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Improved Method for Detection of Methanotrophic Bacteria in Forest Soils by PCR

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Abstract. A primer set was designed for the specific detection of methanotrophic bacteria in forest soils by PCR. The primer sequences were derived from highly conservative regions of the *pmoA* gene, encoding the α-subunit of the particulate methane monooxygenase present in all methanotrophs. In control experiments with genomic DNA from a collection of different type I, II, and X methanotrophs, it could be demonstrated that the new primers were specific for members of the genera *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylobacter*, and *Methylococcus*. To test the suitability of the new primers for the detection of particulate methane monooxygenase (pMMO) containing methanotrophs in environmental samples we used DNA extracts from an acid spruce forest soil. For simple and rapid purification of the DNA extracts, the samples were separated by electrophoresis on a low-melting-point agarose gel. This allowed us to efficiently separate the DNA from coextracted humic acids. The DNA from the melted agarose gel was ready for use in PCR reactions. In PCR reactions with DNA from the Ah soil layer, products of the correct size were amplified by PCR by use of the new primers. By sequencing of cloned PCR products, it could be confirmed that the PCR products represented partial sequences with strong similarity to the *pmoA* gene. The sequence was most related to the *pmoA* sequence of a type II methanotroph strain isolated from the Ah layer of the investigated soils.

It is estimated that about 15–45 Tg y^{-1} atmospheric methane are oxidized in aerobic soils, which amounts to 3–10% of the global CH_4 emission [12, 26]. Microbial CH_4 oxidation is the only known biogenic sink for atmospheric CH_4 [1, 12]. Although biological oxidation of atmospheric CH_4 is widely distributed in different types of upland soils, the responsible organisms are still unknown. A deeper insight into the community structure of soil microorganisms was often limited by the inability to cultivate them. Methanotrophic bacteria responsible for the oxidation of atmospheric methane belong to the uncultivated fraction of soil microorganisms. Molecular biology techniques offer some good opportunities to overcome the limitations for identification of non-cultivable CH_4 oxidizers in natural environments. A promising genetic target for identification of cultivable and non-cultivable CH_4 oxidizers in soils is the membrane-bound methane monooxygenase, which is present in all type I, II, and type X methanotrophs [18]. The pMMO consists of three subunits of 47, 27, and 25 kDa [19, 23] encoded by the *pmoA*, *pmoB*, and *pmoC* genes. The aim of this study was to use the increasing database of *pmoA* gene sequences from cultured and uncultured methanotrophs to design a new primer set which showed enhanced specificity and ability for the detection of known methanotrophs and novel relatives by PCR. It was intended to test the suitability of the new primers with soil from a spruce forest. According to the general knowledge about the occurrence of methanotrophic bacteria in natural environments, forest soils are an unusual habitat for methanotrophs. However, forest soils from the site of this study showed a high capacity to oxidize atmospheric methane [24]. Since the genes encoding the particulate methane monooxygenase and the ammonia monooxygenase share high sequence identity [11, 23], we had to design specific primers for the identification of pMMO-carrying bacteria, suitable to distinguish strictly between methanotrophs and ammonia-oxidizing bacte-

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ria. Moreover, it was aimed to provide an efficient method for the extraction and purification of genomic DNA from forest soils. The techniques were established to facilitate in situ analysis of methanotrophic communities in forest soils participating in the consumption of atmospheric methane.

Materials and Methods

Soil. Experiments were carried out with soil collected from the Ah layer (5 cm of the upper mineral layer) in a stand dominated by *Picea abies* trees located in the Black Forest near the town of Villingen, Germany. The soil is an acid brown earth with pH values between 2.4 and 3.3 in the organic layer (L, Oh, Of) and between 3.2 and 3.7 in the uppermost mineral soil layers (Ah, AhBv) [2]. After sampling, the soils were sieved (3-mm mesh width) and stored at 4°C in the dark until time of analysis.

