Detection of Methanotroph Diversity on Roots of Submerged Rice Plants by Molecular Retrieval of \textit{pmoA}, \textit{mmoX}, \textit{mxaF}, and 16S rRNA and Ribosomal DNA, Including \textit{pmoA}-Based Terminal Restriction Fragment Length Polymorphism Profiling

HANS-PETER HORZ, MERLIN TCHAWA YIMGA, AND WERNER LIESACK*

Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg, Germany

Received 22 December 2000/ Accepted 26 June 2001

The diversity of methanotrophic bacteria associated with roots of submerged rice plants was assessed using cultivation-independent techniques. The research focused mainly on the retrieval of \textit{pmoA}, which encodes the \( \alpha \) subunit of the particulate methane monooxygenase. A novel methanotroph-specific community-profiling method was established using the terminal restriction fragment length polymorphism (T-RFLP) technique. The T-RFLP profiles clearly revealed a more complex root-associated methanotrophic community than did banding patterns obtained by \textit{pmoA}-based denaturing gradient gel electrophoresis. The comparison of \textit{pmoA}-based T-RFLP profiles obtained from rice roots and bulk soil of flooded rice microcosms suggested that there was a substantially higher abundance of type I methanotrophs on rice roots than in the bulk soil. These were affiliated to the genera \textit{Methylomonas}, \textit{Methylobacter}, \textit{Methylococcus}, and to a novel type I methanotroph sub-lineage. By contrast, type II methanotrophs of the \textit{Methylocystis}-\textit{Methylosinus} group could be detected with high relative signal intensity in both soil and root compartments. Phylogenetic treeing analyses and a set of substrate-diagnostic amino acid residues provided evidence that a novel \textit{pmoA} lineage was detected. This branched distinctly from all currently known methanotrophs. To examine whether the retrieval of \textit{pmoA} provided a complete view of root-associated methanotroph diversity, we also assessed the diversity detectable by recovery of genes coding for subunits of soluble methane monooxygenase (\textit{mmoX}) and methanol dehydrogenase (\textit{mxaF}). In addition, both 16S rRNA and 16S ribosomal DNA (rDNA) were retrieved using a PCR primer set specific to type I methanotrophs. The overall methanotroph diversity detected by recovery of \textit{mmoX}, \textit{mxaF}, and 16S rRNA and 16S rDNA corresponded well to the diversity detectable by retrieval of \textit{pmoA}.

The atmospheric trace gas methane (\( \text{CH}_4 \)) is a prominent “greenhouse” gas. Its atmospheric concentration has been increasing until recently at a rate of about 1% a year (8). Up to 70 to 80% of atmospheric \( \text{CH}_4 \) is biogenic (55). Flooded rice fields are one of the major sources of biogenic \( \text{CH}_4 \) (34, 50). Estimations of the annual emission rate from flooded rice fields range between 60 and 110 Tg (8, 21, 45). The upper limit of this emission rate accounts for approximately 25% of the total annual \( \text{CH}_4 \) emission into the atmosphere (8, 21).

Approximately 90% of the \( \text{CH}_4 \) that is emitted from rice paddies escapes through the aerenchyma of the rice plants, whereas only 10% escapes through the floodwater (19, 52). However, the aerenchyma does not merely function as a gas transport system but rather constitutes a dynamic, oxygenated biofilter. The diffusive input of oxygen into the below-ground plant surface area enables aerobic methanotrophs to oxidize \( \text{CH}_4 \). Gilbert and Frenzel (22) showed that the activities of methanotrophs were directly dependent on the oxygen availability in the rice root environment. It was shown that up to 30% of the \( \text{CH}_4 \) produced in rice paddy soil is oxidized by root-associated methanotrophs (5, 9, 15).

Based on phylogenetic, physiological, morphological, and biochemical characteristics, methanotrophs are divided into two major subgroups (27). The \( \gamma \)-proteobacterial type I methanotroph group comprises the genera \textit{Methylomonas}, \textit{Methylolocaldum}, \textit{Methylomicrobium}, \textit{Methylobacter}, \textit{Methylosarcina}, \textit{Methylosphaera}, and \textit{Methylococcus} (also classified as type X) (4, 6, 27, 58), while the \( \alpha \)-proteobacterial type II methanotroph group consists of the genera \textit{Methylocystis} and \textit{Methylosinus} (27) and one more distant species, \textit{Methyllocella palustris} (14).

The methanotrophic diversity in rice field soil has been assessed in detail (28, 30), but knowledge about the diversity of methanotrophic populations associated with rice roots is still limited. Type II strains were isolated from the terminal positive-dilution steps of a most-probable-number dilution series (23). However, whether these results reflect the natural situation on rice roots, i.e., predominance of type II methanotrophs, or instead were the consequence of cultivation bias, is unclear.

