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Quantitative Detection of Methanotrophs in Soil by Novel *pmoA*-Targeted Real-Time PCR Assays

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Methane oxidation in soils is mostly accomplished by methanotrophic bacteria. Little is known about the abundance of methanotrophs in soils, since quantification by cultivation and microscopic techniques is cumbersome. Comparison of 16S ribosomal DNA and *pmoA* (α subunit of the particulate methane monooxygenase) phylogenetic trees showed good correlation and revealed five distinct groups of methanotrophs within the α and γ subclasses of *Proteobacteria*: the *Methylococcus* group, the *Methylobacter*/*Methylosarcina* group, the *Methylosinus* group, the *Methylocapsa* group, and the forest clones group (a cluster of *pmoA* sequences retrieved from forest soils). We developed quantitative real-time PCR assays with SybrGreen for each of these five groups and for all methanotrophic bacteria by targeting the *pmoA* gene. Detection limits were between 10^1 and 10^2 target molecules per reaction for all assays. Real-time PCR analysis of soil samples spiked with cells of *Methylococcus capsulatus*, *Methylomicrobium album*, and *Methylosinus trichosporium* recovered almost all the added bacteria. Only the *Methylosinus*-specific assay recovered only 20% of added cells, possibly due to a lower lysis efficiency of type II methanotrophs. Analysis of the methanotrophic community structure in a flooded rice field soil showed $(5.0 \pm 1.4) \times 10^6$ *pmoA* molecules g^{-1} for all methanotrophs. The *Methylosinus* group was predominant $(2.7 \times 10^6 \pm 1.1 \times 10^6$ target molecules $g^{-1})$. In addition, bacteria of the *Methylobacter*/*Methylosarcina* group were abundant $(2.0 \times 10^6 \pm 0.9 \times 10^6$ target molecules g of soil$^{-1})$. On the other hand, *pmoA* affiliated with the forest clones and the *Methylocapsa* group was below the detection limit of 1.9×10^4 target molecules g of soil^{-1}. Our results showed that *pmoA*-targeted real-time PCR allowed fast and sensitive quantification of the five major groups of methanotrophs in soil. This approach will thus be useful for quantitative analysis of the community structure of methanotrophs in nature.

In soils, methane is oxidized and consumed by methane-oxidizing bacteria (MOB) (6). Based on their main substrates, MOB can be divided into two groups, comprising the autotrophic ammonium-oxidizing bacteria (AAOB) and the methane-assimilating bacteria (MAB), the so-called methanotrophs (25). MAB are affiliated with the α (*Methylocystaceae*; type II) and γ (*Methylococcaceae*; type I) subclasses of *Proteobacteria*, whereas most of the AAOB belong to the β subclass (2, 14). The first step and key reaction of methane oxidation is the introduction of a hydroxyl group by methane monooxygenase (MMO). Two different forms of this enzyme exist: a membrane-bound particulate MMO (pMMO), which is present in almost all methanotrophs isolated so far, and the soluble form (sMMO), which has been found in only some methanotrophs (34). The pMMO is homologous to the ammonium monooxygenase (AMO), the key enzyme of the AAOB (23). AMO catalyzes not only the oxidation of ammonia but also that of CH_4, albeit at a much lower activity (2). In the following report, we focus on methanotrophs (MAB).

Most studies either have determined the number of culturable MAB by most-probable-number (MPN) procedures (11) or have used microscopic techniques such as fluorescence in situ hybridization (FISH) (8, 11). Only the latter method allows targeting of different groups within the MAB by using specific DNA probes (12, 13, 24). In rice field soils, for example, FISH and MPN analyses revealed a dominance of the *Methylocystaceae* over type I methanotrophs (11). A recent study using phospholipid fatty acid (PFLA) analysis showed that both type II and type I methanotrophs were abundant in rice fields over the whole vegetation period, although only the abundance of type II MAB was significantly correlated to soil porewater CH_4 concentrations and rice growth (31). PLFA analysis and FISH cannot be applied to investigate the methanotrophic community in soils in more detail due to limited criteria for distinguishing subgroups or due to the great manual effort required (17, 25). Therefore, quantitative studies of methanotrophic communities in soils are hampered by the lack of adequate methods.

A novel methodological approach to quantifying bacterial abundances in the environment is real-time PCR. This method is used to determine the concentration of target DNA in environmental DNA extracts. In contrast to competitive or MPN-PCR, the measurement is not affected by biases of endpoint analysis where different amounts of PCR amplicons are obtained from the same starting quantity (1, 37). Real-time PCR has already been used for quantification of microorganisms in environmental samples, by targeting either 16S ribosomal DNA (rDNA) or functional marker genes (see, e.g., references 1, 20, 36, 43, and 44). The use of *pmoA* (encoding the α subunit of pMMO) as a marker gene for MAB has clear advantages. In a study by Holmes et al. (25), *pmoA* trees showed congruent clusters of methanotrophic genera compared to phylogenies based on 16S rDNA (44). By comparison, 16S rDNA-based assays may also detect nonmethanotrophic bacteria closely related to known MAB. Assays based on *mxaF* (encoding the α

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subunit of methanol dehydrogenase [MDH]) and *mmoX* (encoding the α subunit of the hydroxylase component of sMMO) have the disadvantage that fewer sequences are available, compromising the formulation of specific primers from the databases. In addition, sMMO is present only in some methanotrophs, such as *Methylocella palustris* (9), *Methylococcus* spp., and a few other species of the type I methanotrophs (35). MDH, on the other hand, is present in all known methylotrophs and thus is not restricted to MAB (32).