Bacterial strains and culture conditions. *Methylococcus capsulatus* (NCIMB 11132), *Methylobacter whittenbury* (NCIMB 11128), *Methylomonas methanica* (NCIMB 11130), and *Methylocystis parvus* (NCIMB 11129) were obtained from the National Collections of Industrial and Marine Bacteria LTD (NCIMB). The methanotrophic strain ID 97-540 was isolated from the Ah soil layer (upper 5 cm of the mineral soil) of the experimental site. This strain is closely related (sequence identity 97.5%) to a type II methanotroph designated as AML-A6 (GenBank accession number AF177299), which was revealed by sequencing partial 16S rDNA and database comparison. The isolates *Methylosinus trichosporium* OB3b, *Methylosinus sporium* and *Methylocystis spec*. were provided by J. Heyer (now at Max-Planck-Institut für Terrestrische Mikrobiologie, Marburg, Germany). Methanotrophic isolates were cultured in a mineral medium at 28°C in an atmosphere containing 20% (vol/vol) CH_4, as previously described [10]. Non-methanotrophic bacteria used in this study were: (i) a methylotrophic bacterial strain isolated from the Ah soil layer of the experimental site. The isolate was classified by partial 16S rDNA sequence analysis (99.9% identity) as *Methylobacterium extorquens*. The isolate was cultured in mineral medium supplemented with 125–250 mM CH_3OH. (ii) The ammonia oxidizer *Nitrosolobus multiformis* AL (NCIMB 11849). Cultivation was performed as recommended in the catalog of strains of the National Collections of Industrial and Marine Bacteria LTD. (iii) *Pseudomonas putida* (DSMZ-1088-260). The strain was grown as described previously [7]. (iv) *Escherichia coli TG1* [25]. The strain was cultivated in LB medium [20].

Construction of a *pmoA* gene primer set. To design PCR primers, known *pmoA* sequences available in GenBank database (accession numbers: AF150803, AF150791, AF150787, AF177328, AF006046, U31651, AF150795, U31653, AF016982, U31654, U89301, U89303, U94337) were aligned and compared by using the Clustalw (1.8) program. From highly conservative regions in the *pmoA* gene the following oligonucleotide sequences were derived. *pmoA*for: 5′-TTCTGGGG(AGCT)TGGAC(AGCT)TA(CT)TT(CT)CC-3′; *pmoA*rev: 5′-TC(AGCT)ACCAT(AGCT)C(GT)(AGT)AT(AG)TA(CT)TC(AGCT)GG-3′. Synthesized oligonucleotides (Roth, Karlsruhe, Germany) were used for PCR in a thermocycler (Biometra, Göttingen, Germany) with 36 cycles (1 min 94°C, 1 min 50°C, 1 min 72°C) and an additional extension step at 72°C for 10 min. The reaction volume was 50 µl containing 2.5 U Tac-polymerase (Gibco BRL), 1 × PCR buffer (Gibco BRL), 5.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide, and about 50 ng of template DNA. Parallel control reactions with the eubacteria-specific primers 27f and 1492r [9] were carried out to assess whether the used template DNA was suitable for PCR amplification. For control reactions the annealing temperature was increased to 60°C.

DNA extraction from soil samples. A rapid and efficient method for direct extraction of genomic DNA from forest soils was developed by combining previously described methods [16, 17, 22] . The main steps were mechanical lysis of the soil bacteria by bead beating, followed by hot SDS lysis. The purification of the extracts was achieved by simple electrophoresis on a low-melting-point agarose gel. Fresh and homogenized soil samples (0.3 g) were weighed in 2-ml reaction tubes and mixed with 0.8 ml extraction buffer (2 × TENS: 100 mM Tris/HCl [pH 8], 40 mM EDTA, 200 mM NaCl, 2% SDS) that contained 0.6 g glass beads (Sigma, Deisenhofen, Germany) of different diameter (100 µm, 600 µm, and 1200 µm). For mechanical cell disruption, the reaction tubes were secured horizontally on a vortex and shaken at maximum speed for 10 min. Afterwards the mixtures were incubated for 10 min at 70°C. The reaction tubes were centrifuged for 10 min at 9000 *g* and 4°C (Eppendorf centrifuge 5402). The supernatants were transferred to a clean reaction tube. The pellets were resuspended in 0.8 ml extraction buffer and centrifuged again (9000 *g*, 4°C). The DNA in the combined supernatants was precipitated by the addition of 1/10 volume of sodium-acetate and 2.5 volume ethanol. The resulting pellets were resuspended in 100 µl aqua dest. Caused by the presence of large quantities of humic acids, the color of the suspensions was deep brown. For quick and simple purification, the samples were separated by gel electrophoresis in portions of 20 µl on a 1% low-melting-point agarose gel. The ethidium-bromide-stained DNA was visualized under a UV lamp. The DNA concentration of the bands was estimated by comparing them with reference bands of a High DNA Mass™ Ladder (Gibco BRL). The agarose-embedded DNA bands were excised from the agarose gel. The DNA-block was melted at 70°C and used directly in the PCR reactions. The quality of the genomic DNA was always tested in PCR reactions with the eubacteria-specific primer pair 27f, 1492r [9]. The described DNA extraction method was evaluated by performing soil DNA extraction with a commercial available soil DNA isolation kit (Mo Bio, Solana Beach, CA, USA). Soil sample size used for DNA extraction with this kit was 0.25 g.