We assessed the methanotrophic diversity associated with roots of submerged rice plants using various cultivation-independent techniques. This assessment was carried out in relation to the methanotrophic diversity detectable in rice paddy bulk soil. Despite the phylogenetic distance between type I and type II methanotrophs, almost all known methanotrophs possess a \textit{pmoA} gene, which encodes the \( \alpha \) subunit (PmoA) of the particulate methane monooxygenase (pMMO). The only exception is \textit{Methyllocella palustris} (13, 14). Consequently, this study focused mainly on the retrieval of \textit{pmoA} using PCR primers described previously (32). These primers also target \textit{amoA}, which encodes the \( \alpha \) subunit (AmoA) of the ammonia monooxygenase in autotrophic ammonia oxidizers. Based on...
the pmoA sequence database created in this study, we established a novel methanotroph-specific community-profiling method using the terminal restriction fragment length polymorphism (T-RFLP) technique (37, 39). The methanotroph diversity detectable by pmoA-based T-RFLP profiling was compared with those detectable by comparative sequence analysis of cloned pmoA and by pmoA-based denaturing gradient gel electrophoresis (DGGE).

To examine the meaningfulness of the pmoA-based results, we also assessed the methanotrophic diversity detectable by retrieval of mmoX (25) and mxaF (42). The mmoX gene encodes the α subunit of the ammonia monooxygenase (MMO) of the hydrogenase component of the soluble methane monooxygenase (sMMO). This monooxygenase is present in most type II methanotrophs, in members of the genus Methylomicrobium, and in some Methylomonas strains (53) but not in most of the other type I methanotrophs (27). The mxaF gene codes for the α subunit of the methanol dehydrogenase, which is present in all methylocytophores. In addition, both 16S ribosomal DNA (rDNA) and 16S rDNA were retrieved using PCR primers specific to type I methanotrophs (57).

**MATERIALS AND METHODS**

**Rice microcosms.** Rice (*Oryza sativa* var. Roma, type japonica) was grown in three flooded, unfertilized microcosms for 70, 84, or 90 days using conditions described previously (20, 26).

Rice roots obtained from the three microcosms were used as source material for the molecular analyses. This material, which we will refer to as root samples M70, M84, and M90, was washed by careful shaking in phosphate-buffered saline (7 mM Na2HPO4, 3 mM NaH2PO4, 130 mM NaCl [pH 7.2]) to remove adhering soil particles. Cores were obtained from the bulk soil matrix by bead beating. After centrifugation, both supernatants were pooled and extracted three times with cold phenol-chloroform (1:1, vol/vol) followed by precipitation of total nucleic acids with 0.1 volume of sodium acetate (3 M; pH 5.2) and 2.5 volumes of ethanol. The pellet was dried and suspended in 100 μl of 0.17 to 0.18 mm and 700 μl of precooled TPM buffer (50 mM Tris-HCl [pH 7.5], 1.7% [wt/vol] polyvinylpyrrolidone, 10 mM MgCl2) (18). This suspension was shaken for 60 s at maximum speed in a bead beater (Dismembrator-S; B. Braun Biotech GmbH, Melsungen, Germany). Glass beads, root particles, and cell debris were pelleted by centrifugation for 5 min at 4°C, and the supernatant was transferred to a new reaction tube. Seven hundred microliters of a phenol-based lysis buffer was added to the pellet, and the bead-beating procedure was repeated. After centrifugation, both supernatants were pooled and extracted three times with cold phenol-chloroform (1:1, vol/vol) followed by precipitation of total nucleic acids with 0.1 volume of sodium acetate (3 M; pH 5.2) and 2.5 volumes of ethanol. The pellet was dried and suspended in 100 μl of Tris-EDTA buffer. For subsequent DNA-based analyses a 50-μl aliquot was stored at −20°C. For preparation of total RNA, the other 50-μl aliquot was mixed with 1 volume of TMC buffer (10 mM Tris-HCl [pH 7.5], 5 mM MgCl2, 0.1 mM CsCl) (18) and 5 U of RNase-free DNase (Promega, Madison, Wis.) and incubated for 1 h at 37°C to remove the DNA. The reaction was stopped by extraction with 1 volume of chloroform. Precipitation and resuspension of total RNA were described above.

**PCR amplification.** The primer sets used in this study are listed in Table 1. For pmoA-based T-RFLP analysis, the 5' primer A189 was labeled with the dye carboxyfluorescein. The reaction mixture contained 1 to 5 ng of DNA, 50 μl of MasterAmp PCR premix F (Eppendorf; Hamburg, Germany), 0.1 μM concentrations of each primer (MWG-Biotech, Ebersberg, Germany), and 2.5 U of Taq DNA polymerase (AmpliTaq; PE Applied Biosystems, Foster City, Calif.). Amplification was performed in a total volume of 100 μl in 0.2-ml reaction tubes, using a DNA thermal cycler (model 2400; PE Applied Biosystems). The thermal PCR profile was as follows: initial denaturation for 2 min at 94°C and 30 cycles consisting of denaturation at 94°C for 45 s, primer annealing for 60 s (annealing temperature specific to each target gene [Table 1]), and elongation at 72°C for 120 s. The final elongation step was 6 min. Aliquots of the amplicons (10 μl) were checked by electrophoresis on a 1% agarose gel.