Therefore, we developed real-time PCR assays targeting the *pmoA* gene in order to analyze the abundances of different groups of methanotrophic bacteria in rice field soil.

MATERIALS AND METHODS

Soil samples. Soil samples were taken from a meadow and rice fields. The meadow soil was located near Giessen, Germany (28). This soil was classified as a Gleysol with the texture of a sandy loam over clay. During the past 50 years the soil has been managed as a meadow and mowed twice a year (28). Samples were taken from the top 10 cm (pH 6.0). The rice field samples were taken from a flooded rice field (*Oryza sativa*, type japonica, variety Korall) at the Italian Rice Research Institute, Vercelli, Italy. Soil characteristics were described by Schütz et al. (38). Soil samples were taken at a distance of ≥20 cm from rice plants and at a depth of 5 to 10 cm.

DNA extraction from soil samples. DNA extraction and purification were performed with the Fast Spin kit (Bio 101, La Jolla, Calif.) in triplicate. The protocol was performed according to the manufacturer's instructions by using a sodium dodecyl sulfate-containing lysis buffer and bead beating. To reduce the humic acid content, DNA extracts were further purified with Sephadex G50 columns (Roche Diagnostics, Mannheim, Germany). DNA extracts were stored in Tris-EDTA (TE) buffer (pH 7.0) at −20°C for further analyses.

Spiking of meadow soil samples. A meadow soil sample from which no *pmoA* could be amplified was chosen. Thus, it was ensured that the measured *pmoA* numbers were retrieved from the cells. An aliquot (4 g) of the soil samples was homogenized in a Stomacher 80 (Sewar Medical Ltd., London, England) at high speed for 1 min. Bacterial numbers of pure cultures of *Methylosinus trichosporium*, *Methylococcus capsulatus*, and *Methylomicrobium album* were determined by microscopy (Axiophot; Zeiss) by analyzing undiluted and 10-fold-diluted bacterial suspensions in a Neubauer cell chamber (Glaswarenfabrik Karl Hecht KG, Sondheim, Germany). Afterwards, 6 ml of a defined mix of these cultures was added to the soil, followed by incubation at room temperature for 1 h. DNA was extracted and purified as described above in four replicates.

Cultivation and DNA extraction of bacterial strains. Bacterial strains were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), the German Culture Collection (DSMZ), or other laboratories (see below). Methanotrophic bacteria were cultivated in 25 ml of ammonium mineral salt medium as modified by Whittenbury et al. (46) in 150-ml gas-tight bottles with a headspace containing 20% (40% for *Methylococcus capsulatus*) CH_4. The cultures were incubated at 25°C (37°C for *Methylococcus capsulatus*) under continuous shaking for 5 days in darkness. Cultures were harvested by centrifugation, and the cell pellet was resuspended in 500 μl of phosphate-buffered saline (pH 7.0). DNA was extracted according to the protocol published by Henckel et al. (16). This procedure was used for *Methylococcus capsulatus* (NCIMB 11853), *Methylocaldum gracile* (NCIMB 11912), *Methylobacter luteus* (NCIMB 11914), *Methylomicrobium album* (NCIMB 11123), *Methylomonas methanica* (NICIMB 11130), *Methylocystis parvus* (NCIMB 11129), *Methylosinus sporium* (NCIMB 11126), and *Methylosinus trichosporium* (NCIMB 11131).

DNA was extracted from lyophilized cells of *Methylopila helvetica* (DSM 6342), *Methylobacterium extorquens* (DSM 1337), and the ammonia-oxidizing strain *Nitrosomonas europaea* (NCIMB 11850) by heating for 10 min at 100°C in 25 μl of TE buffer (pH 7.2), followed by centrifugation (for 10 min at 14,000 rpm in an Eppendorf 5417R centrifuge).

Genomic DNA solutions of the following species or strains were provided by other sources: *Methylocapsa acidiphila* B2 (S. Dedysh, Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia), *Nitrosospira tenuis* (H.-P. Koops, University of Hamburg, Hamburg, Germany), *Nitrosococcus oceani* strain 9 (H.-P. Koops), and *Escherichia coli* (Roche Diagnostics). All DNA solutions were stored at −20°C until use.

