogenetic indicator in affiliating either type I or type II MOB. Recently, *pmoA* has been used in addition to 16S rDNA as a phylogenetic marker for MOB (Auman *et al.,* 2000; Costello and Lidstom, 1999; Murrell *et al.,* 1998). However, the presence of diverse *pmoA* gene copies in single strains may complicate the interpretation of environmental *pmoA* data. In addition, in a recent study, Pacheco-Olivier *et al.* (2000) investigated isolates from arctic soils with unusual *pmoA*/*amoA*

and 16S rDNA sequences. The 16S rRNA sequences of these organisms were closely related to 16S rRNA genes from *Methylosinus* and *Methylocystis*, although they did form a separate branch. However the *amoA*/*pmoA* sequences were affiliated with *amoA* of *Nitrosopira* and were very distant from those *Methylosinus* and *Methylocystis*. Moreover, these sequences were different to those retrieved in our study, so the distribution of *pmoA*-like genes in MOB may be much more complicated than originally thought.

2.5 Southern hybridization

Southern blot analyses were in accordance with PCR results suggesting that PCR were not affected by false positive results. One interesting feature is that we could not detect the novel *pmoA* gene in *Methylococcus capsulatus* and in *Methylosinus trichosporium* OB3b neither by PCR nor by Southern hybridization. These two organisms are model organisms that have extensively been used to study the molecular biology and genetics of MOB (Semrau *et al,* 1995; Stolyar *et al.,* 1999). Our findings may explain why the novel gene had never been detected in these organisms. Moreover, in contrast to *Methylosinus trichosporium* OB3b; we could detect the novel *pmoA* in *Methylosinus trichosporium* SM6. These two organisms are closely related with 16S rDNA sequence similarities of 98.15%. This finding suggests that the distribution pattern of the novel *pmoA* is not only restricted to the species level, but differences can also be observed at the strain level.

2.6 Functional significance of the novel *pmoA*

Northern hybridization and RT-PCR provided evidence that the novel *pmoA* is expressed. However, the putative function (PmoA or AmoA) the gene may have is not clarified by these results. Therefore, we conducted a search for amino acid residues that may indicate whether the enzyme is adapted to methane or to ammonia as a substrate. An amino acid was considered a putative signature if it fulfilled the following criteria: it had to be universally conserved in all type I and type II MOB, and at the same position in the alignment there had to be universal conservation of a different residue in all known autotrophic ammonia oxidizers of the β-*Proteobacteria* (Holmes *et al.*, 1995). Sites fulfilling these criteria were found distributed throughout the gene, suggesting that the protein expressed by the novel *pmoA* gene type will also show strong structural conservation. Residues that are identical for all MOB and AOB may reflect adaptation to AMO or pMMO function. Figure 16 shows an alignment

of the predicted protein sequences for PmoA and AmoA. Residues conserved within each of the identity groups and those that are universally conserved are highlighted. The novel *pmoA* cluster sequences contained 93.3% (42 of 45) universally conserved monooxygenase (pMMO and AMO) signatures, 76.8% (11 of 14) of the putative MMO signatures and 0.0% of the putative AMO signatures. The strong conservation of MMO (and especially pMMO) signatures is an indication that the novel gene codes for the active site of a particulate methane monooxygenase. Both pMMO and AMO are key enzymes in major biogeochemical cycles and are of potential significance in bioremediation programs. However, further studies are needed to understand the role and adaptation of the novel *pmoA* to methanotrophs.