**RT-PCR of 16S rRNA.** Ribosomal copy DNA (rcDNA) of type I methanotrophs was synthesized from total RNA using primer MethT1cR (Table 1), Moloney murine leukemia virus reverse transcriptase (RT) (RNase H minus; Promega, Mannheim, Germany), and a previously described protocol (38). PCR of 16S rcDNA was carried out as described above.

**pmoA-based T-RFLP analysis.** T-RFLP analysis was performed for each total DNA extract in triplicate using a protocol reported previously (38, 39). The pmoA was amplified by PCR as described above. After purification with Qiagen spin columns (Qiagen, Hilden, Germany), approximately 100 ng of the amplicons was digested with 10 U of the restriction endonuclease MspI (Promega). The digestion was carried out in a total volume of 10 μl for 3 h at 37°C. Aliquots (2.5 μl) of the digested amplicons were mixed with 2.0 μl of formamide and 0.5 μl of an internal lane standard (GeneScan-1000 ROX; PE Applied Biosystems). After digestion, the mixture was denatured at 100°C for 3 min and then chilled on ice. Electrophoresis on a polyacrylamide gel (6%) was performed using an automated DNA sequencer (model 373; PE Applied Biosystems) for 6 h at the

| Target gene (fragment length in bp) | Primer set | Sequence (5' to 3') | Annealing temperature (°C) | Reference |
|-------------------------------------|------------|----------------------|---------------------------|-----------|
| pmoA (531)                          | A189f      | GGNGACTGGGCCATCTCTGG | 62 to 52 touchdown         | 32        |
|                                     | A682r      | GAASGCAGAAAGAAGGCC   |                           |           |
| mmoX (863)                          | 534fr      | CCGCTGGAGAGGCGATGAA  | 62 to 52 touchdown         | 25        |
|                                     | 1393rt     | CACCTGAGCGCCGTCG     |                           |           |
| mxaF (557)                          | f1003      | GGGCAGACACACTGGGCTGTG| | 55        |
|                                     | r1561      | GGGGCACGATGAAAGGCTCCC| | 42        |
| 16S rDNA (922) and 16S rcDNA (556)   | MethT1dF   | CTCCTCAGGCGGCTG     | | 56        |
|                                     | MethT1bR   | GATCCYMTGATGTCAAGG   | | 57        |
|                                     | MethT1cR   | ATCCAAATCAGGTTCCCCAGGTAAGCCC | |          |
following settings: 2.500 V, 40 mA, and 27 W (24-cm gel length). After electro- 
photographs, the sizes of the 5'-terminal restriction fragments (T-RFs) and the intensi- 
ties of their fluorescence emission signals (i.e., signal intensities) were automatically 
calculated by the GeneScan Analysis software, version 2.1 (PE Applied Biosystems). 
The reliability of signal intensification between replicates was ±1 bp. The relative signal 
intensity of each T-RF was calculated based on the signal intensity of the individual 
T-RF in relation to the total signal intensity of all T-RFs (including the 531-bp 
fragment) detected in the respective T-RFLP community profile. The 531-bp 
fragment corresponds to pmoA amplicons without any restriction site for MspI. 

pmoA-based DGGE. PCR amplification of pmoA and DGGE in the Dode 
System (MB-Red, Munich, Germany) was carried out as described by Henkel 
et al. (28). In brief, PCR products were separated in 1-mm thick polyacrylamide 
gels (6.5% [wt/vol] acrylamide-bisacrylamide [37:5:1]) using a linear denaturing 
gradient that ranged from 35 to 35%. A denaturing gradient of 80% corre- 
sponded to 6.5% acrylamide, 5.6 M urea, and 32% deionized formamide. The 
electrophoresis was performed in 0.5X TAE buffer (0.04 M Tris-base, 0.02 M 
sodium acetate, 1 mM EDTA [pH 7.4]) for 15 h at a constant voltage of 48 V at 70°C. Gels were stained with 1:10,000 (vol/vol) SYBR-Green I (Biozym, Hes- 
tisch-Oldendorf, Germany) for 45 min and scanned with a Storm 860 Phosphor-
imager (Molecular Dynamics, Sunnyvale, Calif.).

Cloning and sequencing. PCR products of pmoA, mmoX, mxaF, 16S rDNA, and 
and 16S rDNA and rcDNA were cloned using the TOPO TA cloning kit (Invitrogen Corp., 
San Diego, Calif.) as recommended by the manufacturer. The preparation of 
plasmid DNA of randomly selected clones, PCR amplification of cloned inserts, and 
noradioactive sequencing were carried out as described previously (48). In 
addition, oligonucleotide primers targeting internal regions of the cloned inserts 
were used for sequencing of mmoX and 16S rDNA and rcDNA.