A *pmoA* sequence related to published forest clone sequences, MForest (AJ496664), was obtained from a deciduous forest near Marburg, Germany, as previously described by Henckel et al. (17). The *pmoA* gene was amplified from a soil DNA extract with the universal primers A189 forward (with GC clamp) and A682 reverse. The mixed PCR products were separated by denaturing gradient gel electrophoresis (DGGE). An individual band was excised with sterile needles and reamplified by PCR for sequencing according to the work of Henckel et al. (16). The phylogenetic affiliation of this *pmoA* sequence, MForest (165 amino acid residues; 495 bp), was analyzed by calculating a distance matrix. The amino acid sequence was identical to the *pmoA* sequence with accession number AF368372. Furthermore, MForest had high identities (96.8 and 98.7%) to the forest clones Rold5 (AF148527) and RA14 (AF148521), respectively. The closest relation to a bacterial isolate was that to *Methylocapsa acidiphila* B2 (70.1% identity). The reamplified PCR product of MForest was stored at −20°C.

Primer development and phylogenetic reconstructions. To design *pmoA* group-specific primers, the "Probe design" tool of the ARB software package (42) was used in a database consisting of *pmoA* and *amoA* nucleic acid and translated protein sequences. This database contained all publicly available *pmoA* sequences (updated April 2002 from GenBank) and 48 additional type II-related sequences (21), for a total of 354 sequences.

The following primers were used for the assays presented in Table 1: A189 F (GGN GAC TGG GAC TTC TGG) (23), Mb661 R (GGT AAR GAC GTT GCN CCG G) (7), II223 F (CGT CGT ATG TGG CCG AC) (this study), Mb601 R (ACR TAG TGG TAA CCT TGY AA) (this study), Mc468 R (GCS GTG AAC AGG TAG CTG CC) (this study), II646 R (CGT GCC GCG CTC GAC CAT GYG) (this study), Mcap630 R (CTC GAC GAT GCG GAG ATA TT) (this study), and Forest675 R (CCY ACS ACA TCC TTA CCG AA) (this study).

To analyze the phylogeny of MOB, quartet PUZZLE trees were calculated (41) using the PHYLIP software package (version 3.6a2; J. Felsenstein, Department of Genetics, University of Washington, Seattle; available at http://evolution.genetics.washington.edu/phylip.html). Trees were confirmed by maximum-likelihood analysis (FastDNAML, ARB software package, and PROTML, Institute Pasteur [http://bioweb.pasteur.fr/seqanal/interfaces/molphy.html]). For the PUZZLE tree based on 16S rDNA, the HKY model (Hasegawa substitution model [14a]) and a 50% base frequency filter resulting in 1,221 valid nucleotide positions were used. The *pmoA*- and *amoA*-based tree was calculated with the VT (33a) evolution model using 103 valid positions of amino acid residues. Substitution rates of each site were estimated from the data sets. In the puzzling step of both trees, 2,000 replicated trees were created and reconstructed as "unrooted."

Sequences of the following organisms were obtained from the GenBank sequence database (http://www.ncbi.nlm.nih.gov/GenBank), aligned with the alignment tools of the ARB software package, and used for phylogenetic reconstructions (GenBank accession numbers of 16S rRNA [first number] and of *pmoA* or *amoA* DNA sequences [second number] are given in parentheses): *Methylomicrobium album* (X72777/U31654), *Methylomicrobium pelagicum* (L35540/U31652), *Methylomonas methanica* (AF304196/U31653), *Methylobacter* sp. strain BB5.1 (AF016981/AF016982), *Methylocaldum szegediense* (U89300/U89303), *Methylocaldum gracile* (U89298/U89301), *Methylocaldum tepidum* (U89297/U89304), *Methylococcus capsulatus* (X72771/L40804), *Methylococcus thermophilus* (X73819/not available), "*Methylothermus*" strain HB (U89299/not available), *Methylosphaera hansonii* (U67929/not available), *Nitrosococcus oceani* (M96395/AF047705), *Nitrosococcus halophilus* (none/AF272521), *Methylosarcina fibrata* (AF177296/AF177325), *Methylosarcina quisquiliarum* (AF177297/identical with *pmoA* sequence of *Methylosarcina fibrata*), *Methylocystis parvus* (M29026/U31651), *Methylocystis* sp. strain LW5 (not available/AF150791), *Methylosinus sporium* (M95665/AF458994), *Methylosinus trichosporium* Ob3b (M29024/S81887), *Beijerinckia indica* (M59060/no pMMO), *Methylocella palustris* (Y17144/has only sMMO), *Methylocapsa acidiphila* (AJ278726/AJ278727), *pmoA* DNA (forest clone) sequences of Rold5 (AF148527) and Maine6 (AF148528), *Nitrosomonas europaea* (AF353160/Z97861), *Nitrososmonas communis* (Z46981/AF2399), *Nitrosomonas halophila* (Z46987/AF2398), and *Nitrosospira tenuis* (M96405/U76552).