Cloning, sequencing, and DNA isolation from bacterial strains. DNA isolation, transformation, and restriction analysis were performed by conventional techniques [20]. PCR products were ligated into the pCR 2.1 vector by using the TA cloning Kit (Invitrogen, Carlsbad, CA). Plasmids were isolated from selected clones with the Qiagen plasmid mini purification kit (Qiagen, Hilden, Germany). Both strands of the inserts were sequenced by using cycle sequencing dideoxy chain termination reactions with Big Dye Terminators (PE Applied Biosystems, Weiterstadt, Germany). The sequences were analyzed on an ABI PRISM-System 310 (PE Applied Biosystems).

Comparative and phylogenetic PCR product analysis. DNA sequences were compared with *pmoA* sequences available in GenBank database by using the NCBI (National Center for Biotechnology Information) tool BLAST (Basic Local Alignment Search Tool). Afterwards, the DNA sequences were aligned to representative sequences with close relation, by using the Clustalw 1.8 program. Phylogenetic trees were constructed by using the Paup 3.0 program.

Results and Discussion

In order to identify a broad selection of type I, II, and X methanotrophs by PCR, a new primer set for targeting the *pmoA* gene was designed. Initially, *pmoA* amino acid sequences from cultured and uncultured methanotrophs

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Methylosinus sp. PW1                VNFWGWTYFPISLVFPSALVVPALWLDIILLLSGSYVITAVVGALGWGLLFYPNNWPTIAAFHQATEQHGQLMSLADLIGLHFVRTSMPEYIRMVERG
Methylocystis sp. LW5               VNFWGWTYFPISLVFPSALIVPAIWLDVILLLSGSYVITAVVGSLGWGLLFYPNNWPAIAAFHQATEQHGQLMTLADLIGFHFVRTSMPEYIRMVERG
Methylosinus sp. LW2                VNFWGWTYFPISLVFPSALIVPAIWLDVILLLSGSYVITAVVGSLGWGLLFYPNNWPAIAAFHQATEQHGQLMTLADLIGLHYVRTSMPEYIRMVERG
type II meth. AML-A6                VNFWGWTYFPISLVFPSALIVPAIWLDVILLLSGSYVITAIVGSLGWGLLFYPNNWPAIAAFHQATEQHGQLMTLADLIGFHFVRTSMPEYIRMVERG