Phylogenetic analysis. Based on sequence information deposited either in 
public-domain databases or generated in the course of this study, we established 
sequence databases for pmoA, mxaF, and mmoX. Each of these sequence data- 
bases was integrated into the ARB program package (developed by O. Strunk 
and W. Ludwig; Technische Universität München [http://www.arb-home.de]) and 
was manually put into an aligned format. The 16S rDNA and rcDNA clone 
sequences were added to a database of about 14,000 complete or partial bacterial 
16S rRNA sequences. Evolutionary distances (ARB and PHYLIP [17]) between 
pairs of inferred amino acid sequences (pmoA, mmoX, and mxaF) were calcu-
lated using various models (11, 36). Evolutionary-distance values between pairs of 
16S rDNA and rcDNA clone sequences were calculated by applying the 
Jukes-Cantor correction (35). The trees were constructed using the neighbor-
joining method (49). The statistical significance levels of interior nodes were 
determined by performing bootstrap analyses by the neighbor-joining method 
(1000 data resamplings). To exclude obvious chimeric primary structures from the 
pmoA, mmoX, mxaF, and 16S rDNA and rcDNA sequence databases, separate 
treeing analyses of the 5’ and 3’ halves of the respective sequence data sets were 
carried out.

Nucleotide sequence accession numbers. The environmental pmoA (plus 
amoA and sequence types of uncertain affiliation), mmoX, mxaF, 16S rDNA, and 
and 16S rDNA clone sequences recovered in this study from rice roots of flooded 
rice roots of Methylococcus capsulatus. Cluster II (10 clones) formed a separate lineage without any clear 
affiliation to any of the known genera. Cluster III (10 clones) exhibited a moderate relationship to Methylophilus capsulatus. 

Only two pmoA clones could be assigned to the Methylocys-
tis-Methylosinus group (type II methanotrophs). Four se- 
quence types grouped with ammonia oxidizers of the 
β-proteobacterial Nitrosomonas-Nitrosospira group. Five se- 
quence formed the lineages A, B, and C (Fig. 1A), which 
branching distinctly from all currently known methanotrophs 
or autotrophic ammonia oxidizers.

Separate treeing analysis of the 5’ and 3’ halves of the respective sequence types suggested that the clone sequences 
assigned to the lineages A, B, and C were of natural origin, i.e., 
separate treeing analysis did not provide evidence that any of 
these clones were chimeric. We therefore determined amino 
acid signature residues for the inferred peptide sequences 
of the lineages A, B, and C (Table 2) (33). These are either 
universal to PmoA (methanotrophs) and AmoA (autotrophic 
ammonia oxidizers) or specific for either PmoA or AmoA 
(substrate-diagnostic residues). Based on this approach, Holmes 
et al. (33) assigned a newly detected cluster of sequence types 
(forest clones) (Table 2) to an uncharacterized group of meta-

One of the sequences was assigned to the type I 
mechanisms of methanotrophs and environ-
mental samples, we predicted that the tetrameric restriction enzyme MspI would be the restriction enzyme most appropri- 
ate to analyze the genetic diversity of methanotrophic communities in a single electrophoretic profile. Defined mixtures of genomic DNA from cultured methanotrophs subjected to 
MspI-based T-RFLP analysis exactly produced those T-RFs 
predicted based on our pmoA sequence database (data not shown). Consequently, MspI was used for pmoA-based T-RFLP 
analysis of methanotrophic communities. Extraction of total 
DNA from the same root sample in triplicate followed by PCR 
applification of pmoA and T-RFLP analysis produced highly 
similar T-RFLP community profiles. The coefficients of vari- 
ation of the relative signal intensities between these profiles were 
between 5.4 and 12.3% for the major peaks, i.e., those with sizes 
of 80, 245, 350, 440, 505, and 531 bp (Fig. 1B; see also Fig. 2). 
The coefficients of variation for the major peaks between T- 
RFLP community profiles generated in triplicate from individ- 
ual DNA extracts ranged from 1.7 to 7.6%. This analysis 
showed that the T-RFLP technique was reliable for a rapid PCR-
based fingerprinting of methanotrophic communities. High 
reproducibility of the T-RFLP technique has been reported 
previously (e.g., for 16S rDNA-based T-RFLP analysis [39, 
44]).
FIG. 1. (A) Distance dendrogram constructed for partial pmoA and amoA gene sequences based on 165 derived amino acid sites in relation to pmoA-based T-RFLP (B) and DGGE (C) community patterns. The two patterns and most of the pmoA clone sequences were obtained from sample M84. (A) The dendrogram shows environmental pmoA and amoA (plus other putative monooxygenase) sequences retrieved from roots of submerged rice plants (M84, M90) in relation to pmoA of cultured type I and type II methanotrophs, environmental pmoA clone sequences, and amoA sequences of the β-proteobacterial *Nicrosoma* and *Nitosospira* group. The environmental pmoA sequences used for reference were retrieved from various habitats as follows: beech forest in Denmark (RA14 [AF148521], RA21 [AF148522], RokI [AF148523], Rold4 [AF148526], Rold5 [AF148527]), rain forest in Brazil (Pantanal13 [AF148525]), mixed hardwood forest in the United States (Maine6 [AF148528], Maine9 [AF148531]) (33), deciduous forest soil near Marburg, Germany (MR2 [AF200726], MR16 [AF200729]) (29), rice soil incubations (He-I [AF126908], He-II [AF126913], He-VI [AF126911]) (28), and blanket peat bog (PE9 [AF006050], PD2 [AF006047]) (41). The numbers I, II, and III refer to three distinct pmoA sequence clusters of type I methanotrophs, which have been retrieved from rice roots. The numbers at the nodes indicate the percentage of recovery in 500 bootstrap resamplings. Only bootstrap values ≥50 are shown. Scale bar, 0.1 substitution per amino acid site. Database accession numbers of reference organisms are as follows: *Methylocystis* sp. strain M, U81596; *Methylocystis parvus*, U31651; *Methylosinus trichosporium*, U31550; *Methylomonas* sp. strain BBS1, AF016982; *Methylomonas pelagicum* album, U31654; *Methylomonas pelagicum* pelagicum, U31652; *Methylomonas* methylare, U31653; *Methylomonas gracile*, U89301; *Methylomonas* szegediense, U89303; *Methylomonas* caput, U89304; *Methylomonas* caput, L40804; strain HB, U89302; and *Nicrosoma europae*, AF037107. (B) pmoA-based T-RFLP profile. The x axis shows the lengths (in base pairs) of the T-RFs, and the y axis shows the intensities of the fragments in arbitrary units. The numbers in boxes indicate the sizes of T-RFs which could be assigned to phylogenetically defined methanotroph populations or to autotrophic ammonia oxidizers (see arrows). (C) pmoA-based DGGE pattern. For comparative sequence analysis, predominant DGGE bands were excised, reamplified, and reanalyzed by DGGE to verify band purity. Affiliation of these bands to distinct pmoA clusters is indicated (compare with Fig. 1A).
TABLE 2. Signature residues of predicted amino acid sequences*