Real-time PCR assays. Data analysis was carried out with iCycler software (version 2.3.1370; Bio-Rad) as described by Stubner (43). The cycle at which the fluorescence of a certain target molecule number exceeded the background fluorescence (threshold cycle [C_T]) was determined from dilution series of target DNA with defined target molecule amounts. C_T was proportional to the logarithm of the target molecule number. Thus, a C_T measured in a sample could be converted to a target molecule number. More details of real-time PCR are explained by Raeymaekers (37) and Suzuki et al. (44). PCR was performed in 40-μl volumes using Thermo-fast 96 PCR plates (PeqLab, Erlangen, Germany), which were sealed with iCycler IQ optical quality tapes (Bio-Rad) on an iCycler IQ thermocycler (Bio-Rad). Each measurement was performed in four replicates.

TABLE 1. Conditions of real-time PCR assays for quantitative detection of MAB

Assay	Forward/reverse primer	Concn[a] of forward/reverse primer (nM)	Target group	Length of amplicon (bp)	Annealing temp (°C)	Data acquisition temp (°C)
MBAC	A189 F/Mb601 R	667/667	*Methylobacter*/*Methylosarcina* group	432	54.0	82.0
MCOC	A189 F/Mc468 R	1,000/333	*Methylococcus* group	299	64.0	82.0
TYPEII	II223 F/II646 R	667/667	*Methylosinus* group	444	69.5	83.0
MCAP	A189 F/Mcap630	667/667	*Methylocapsa*	461	68.7	82.0
FOREST	A189 F/Forest675 R	1,000/1,000	Forest clones	506	67.0	82.0
MTOT	A189 F/Mb661 R	667/667	*Methylobacter*/*Methylosarcina, Methylococcus, Methylosinus* group, *Methylocapsa, Nitrosococcus*,	491	65.5	NA[b]

[a] Final concentration.
[b] NA, not applicable; no differentiation of primer dimers and amplicon was possible.

Five microliters of DNA template was added to 35 μl of Master mix containing 4 μl of PCR buffer (Invitrogen), 3.2 μl of $MgCl_2$ (final concentration, 4 mM; Invitrogen), 4 μl of fatty acid-free bovine serum albumin (5 μg/μl; Sigma-Aldrich), 4 μl of mixed deoxyribonucleoside triphosphates (2 μM each; PeqLab), 0.4 μl of SybrGreen I (500-fold diluted in H_2O), 0.4 μl of each primer (MWG Biotech AG, Boersberg, Germany) (see Table 1 for final concentrations), 0.32 μl of PLATINUM DNA polymerase (5 U/μl; Invitrogen), and 18.7 μl of double-distilled water (Sigma-Aldrich). The addition of bovine serum albumin as described by Kreader (29) reduced inhibition by humic substances.

Assays were performed with a four-step thermoprofile: denaturation of DNA (25 s at 94°C), annealing of primers under stringent conditions (20 s at an assay-specific temperature) (Table 1), elongation (45 s at 72°C), and fluorescence data acquisition during an additional temperature step (10 s at a temperature above the melting point of primer dimers). The latter, assay-specific temperature was determined and verified by melting curve analysis (data not shown). As calibration standards for the real-time PCR assays, dilution series of positive-control DNA were used. DNAs from *Methylococcus capsulatus* (MCOC), *Methylomicrobium album* (MBAC and MTOT), *Methylosinus trichosporium* (TYPEII), *Methylocapsa acidiphila* (MCAP), and the DGGE band Mforest (FOREST) were the targets for *pmoA*-specific PCR. The positive-control DNA extracts were amplified with the assay primers (Table 1). The resulting amplicons were purified with a Qiaquick PCR purification kit as recommended by the manufacturer (Qiagen, Hilden, Germany) and cloned into pGEM-T. After reamplification with vector-specific primers according to the manufacturer's instructions (Promega, Madison, Wis.), the PCR products were purified as described above. The PCR products obtained were then quantified with the PicoGreen dsDNA quantitation kit (Molecular Probes, Leiden, The Netherlands) using fluorimetry. The measured DNA amount could be converted to target molecule numbers per microliter, and the *pmoA* standards were adjusted to 10^{10} target molecules μl^{-1} for storage at −20°C.

Before quantification, the DNA extracts were tested for inhibitory effects of coextracted substances by determining *pmoA* target molecule numbers in dilution series of environmental DNA extracts according to the work of Stubner (43). The lowest dilution not inhibited was used for further measurements (also discussed in reference 43).

Statistical data analysis. A pairwise *t* test was performed to evaluate significant differences between target molecule and cell numbers. Calculations were performed with Excel, version 7.0 (Microsoft).

RESULTS

Phylogeny of methanotrophic *Proteobacteria*. Since additional methanotrophic genera have been described since the study of Holmes et al. (25), we compared the phylogenies of *pmoA* amino acid sequences and 16S rDNA sequences. For this analysis we used mainly MOB, for which both *pmoA* and 16S rDNA sequences are presently available in databases. The phylogenetic trees based on these two genes are very similar (Fig. 1). For *Methylosphaera hansonii* only 16S rDNA sequences were available. Despite the fact that *Methylocella palustris* is the most closely related MAB, it cannot be included in this group because it does not posses a pMMO (9). The forest clones represent a *pmoA* sequence cluster retrieved only from environmental studies. Therefore, no 16S rDNA sequences are known.