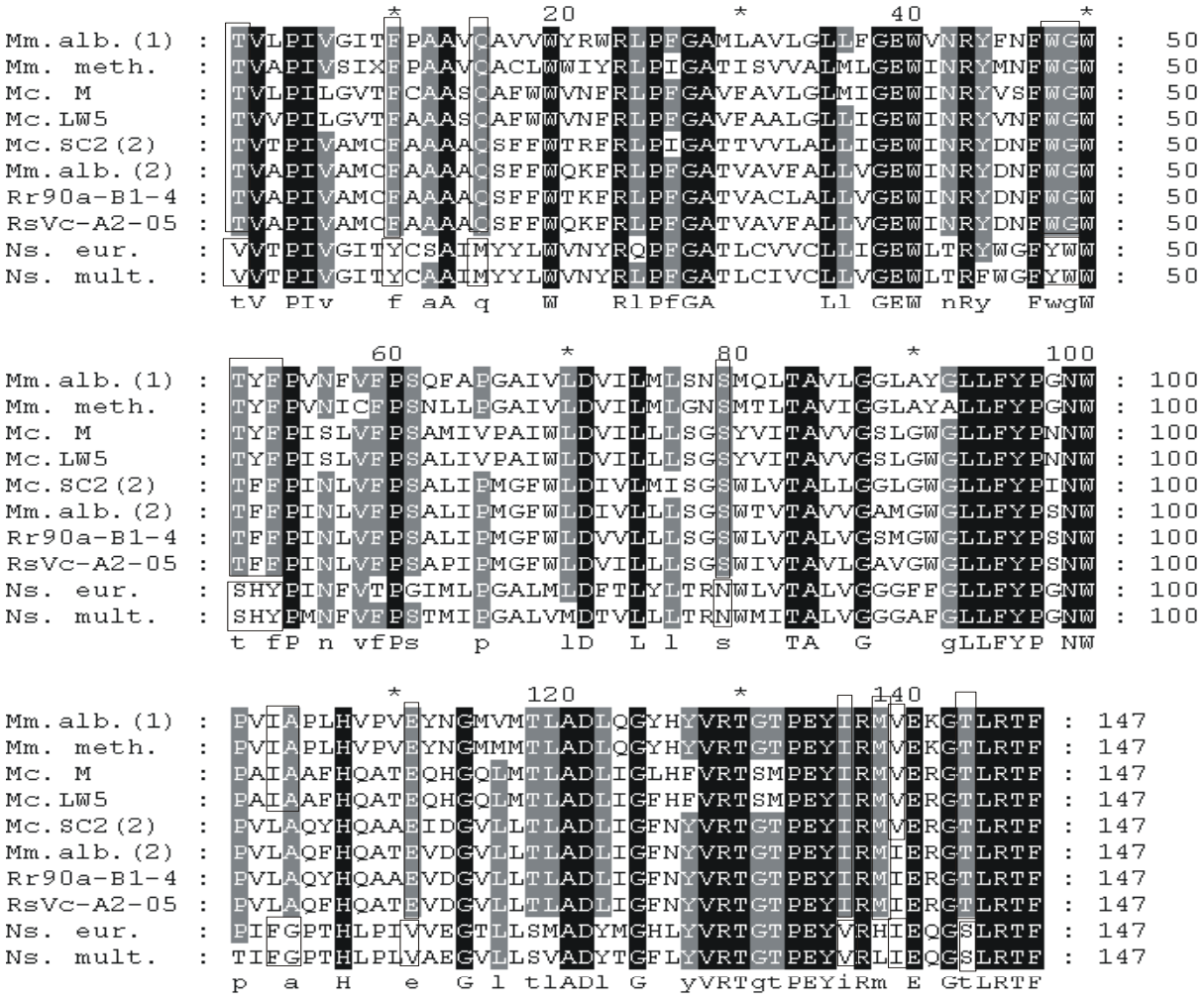

Fig. 16. Alignment of inferred amino acid sequences of *pmoA* and *amoA* from representative methanotrophic and nitrifying bacteria as well as clones Rr90a-B1-43 and RsVc-A2-05 and *pmoA2* of *Methylocystis* strain SC2 and *Methylomicrobium album*. Residues, that are universally conserved in extant MOB and AOB are highlighted in black. Residues boxed are putatives MMO or AMO signatures. The sequences shown are as follow: Mm. alb. (1), *Methylomicrobium album* (*pmoA1*); *Methylomonas methanica*; Mc. M, *Methylocystis* sp. M; Mc. LW5, *Methylocystis* sp. LW5, Mc. SC2 (2), *Methylocystis* sp. SC2 (*pmoA2*); Mm. alb. (2), *Methylomicrobium album* (*pmoA2*); clone Rr90a-B1-43; clone RsVc-A2-05; Ns. eur. *Nitrosomonas europaea*; Ns mult., *Nitrosospira multiformis*

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