| Sequence types | MMO (n = 21) | AMO (n = 21) | Universal† (n = 52) |
|----------------|--------------|--------------|--------------------|
| Lineage A      | 17 (81)      | 1 (5)        | 49 (94)            |
| Lineage B      | 7 (33)       | 4 (19)       | 43 (83)            |
| Lineage C      | 9 (43)       | 6 (29)       | 40 (77)            |
| Forest clones  | 16 (76)      | 2 (10)       | 49 (94)            |

* Amino acids were considered to be putative substrate-diagnostic signature residues only if they fulfilled the following two criteria: (i) residues were conserved in all currently known pmoA sequence types of type I and type II methanotrophs (putative MMO signature residues), and (ii) all amoA sequence types also exhibited at the same alignment positions a conserved amino acid residue but different from that of the pmoA sequence types (putative AMO signature residues). Twenty-one of 165 amino acid positions met these two criteria. MMO, methane monooxygenase; AMO, ammonia monooxygenase.

† Residues which are universally conserved in both pmoA and amoA sequence types.

Root-associated methanotroph diversity detectable by T-RFLP analysis was compared to that detectable by DGGE using the same extract of total DNA as the starting material. DGGE revealed the presence of only pmoA sequence types affiliated with type II methanotrophs and pmoA cluster III (Fig. 1C).

The community profiles obtained by pmoA-based T-RFLP analysis from root samples M70, M84, and M90 showed similar relative abundances of the major T-RFs, i.e., of T-RFs with sizes of 80, 245, 350, 440, 505, and 531 bp. The T-RFLP profiles generated from the anoxic bulk soil were characterized mainly by the 245-bp T-RF of type II methanotrophs, but minor peaks indicative of type I methanotrophs (80- and 350-bp T-RFs) and ammonia oxidizers (47-bp T-RF) were also present. The comparison of T-RFLP profiles obtained from bulk soil versus rice roots is shown for rice microcosm M84 (Fig. 2).

**mmox**. Five of 15 clones analyzed were closely related to the mmox of Methylocystis sp. strain LR1 (Fig. 3). This type II methanotroph was isolated in Canada (16). Three mmox clones were assigned to Methylocystis sp. strain M. The treeing analysis suggested that clone M84-S38 was affiliated to the mmox of the acidophile Methylocella palustris (13, 14). However, the dissimilarity values of the predicted peptide sequence of clone M84-S38 with MмоX of Methylocella palustris (15.7%), the Methylocystis-Methylosinus group (17.1 to 18.5%), and Methylococcus capsulatus Bath (17.5%) were all similar. The MмоX sequence types of the phylogenetically distinct type I methanotrophs Methylosinus sp. strain KSWII and Methylococcus capsulatus Bath differ by 11.3% (53). Taking into account these dissimilarity values, it can only be speculated that clone M84-S38 corresponds to a novel methanotrophic bacterium which harbors sMMO. The remaining six clones were false positives containing non- MMOX sequence types.

**mxaF**. Twenty-four of 50 clones analyzed formed a coherent mxaF sequence cluster related to Methylomonas methanica and Methylomicrobium album. One sequence type each could be assigned to Methylocystis sp. strain LR1 (16), strain LK6 (Fig. 4), and Hyphomicrobiurn sp. strain CM2 (data not shown).
remaining 23 clones were false positives containing non-mxaF sequence types.