The 16S rDNA tree (Fig. 1A) revealed four groups within the α and γ subclasses of *Proteobacteria*. To the α subclass belong the *Methylosinus* group (classical type II; *Methylocystaceae*) (14, 30) and the *Methylocapsa* group. The methanotrophic bacteria of the γ subclass of *Proteobacteria* include the *Methylobacter*/*Methylosarcina* group (*Methylosarcina*, *Methylomonas*, *Methylobacter*, *Methylomicrobium*, and *Methylosphaera*; classical type I methanotrophs) and the *Methylococcus* group (*Methylococcus*, *Methylocaldum*, and "*Methylothermus*" strain HB (3). Within the *Methylobacter*/*Methylosarcina* group the genera *Methylobacter* and *Methylomicrobium* could not be clearly affiliated, resulting in a tree with multiple branchings. In agreement with the description of Bowman et al. (5), *Methylosphaera* belongs to the *Methylobacter*/*Methylosarcina* group.

The phylogenetic analysis of *pmoA* amino acid sequences showed the same four groups as that of the 16SrDNA sequences (Fig. 1B). However, a fifth group comprising the forest clones was found in addition. This group affiliates with *pmoA* of MAB within the α subclass of *Proteobacteria*. It consists only of molecularly retrieved sequences from oxic forest soils (17, 25). On the basis of the combined phylogenetic analyses, all MAB can be divided into the *Methylococcus* group, the *Methylobacter*/*Methylosarcina* group, the *Methylosinus* group, the *Methylocapsa* group, and the forest clone group.

***pmoA* targeted real-time PCR assays.** Real-time PCR assays were developed to detect the phylogenetic groups defined in Fig. 1B by using *pmoA* as a marker gene (Table 1). For most assays the forward primer A189F, which aligns with all known *pmoA* sequences, was chosen. Group-specificity was achieved for most assays by designing group-specific reverse primers (Table 1). A general assay for MAB, the MTOT assay, was established using primer Mb661R (7).

The Probematch analysis in ARB showed that the MTOT assay detects almost all methanotrophic bacteria but not the sequences of *Methylomonas*, *Methylocaldum*, or the forest clones. MTOT furthermore excludes all known *amoA* sequences of AAOB (except for *Nitrosococcus*). MBAC targets all sequences within the *Methylobacter*/*Methylosarcina* group. The closest related nontarget organisms are *Methylocaldum* spp. (three mismatches), while for *Methylosphaera hansonii* no sequence data are available. The target group of MCOC is