unidentified eubacterium            VNFWGWTYFPISLVFPSALIVPAIWLDVILLLSGSYVITAVVGSLGWGLLFYPNNWPAIAAFHQATEQHGQLMSLADLIGLHFVRTSMPEYIRMVERG
Methylocystis parvus                VNFWGWTYFPISLVFPSALIVPAIWLDVILLLSGSYVITAVVGSLGWGLLFYPNNWPAIAAFHQATEQHGQLMTLADLIGLHYVRTSMPEYIRMVERG
Methylomonas sp. LW15               MNFWGWTYFPVNICFPSNLLPGAIVLDVILMMGNSMTLTAVVGGLAYGLLFYPGNWPVIAPLHVPVEYNGMMMTLADLQGYHYVRTGTPEYIRMVEKG
Methylomonas methanica              MNFWGWTYFPVNICFPSNLLPGAIVLDVILMLGNSMTLTAVIGGLAYALLFYPGNWPVIAPLHVPVEYNGMMMTLADLQGYHYVRTGTPEYIRMVDKG
Methylobacter sp. B5.1              LNFWGWTYFPVNFCFPSNLMPGAIVLDVVLMLSNSMTLTAVVGGMAWGLLFYPGNWPIIAPLHIPVEYNGMMFTLADLQGYHYVRTGTPEYIRMVEKG
Methylobacter albus                 FNFWGWTYFPVNFVFPSQFAPGAIVLDVILMLSNSMQLTAVLGGLAYGLLFYPGNWPVIAPLHVPVEYNGMVMTLADLQGYHYVRTGTPEYIRMVEKG
Methylocaldum gracile               FNFWGWTYFPINFVFPAVLVPGAILLDTFLMLSGSYLFTAIVGGMAWGLVFYPGNWPMIAPLHVPVEYNGMLMSIADIQGYNYVRTGTPEYIRMVEKG
Methylocaldum szegediense           FNFWGWTYFPINFVFPALLVPGAILLDTFLMLSGSYLFTAIVGGLAWGLIFYPSNWPMIAPLHVPVEYNGMLMSIADIQGYHYVRTGTPEYIRMVEKG
Methylococcus capsulatus            FNFWGWTYFPINFVFPASLVPGAIILDTVLMLSGSYLFTAIVGAMGWGLIFYPGNWPIIAPLHVPVEYNGMLMSIADIQGYNYVRTGTPEYIRMVEKG
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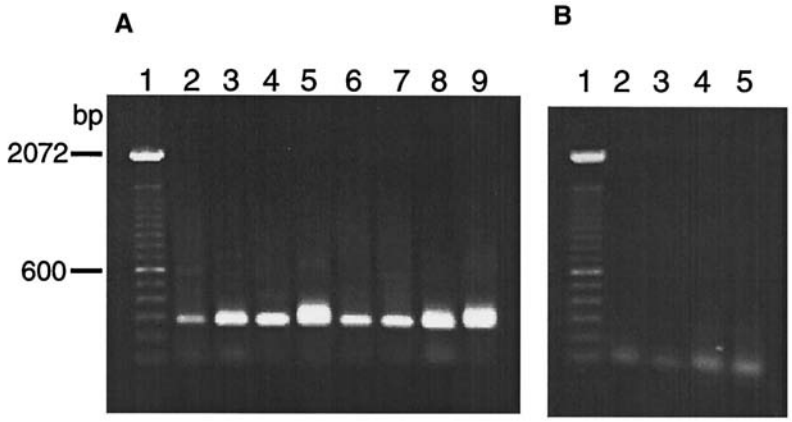
Fig. 1. Partial alignment of the deduced amino acid sequences of *pmoA* genes. Sequences shown in the alignment are from *Methylosinus* sp. PW1 (AF150803), *Methylosinus* sp. LW5 (AF150791), *Methylosinus* sp. LW2 (AF150787), type II methanotroph AML-A6 (AF177328), unidentified eubacterium (AF006046), *Methylocystis parvus* (U31651), *Methylomonas methanica* (U31653), *Methylobacter* sp. B5.1 (AF016982), *Methylobacter albus* (U31654), *Methylocaldum gracile* (U89301), *Methylocaldum szegediense* (U89303). The calculated (ClustalW version 1.8) identities (*), conservative exchanges (:), and similarities are shown below the alignment. Regions that were selected for the primer design are inverted.