16S rDNA and 16S rRNA analysis of type I methanotrophs. 16S rDNA clone libraries were generated from samples M70, M84, and M90. An rDNA clone library was created only from freshly prepared roots of sample M70. Because RT-PCR was unsuccessful with the primer set MethT1dF and MethT1bR, we replaced MethT1bR with primer MethT1cR (Table 1). The use of MethT1cR resulted in an amplicon of the predicted size (556 bp). The retrieval of both 16S rDNA and 16S rRNA led to the identification of a diverse community of type I methanotrophs (Fig. 5). No false-positive clone sequences were detected in a set of 23 16S rDNA and 6 16S rRNA clones randomly selected for analysis. This underlines the target specificity of these PCR primers for type I methanotrophs (57).

**DISCUSSION**

Root-associated methanotroph diversity assessed by comparative analysis of pmoA, mmoX, mxaF, and 16S rRNA and rDNA sequences. The branching pattern of the pmoA tree showed a remarkable congruence with that of the 16S rDNA and rDNA tree (Fig. 1A and 5). Therefore, it is highly likely for example that pmoA cluster III corresponds to the 16S rDNA branch characterized by the clones M90-D37, M84-D38, and M84-D36. Although it is difficult to deduce a close phylogenetic correspond-
FIG. 5. Distance dendrogram showing the 16S rDNA clone sequences retrieved from roots of submerged rice plants (samples M70, M84, and M90) in relation to type I methanotrophs and nonmethanotrophic members of Proteobacteria. The environmental 16S rDNA sequences encompass the clones M70-D2 to M90-D34. Due to limited sequence length (556 bp), the 16S rDNA clones M70-R5 to M70-R40 (R = 16S ribosomal copy DNA recovered from total RNA of sample M70) have been inserted into the distance dendrogram using parsimony methods. RR11 (AF179603) was retrieved from rhizosphere soil of a flooded rice microcosm (3). The 16S rDNA sequences of α-proteobacterial type II methanotrophs were used to root the tree. The numbers at the nodes indicate the percentage of recovery in 500 bootstrap resamplings. Only bootstrap values ≥50 are shown. Scale bar, 0.1 substitution per nucleotide sequence position. Database accession numbers of reference organisms are as follows: Methylocystis sp. strain M, U81595; Methylocystis parasum, Y18945; Methylobacter sp. strain BB5.1, AF016981; Methylobacter bosi, L20839; Methylobacter capsulatus, L20843; Methylobacter luteus, M95657; Methylobacter psychrophilus, AF152597; Methylobacter vinelandii, L20841; Methylophilus whittenburyi, X72773; Methylocorbiium agile, X72767; Methylocorbiium album, M95659; Methylocorbiium pelagicum, L35540; Methylocorbiium aurantiaca, X72776; Methylocorbiium methanica, AF50806; Methylocorbiium nodinum, X72778; Methylocorbiium rubra, M95662; Methylocorbiium capsulatum, M95657; Methylocorbiium scituli, UE9300; Methylocorbiium tepidum, U89297; Methylocorbiium capsulatum, L20842; Escherichia coli, V00348; Erwinia carotovora, M95149; Vibrio cholerae, O11197; Pseudomonas flascens, U01916; and Legionella steigerwaltii, X73400.
diversity detectable by T-RFLP analysis was clearly higher than that detectable by DGGE (Fig. 1B versus C). Explanations for this finding might be that sequence types are separated in T-RFLP analysis and DGGE by different methodological principles and staining of DGGE gels is less sensitive than fluorescence detection in T-RFLP analysis. Taking these observations together, it can be concluded that for cultivation-independent assessment of methanotroph diversity pmoA-based T-RFLP analysis represents an important tool to complement the pmoA-based cloning approach and DGGE.

Ecological significance of root-associated methanotrophic diversity. The analysis of phospholipid ester-linked fatty acids (PLFA) recovered from rhizosphere soil of flooded rice microcosms indicated an increased abundance of type I methanotrophs after NH₄⁺ fertilization (ninefold increase in the type-I-specific PLFA biomarker), while the type-II-specific PLFA biomarker increased only two- to threefold after NH₄⁺ fertilization (3). These results led to the conclusion that a high ammonium concentration is essential for growth of type I methanotrophs. In a control experiment based on molecular retrieval of 16S rDNA and DGGE, type I methanotrophs were also detected in rhizosphere soil of unfertilized rice microcosms. However, type I methanotrophs were not detected in the bulk soil of these microcosms. Based on these preliminary data, it was concluded that the rice plant itself also favors growth of type I methanotrophs (3).

The latter conclusion is clearly supported by this study. The T-RFLP profiles suggest a substantially higher relative abundance of type I methanotrophs on rice roots than in the bulk soil (Fig. 2). The detection of rRNA from type I methanotrophs (Fig. 5) provides evidence that these species are metabolically active and thus further supports the idea that rice roots are an important habitat for type I methanotrophs. The promotion of methanotrophic activity by rice plants might be of twofold nature. The diffusional input of oxygen into the root environment directly affects the activities of the obligately aerobic methanotrophs. An indirect effect on NH₄⁺ availability might be mediated by the escape of oxygen from the root tissue into the rhizosphere soil, which can increase the redox potential in the root vicinity and, as a consequence, lead to the desorption of fixed NH₄⁺ ions from clay minerals (51). Increased availability of ammonium should especially favor proliferation of type I methanotrophs (3, 27).