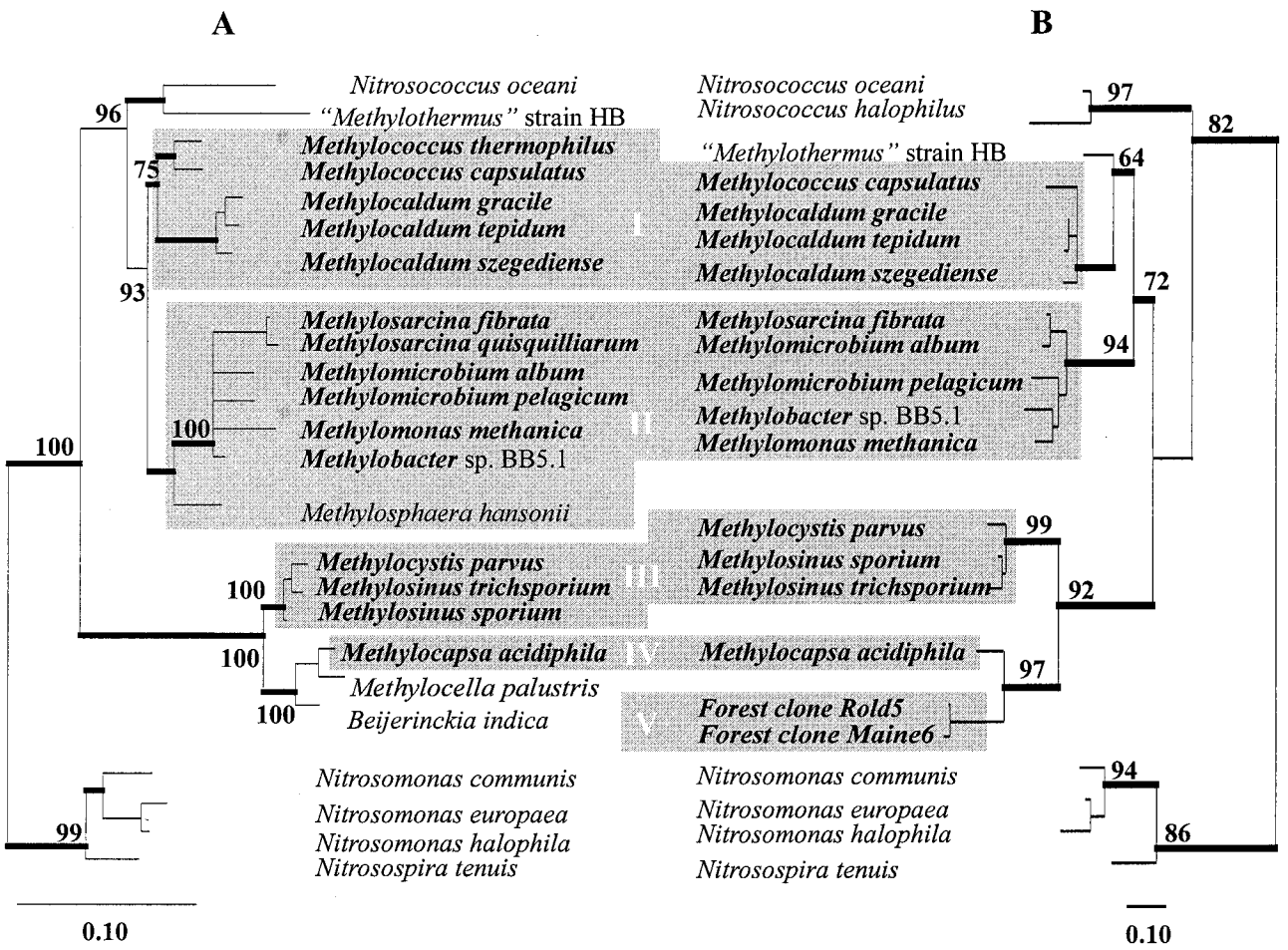

FIG. 1. Comparison of phylogenies based on 16S rDNA and *pmoA* and *amoA*. Trees A (16S rDNA) and B (*pmoA*) are quartet PUZZLE trees. Thick branches are those confirmed by maximum-likelihood analysis. Bars represent 10% sequence divergence. Shaded areas indicate groups defined in the text (I, *Methylococcus* group; II, *Methylobacter/Methylosarcina* group; III, *Methylosinus* group; IV, *Methylocapsa* group; V, forest clones). Names of species targeted by the real-time PCR assays are boldfaced.

the *Methylococcus* group. The closest nontarget organism is a *Methylosinus*-affiliated sequence (one mismatch). The TYPEII assay covers all sequences affiliated with the *Methylosinus* group. The closest nontarget sequences belong to forest clones (two mismatches). The MCAP assay detects the *pmoA* sequence of *Methylocapsa acidiphila* strain B2. It has two mismatches to sequences of the *Methylosinus* group and the *Methylobacter/Methylosarcina* group. The FOREST assay detects sequences from the forest clone sequence group. It has at least three mismatches to sequences of the *Methylococcus* group and of *Methylocapsa*. Recently, potential paralogues of *pmoA* have been found in several methanotrophic strains (10). Probe-match analysis in ARB revealed that the assays developed discriminate against these sequences.

For each assay, primer concentrations and annealing temperatures were experimentally optimized to obtain specific amplification (Table 1). The resulting conditions were experimentally checked by using genomic DNAs of positive- and negative-control strains. Amplification of PCR products of the correct sizes was obtained only from target organisms, not from the nontarget organisms tested (Table 2). Detection limits were determined from at least four independent measurements from dilution series of positive-control DNA (10^7 to 10^1 target molecules per reaction). A minimum sensitivity of 10^1 to 10^2 target molecules per reaction for each assay was achieved.

To measure the impact of nonspecific genomic DNA on the real-time PCR measurement, target DNA was mixed with DNA of nontarget organisms. A total of 5×10^6 or 5×10^4 target molecules of assay MBAC were mixed with genomic DNA of *E. coli* (330 ng μl^{-1}) and, in a second case, with DNA of *Methylococcus capsulatus* (30 ng μl^{-1}). As a control the same amount of target molecules was measured without the addition of genomic DNA. It was found that genomic DNA from *E. coli* had no effect on the measurement of target DNA, even if there were 100-fold more genomes than target molecules in the assays (data not shown). The presence of genomic DNA of the methanotrophic nontarget organism also did not influence the measurement (data not shown).

Correlation of target molecule numbers with cell numbers. To evaluate the correlation of *pmoA* target molecule numbers with cell numbers, a soil was spiked with a mixed methanotrophic culture with defined numbers of cells. The DNA extracts were afterwards analyzed with assays TYPEII, MCOC, and MBAC (Table 3). We assume that at least 2 copies of *pmoA*

TABLE 2. Specificity of the real-time PCR assays[a]