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                                    pmoAfor (5´-3´)          pmoArev (5´-3´)
                                    TTCTGGGGNTGGACNTAYTTYCC CCNGARTAYATHMGNATGGTNGA
Nitrosolobus multiformis            TCTTTGGCCC-GACCCACCTGCC CCTGAGTACGTGCGCCTGATCGA
Nitrosospira sp. L13                TCTTCGGGCC-GACCCACCTGCC CCTGAGTACGTGCGACTGATCGA
Nitrosospira briensis               TCTTTGGCCC-GACCCACCTGCC CCCGAGTACGTACGCAACATCGA
Nitrosomonas sp. TK794              TCTTTGGCCC-GACCCATCTGCC CCTGAGTATGTTCGTCATATTGA
Nitrosomonas eutropha               TCTTTGGCCC-GACCCATCTGCC CCTGAGTATGTTCGTCATATTGA
Nitrosomonas halophila              TTTTTGGACC-GACCCACCTGCC CCGGAATACACCCGTCTGATCGA
Nitrosomonas marina Nm22            TATTTGGTCC-GACCCATCTGCC CCGGAGTATGTGAGGCTGATTGA
Nitrosomonas urea                   TTTTTGGTCC-AACCCACTTGCC CCGGAATATGTTCGCCTGATTGA
Nitrosococcus mobilis               TTTTTGGACC-AACCCATTTGCC CCCGAGTATGTTCGTCATATTGA
Nitrosomonas europaea               TTTTTGGACC-AACCCATTTGCC CCCGAGTATGTTCGTCATATTGA
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Fig. 2. (A) Amplification products supplied by PCR with template DNA from different type I, II, and X methanotrophs, using the *pmoA* for–*pmoA*rev primers. 1 = 100 bp ladder (Gibco BRL, Berlin, Germany); 2 = *Methylococcus capsulatus*; 3 = *Methylomonas methanica*; 4 = *Methylobacter whittenbury*; 5 = type II methanotroph ID 97-540; 6 = *Methylosinus sporium*; 7 = *Methylocystis* sp.; 8 = *Methylocystis parvus*; 9 = *Methylosinus trichosporium*. (B) Results from PCR with template DNA from different non-methantrophs used as negative controls. 1 = 100 bp ladder, 2 = *E. coli*, 3 = *Pseudomonas putida*, 4 = *Methylobacterium extorquens*, 5 = *Nitrosolobus multiformis*. (C) Comparison of the *pmoA* primer sequences (*pmoA*for and *pmoA*rev) with the nucleotide sequences of *amoA* genes. Partial gene sequences are from *Nitrosolobus multiformis* (AF042171), *Nitrospira* sp. L13 (AJ238542), *Nitrospira briensis* (U76553), *Nitrosomonas* sp. TK794 (AB031960), *Nitrosomonas eutropha* (U51630), *Nitrosomonas halophila* (AJ238541), *Nitrosomonas marina Nm22* (AJ388587), *Nitrosomonas urea* (AJ388585), *Nitrosococcus mobilis* (AF037108) and *Nitrosomonas europaea* (AF037107). Sequence identities are inverted.

obtained from the GenBank database were aligned, and highly conserved regions were identified (Fig. 1). From these regions a degenerative forward 23mer and a degenerative reverse 23mer were derived (Methods). The expected amplification product is a 281-bp segment of the *pmoA* gene. The specificity of the new primers was

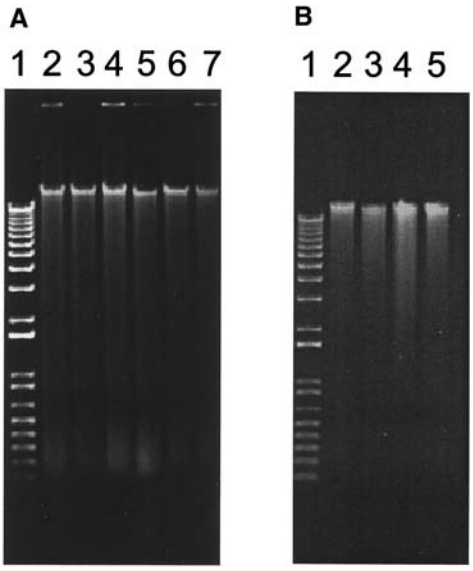

Fig. 3. DNA isolated from forest soil. (A) LMP Agarose gel (1%) of genomic DNA extracted by the newly developed method. Lanes: 1 = 1 kbp ladder (Gibco BRL, Berlin, Germany); 2–4 = genomic DNA extracted from the organic layer (three independently taken soil samples); 5–7 = genomic DNA extracted from the Ah layer (three independently taken soil samples). (B) LMP Agarose gel (1%) of genomic DNA extracted by using the soil DNA isolation kit. Lanes: 1 = 1 kbp ladder; 2–3 = genomic DNA extracted from the Ah soil layer (two independently taken soil samples); 4–5 = genomic DNA extracted from the organic layer (two independently taken soil samples).