The T-RFLP profiles obtained from rice roots and bulk soil are characterized by high relative signal intensities of the type II methanotroph-specific 245-bp T-RF (35.4 and 67%, respectively). In the anoxic bulk soil, 16S rRNA genes extracted from desiccation-resistant exospires and lipid cysts formed by Methylocaldum spp. and Methylocystis spp., respectively, as well as from vegetative cells present in a stage of anaerobic dormancy (46, 47), might have contributed to the high relative signal intensity.

The cultivation-independent characterization of type I methanotrophs in unfertilized rhizosphere soil by Bodelier et al. (3) resulted in the detection of only one distinct cluster of highly similar 16S rDNA sequence types related to Methylobacter spp. (Fig. 5, sequence type RR11). By contrast, our results of comparative sequence analysis of cloned pmoA and T-RFLP community profiling revealed a more complex population structure encompassing type I methanotrophs affiliated with the genera Methylophilus, Methylobacter, Methylophilus, and Methylococcus and a novel type I methanotroph sublineage. The considerable number of distinct methanotrophic populations that colonized the root compartment is also illustrated by the recovery of various 16S rDNA sublineages of type I methanotrophs. The presence of such a highly diverse methanotrophic community might indicate that there are a large number of ecological micronesches characterized by spatiotemporal variations in the mixing ratios of CH₄ and O₂ (1, 30, 31) and in the availability of nitrogen (3, 24).

The hypothesis that the CH₄/O₂ mixing ratio is an important regulator of root-associated methanotrophic diversity is supported by the close correspondence between mxaF sequence types obtained in a previous study (clones Mo1/Mo2 [31]) and the mxaF cluster detected on rice roots (Fig. 4). Mo1 and Mo2 were detected by pmoA-based DGGE in a methanotrophic consortium enriched from rice field soil under a high CH₄/low O₂ mixing ratio, while enrichment conditions using low CH₄/ high O₂ and low CH₄/low O₂ mixing ratios did not favor growth of these type I methanotrophs.

Final conclusions. The comparison of data obtained by retrieval of pmoA with those obtained by recovery of mmoX, mxaF, and 16S rDNA and rRNA clearly indicates that pmoA represents an excellent functional gene marker for cultivation-independent analysis of methanotrophic diversity. However, the data also indicate that a comprehensive view of methanotrophic diversity can be obtained only by a combined use of various molecular techniques, i.e., by cloning and sequencing and by cloning-independent fingerprinting. Within this framework, the pmoA-based T-RFLP analysis proved to be a suitable tool to rapidly assess methanotrophic diversity. Taking all molecular data together, a highly diverse community of type I and type II methanotrophs was detected. Except for Methylophilus album, type I methanotroph populations were detected in the root environment with clearly higher relative signal intensities than in the bulk soil. Thus, the data as a whole agree well with the hypothesis that type I methanotrophs are predominant in environments that allow rapid growth of methanotrophic bacteria, while type II methanotrophs are more abundant in environments where growth rates are periodically restricted (27, 54).

ACKNOWLEDGMENTS

We are grateful to Sonja Fleissner for excellent technical assistance. This work was supported by a grant from the European Community RTD Programme Biotechnology (contract BIO-CT96-0419). M.T.Y. thanks the Deutschen Akademischen Austauschdienst for financial support in the form of a PhD scholarship.

REFERENCES

1. Amaral, J. A., and R. Knowles. 1995. Growth of methanotrophs in methane and oxygen counter gradients. FEMS Microbiol. Lett. 126:215–220.
2. Auman, A. J., S. Stolar, A. M. Costello, and M. E. Lidstrom. 2000. Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl. Environ. Microbiol. 66:5259–5266.
3. Bodelier, P. L. E., P. Roslev, T. Henczel, and P. Frenzel. 2000. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403:421–424.
4. Bodrossy, L., E. M. Holmes, A. J. Holmes, K. L. Kovacs, and J. C. Murrell. 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, Methylocaldum gen. nov. Arch. Microbiol. 168:993–993.
5. Bosse, U., and P. Frenzel. 1997. Activity and distribution of methane-oxidizing bacteria in flooded rice soil microcosms and in rice plants (Oryza sativa). Appl. Environ. Microbiol. 63:1199–1207.
6. Bowman, J. P., S. A. McCammon, and J. H. Skerratt. 1997. Methylophilus
hansoni gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143:1451–1459.

7. Curry, D. L. N., D. V. Laidler, G. P. C. Salmon, and J. C. Murrell. 1991. Molecular analysis of the methanomonocarboxygenase (MMO) gene cluster of Methylosinus trichosporium OB3b. Mol. Microbiol. 5:333–342.

8. Ciccone, R. J., and R. S. Oremund. 1988. Biogeochemical aspects of atmospheric methane. Global Biogeochem. Cycles 2:299–327.

9. Conrad, R., and T. J. M. Tiedje. 1991. The methanotroph in the soil surface layer of a flooded rice field and the effect of ammonium. Biol. Fertil. Soils 12:28–32.