Organism	Result[b] for the following assay:					
	MCOC	MBAC	TYPEII	MCAP	FOREST	MTOT
Methylococcus capsulatus	+	−	−	−	−	+
Methylocaldum gracile	+	−	−	−	−	−
Methylobacter luteus	−	+	−	−	−	+
Methylomicrobium album	−	+	−	−	−	+
Methylomonas methanica	−	+	−	−	−	−
Methylocystis parvus	−	−	+	−	−	+
Methylosinus sporium	−	−	+	−	−	+
Methylosinus trichosporium	−	−	+	−	−	+
Methylocapsa acidiphila	−	−	−	+	−	+
DGGE band affiliated with forest clones	−	−	−	−	+	−
Methylopila helvetica	−	−	−	−	−	−
Methylobacterium extorquens	−	−	−	−	−	−
Nitrosococcus oceani strain 9	−	−	−	−	−	+
Nitrospira tenuis	−	−	−	−	−	−
Nitrosomonas europaea	−	−	−	−	−	−
Escherichia coli	−	−	−	−	−	−

[a] Assays were tested with genomic DNA extracts from a set of methanotrophic and other *Proteobacteria* in at least two independent experiments.
[b] −, no signal after 45 cycles; +, signal.

per cell can be expected (39). With this assumption, cell numbers can be calculated from target molecule numbers. Measurements of four independent DNA extracts with the MCOC and MBAC assays resulted in cell numbers that were not statistically different from the numbers of cells added prior to DNA extraction (Table 3). However, the number of *Methylosinus trichosporium* cells determined by the TYPEII assay was equivalent to only 20% ($P < 0.01$) of the number of cells added (Table 3).

Analysis of the methanotrophic community in samples from a flooded rice field. Soil samples of a flooded rice field were extracted in triplicate and analyzed by real-time PCR (Table 4). The community analysis showed that bacteria of the *Methylosinus* group (type II MAB) and of the *Methylobacter*/*Methylosarcina* group (type I MAB) were the dominant MAB in the soil samples investigated (Table 4). MAB of the *Methylococcus* group, on the other hand, were less abundant (about 6% of all MAB detected). MAB of the *Methylocapsa* group and forest clones were below the detection limit ($<1.9 \times 10^4$ *pmoA* molecules g of soil^{-1}). The sum for the groups of MAB detected ($5.0 \times 10^6 \pm 1.4 \times 10^6$ *pmoA* molecules g^{-1}) was significantly higher ($P < 0.05$) than that obtained with the MTOT assay ($0.9 \times 10^6 \pm 0.3 \times 10^6$ *pmoA* molecules g^{-1}).

DISCUSSION

The community ecology of methanotrophs is important for understanding the contribution of distinct MAB to methane oxidation in a natural habitat. Existing methods such as the MPN technique or quantitative FISH are biased by selective culture conditions or require too much manual effort to obtain statistically significant values in soil, where numbers of MAB are typically on the order of <1% of total bacteria. Therefore, the development of real-time PCR assays targeting MAB is a promising way to elucidate the structure of methanotrophic communities and to evaluate the role of specific groups (taxocenes, defined by Hutchinson [27]) of MAB with respect to their ecological function. The congruence of *pmoA*- and 16S rDNA-based phylogenies is a precondition for using *pmoA* as a target for the molecular quantification of methanotrophs.

With the MBAC, MCOC, TYPEII, MCAP, and FOREST assays, it is possible to detect the known diversity of MAB with the exceptions of *Methylocella* and "*Methylothermus*." All the assays discriminate against homologous *amoA* sequences (23) of the nitrifying *Proteobacteria* and against nonspecific background sequences. MTOT was designed to discriminate especially against AAOB and detects most, but not all, methanotrophs (Table 2).

SybrGreen (SG) was used as the detection system as discussed by Stubner (43). SG allows application of any primer system to real-time PCR without developing further probes. Of course, all assays have to be checked and optimized for specificity (annealing temperature, correct PCR products). The detection limits of the assays we designed are similar to those of probe-based assays such as TaqMan or frequence resonance energy transfer probe assays (see, e.g., references 4, 44, and 45). Hein et al. (15) compared an SG protocol with a TaqMan assay using the same primer set. They observed a relatively lower sensitivity in the SG assay. Therefore, they preferred the probe-based approach, to avoid the codetection of primer

TABLE 3. Recovery of *pmoA* from soil spiked with pure cultures[a]

Recovered pure culture (assay)	Mean no. of target molecules in individual extraction[b]				No. of:		
	1	2	3	4	Target molecules	Cells[c]	
						Calculated	Added
Methylosinus trichosporium (TYPEII)	0.9 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1[d]	1.5 ± 0.3 ($n = 11$)
Methylococcus capsulatus (MCOC)	1.5 ± 0.5	4.6 ± 0.8	2.2 ± 0.6	3.1 ± 0.6	2.9 ± 0.7	1.4 ± 0.3[e]	1.6 ± 0.2 ($n = 13$)
Methylomicrobium album (MBAC)	0.7 ± 0.2	0.7 ± 0.1	0.2 ± 0.1	0.9 ± 0.1	0.6 ± 0.2	0.3 ± 0.1[e]	0.4 ± 0.1 ($n = 13$)

[a] Soil was spiked with cells of *Methylosinus trichosporium*, *Methylococcus capsulatus*, or *Methylomicrobium album*.
[b] All values are means ± SEs and represent target molecules or cells (10^7) per milliliter of spiked soil slurry.
[c] Numbers of cells were calculated from mean molecule target numbers; 2 *pmoA* copies per cell were assumed.
[d] Calculated cell numbers deviated significantly from added cell numbers ($P < 0.01$).
[e] Calculated numbers of cells were not significantly different from added numbers of cells ($P > 0.005$).