10. Costello, A. M., and M. E. Lidstrom. 1999. Molecular characterization of functional and phylogenetic genes from natural methanotrophs in lake sediments. Appl. Environ. Microbiol. 65:5066–5074.

11. Dayhoff, M. O., R. M. Schwartz, and B. C. Orcutt. 1978. A model of evolutionary change in proteins, p. 345–352. In M. O. Dayhoff (ed.), Atlas of protein sequence and structure, vol. 5, suppl. 3. National Biomedical Research Foundation, Silver Spring, Md.

12. Dedysh, S. N., N. S. Panikov, and J. M. Tiedje. 1998. Acidophilic methanothanolic communities from Sphagnum peat bogs. Appl. Environ. Microbiol. 64:922–929.

13. Dedysh, S. N., N. S. Panikov, W. Liesack, R. Großkopf, J. Zhou, and J. M. Tiedje. 1998. Isolation of acidophilic methane-oxidizing bacteria from north-chinese peat wetlands. Science 282:281–284.

14. Dedysh, S. N., W. Liesack, V. N. Khmelevina, E. A. Trosenko, J. D. Semrau, A. B. Barnes, N. S. Panikov, and J. M. Tiedje. 2000. Methylotoga palustris gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bog representing a novel subtype of serine-pathway methanotrophs. Int. J. Syst. Evol. Microbiol. 50:955–960.

15. Denier van de Gon, H. A. C., and H. U. Neue. 1991. Methane oxidation in the soil surface of rice microcosms and their effect on porewater methane concentration. FEMS Microbiol. Ecol. 68:1–14.

16. Dedysh, S. N., N. S. Panikov, and J. M. Tiedje. 1998. Isolation of acidophilic methane-oxidizing bacteria from north-chinese peat wetlands. Science 282:281–284.

17. Dedysh, S. N., W. Liesack, V. N. Khmelevina, E. A. Trosenko, J. D. Semrau, A. B. Barnes, N. S. Panikov, and J. M. Tiedje. 2000. Methylotoga palustris gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bog representing a novel subtype of serine-pathway methanotrophs. Int. J. Syst. Evol. Microbiol. 50:955–960.

18. Denier van de Gon, H. A. C., and H. U. Neue. 1991. Methane oxidation in the soil surface of rice microcosms and their effect on porewater methane concentration. FEMS Microbiol. Ecol. 68:1–14.

19. Conrad, R., and F. Rothfuss. 1998. Diversity and structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol. Ecol. 26:25–36.

20. McDonald, I. R., and J. C. Murrell. 1995. Detection of methanotrophic bacteria in environmental samples with PCR. Appl. Environ. Microbiol. 61:116–121.

21. McDonald, I. R., J. R., and J. C. Murrell. 1997. The methyl deuteregenase structural gene mxaF and its use as a functional gene probe for methanotrophs. FEMS Microbiol. Lett. 156:205–210.

22. Osborne, A. M., R. B. Moore, and K. N. Timmis. 2000. Detection and isolation of methanotrophic bacteria possessing soluble methyl-monoxygenase (sMMO) genes using the polymerase chain reaction (PCR). Microb. Ecol. 32:21–31.

23. Saitou, N., and M. Nei. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. J. Mol. Evol. 26:406–425.

24. Sass, R. L. 1994. Short summary chapter for methane, p. 1–8. In H. N. Munro (ed.), Evolution of genes and proteins. Sinauer, Sunderland, Mass.

25. Shigematsu, T., S. Hanada, M. Eguchi, Y. Kamagata, T. Kanagawa, and R. Kurane. 1999. Soluble methane monooxygenase gene clusters from trichloroethylene-degrading Methylomonas sp. strains and detection of methanotrophs surviving in situ bioremediation. Appl. Environ. Microbiol. 65:5199–5206.

26. Watanabe, A., T. Nii, and M. Nei. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

27. Sato, N., and L. J. Shinkets. 1999. Methanotroph diversity in flooded rice soils as affected by redox potential. Eur. J. Agron. 8:179–187.

28. Schütz, H., W. Seiler, and R. Conrad. 1989. Processes involved in formation and emission of methane in rice paddies. Biogeochemistry 7:53–55.

29. Shigenobu, T., T. Nakada, T. Nakagawa, T. Kojima, and K. Kurane. 1999. Soluble methane monooxygenase gene clusters from trichloroethylene-degrading Methylomonas sp. strains and detection of methanotrophs surviving in situ bioremediation. Appl. Environ. Microbiol. 65:5199–5206.

30. Vecherskaya, M. S., V. F. Galchenko, E. N. Sokolova, and V. A. Samarkin. 1992. Climate change, p. 3312–3318. In H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York, N.Y.

31. Wise, M. G., J. V. McArthur, and L. J. Shinkets. 2000. Methanotrophic diversity in flooded rice soils as affected by redox potential. Eur. J. Agron. 8:179–187.

32. Schütz, H., W. Seiler, and R. Conrad. 1989. Processes involved in formation and emission of methane in rice paddies. Biogeochemistry 7:53–55.