TABLE 4. Analysis of the methanotrophic community structure by real-time PCR in a flooded rice field soil

Methanotrophic group (assay)	Target molecules[b] in rice field soil (Vercelli, 1999)	Subclass of *Proteobacteria*
Total methanotrophs (MTOT)[a]	0.9 (±0.3)*	α and γ
Methylosinus group (TYPEII)	2.7 (±1.1)	α
Methylocapsa group (MCAP)	ND	α
Forest clones (FOREST)	ND	α
Methylococcus group (MCOC)	0.3 (±0.1)	γ
Methylobacter/*Methylosarcina* group (MBAC)	2.0 (±0.9)	γ
Sum of groups	5.0 (±1.4)*	α and γ

[a] Measurement with MTOT gives a result significantly different from the sum of group-specific assays ($P < 0.05$).

[b] Expressed as 10^6 target molecules g (fresh weight) of soil^{-1} (±SE). ND, not detectable; $<1.9 \times 10^4$ targets g of soil^{-1}. Asterisks indicate that numbers deviate statistically significantly ($P < 0.05$).

dimers. In our study, it was possible to discriminate against the primer dimer fluorescence by acquiring data at a temperature above the melting point of these by-products. This temperature can be determined and verified by melting curve analysis (data not shown). Therefore, it was possible to reach sensitivities of 10 target molecules per reaction.

Cell numbers determined by real-time PCR analysis in spiked soil samples showed a good match with the numbers of added cells. Theoretically, *pmoA* copy numbers should be twice as high as cell numbers because of the existence of 2 *pmoA* copies per cell (39, 40). Furthermore, growing cells might have more than 1 genome copy per cell, thus further increasing the *pmoA* gene copy number in relation to the cell number. On the other hand, a lysis efficiency presumably below 100% should result in underestimation. For example, *Methylosinus* is well known to resist standard lysis techniques, probably because of capsule material (14, 33). This might provide an explanation for the low bacterial numbers found with the TYPEII assay. Measurements with the MCOC and MBAC assays, on the other hand, showed a good correlation with added cell numbers. In these measurements, the biases mentioned apparently were not important or cancelled each other out within the accuracy of the measurements. The standard error (SE) of the analysis of the spiked soil was relatively high (up to 33%). However, the SE of a single real-time PCR measurement was in general not higher than the SE in four replicate DNA extracts.

Determination of the MAB community structure in rice field soil identified the *Methylosinus* group (type II methanotrophs) [$(2.7 \pm 1.1) \times 10^6$ *pmoA* molecules g^{-1}] and members of the *Methylobacter*/*Methylosarcina* group (type I methanotrophs) [$(2.0 \pm 0.9) \times 10^6$ *pmoA* molecules g^{-1}] as the predominant groups (Table 4). Several studies have already evaluated the diversity of methanotrophs in rice field soil, including comparative sequence analysis of ribosomal and functional phylogenetic marker genes as well as terminal restriction fragment length polymorphism, FISH, MPN, and PFLA analyses (16, 18, 19, 22, 26, 31). All these data indicate that MAB of the *Methylosinus* group and the *Methylobacter*/*Methylosarcina* group are the predominant methanotrophs in rice field soils. Our study, however, is the first accurate quantification of MAB in general and of specific taxocenes of MAB. Such a quantification was not possible in a rice field soil by FISH or other methods. All MAB groups together represented about 5×10^6 *pmoA* molecules g of soil^{-1}, equivalent to about 2.5×10^6 MAB g of soil^{-1}. This number represents about 0.6% of the total community of *Bacteria* in the soil (43). The general assay MTOT detected significantly ($P < 0.05$) less MAB [$(0.9 \pm 0.3) \times 10^6$ *pmoA* molecules g^{-1}] than the sum of all group-specific assays (Table 4). This may be due to the fact that MTOT does not target all known MAB (Table 2) and thus may result in underestimation of total cell numbers. *Methylomonas* (type I)-affiliated sequences, for example, which were found on roots of submerged rice plants (26), are detected only by the MBAC assay, not by the MTOT assay (Table 2). Hence, we think the sum of the subgroup-specific assays better reflects the real number of methanotrophs in this soil.

In conclusion, the newly developed *pmoA*-targeted real-time PCR assays may facilitate detection of MAB in soils and other environments. It is possible to analyze the structure of the methanotrophic community by distinguishing five distinct groups, even when MAB are present at a relatively low abundance. The MAB community structure in rice field soil, for example, consisted of type I and type II MAB, which together represented only 0.6% of the total bacterial community.

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