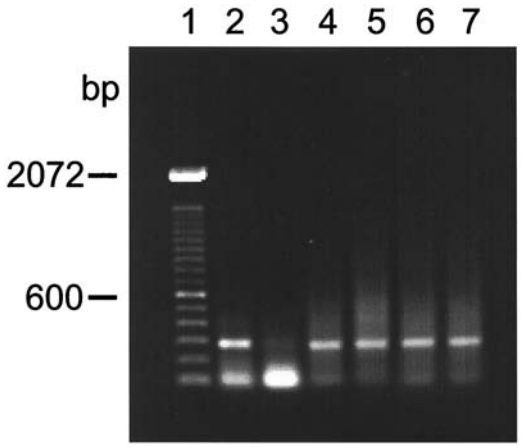

Fig. 4. Amplification products derived from PCR with template DNA from forest soil using the *pmoA*for–*pmoA*rev primers. Lanes: 1 = 100 bp ladder (Gibco BRL, Berlin, Germany); 2 = template DNA from the Ah soil layer; 3 = template DNA from the organic layer; 4–7 = template DNA obtained from four independent extractions of soil from the Ah layer.

tested in PCR reactions with genomic DNA from a selection of well-characterized control organisms (Fig. 2a and 2b). PCR products of the correct size (281 bp) were obtained with template DNA from all selected type I, II, and X methanotrophs (Fig. 2a). By sequence analysis it was confirmed that the PCR products represent partial sequences of the *pmoA* gene. It was proven that PCR with template DNA from the negative control organisms *E. coli*, *Pseudomonas putida*, *Methylobacterium extorquens* and the ammonia oxidizer *Nitrosolobus multiformis* did not yield any amplification products (Fig. 2b). To establish that the new primers were suitable to distinguish between *pmoA*-containing methanotrophs and *amoA*-containing nitrifiers, an alignment between the primer sequences and the available *amoA* sequences was performed (Fig. 2c). Similarities between the reverse primer sequence and *amoA* gene sequences reached values up to 87%. But the level of similarity to the forward primer sequence was only 65% at most. The fact that template DNA from *Nitrosolobus multiformis* supplied no PCR products (Fig. 2b) proved that the nucleotide differences are sufficient to exclude unspecific amplification of *amoA* sequences.

After the successful use of the newly designed primers in PCR reactions with known methanotrophs, it was intended to test the new primers with template DNA from a forest soil that shows CH_4 oxidation activity [24]. For efficient and rapid DNA extraction, we used a protocol that was established by combining and modifying previously described methods [16, 17, 22]. The major extraction steps were based on mechanical lysis by bead beating with a vortex, followed by a chemical hot SDS lysis. The color of the DNA extracts that were obtained after a final ethanol/sodium-acetate precipitation step was deep brown, caused by the presence of excessive amounts of humic acids. The contaminants were coextracted together with the DNA. In order to obtain template DNA with sufficient purity for PCR, we separated the extracts by gel electrophoresis in a low-melting-point agarose gel (Fig. 3A). From the brown smear in the gel it appears that the humic acids migrate faster than the DNA. A comigration of humic acids and DNA, as mentioned by [27], cannot be completely excluded, but was negligible, because it did not interfere with the following procedures. Therefore, no polyvinylpyrrolidone (PVPP) was added to agarose gels to remove humic acids by the formation of PVPP-humic acids complexes [27]. The resulting DNA bands (>15 kb in size) were excised from the gel, avoiding smeared areas of sheared DNA. The DNA yield varied for soil samples from the organic layer between 4 and 7 μg DNA g^{-1} soil (fresh weight), and for samples from the Ah-layer between 1.5 and 4 μg DNA g^{-1} soil (fresh weight). The quality of the DNA from melted agarose blocks was tested in PCR with the eubacterial primers 27f and 1492r [9]. PCR with DNA from both soil layers supplied 16S rDNA amplification products of the expected size and of relatively high quantity,

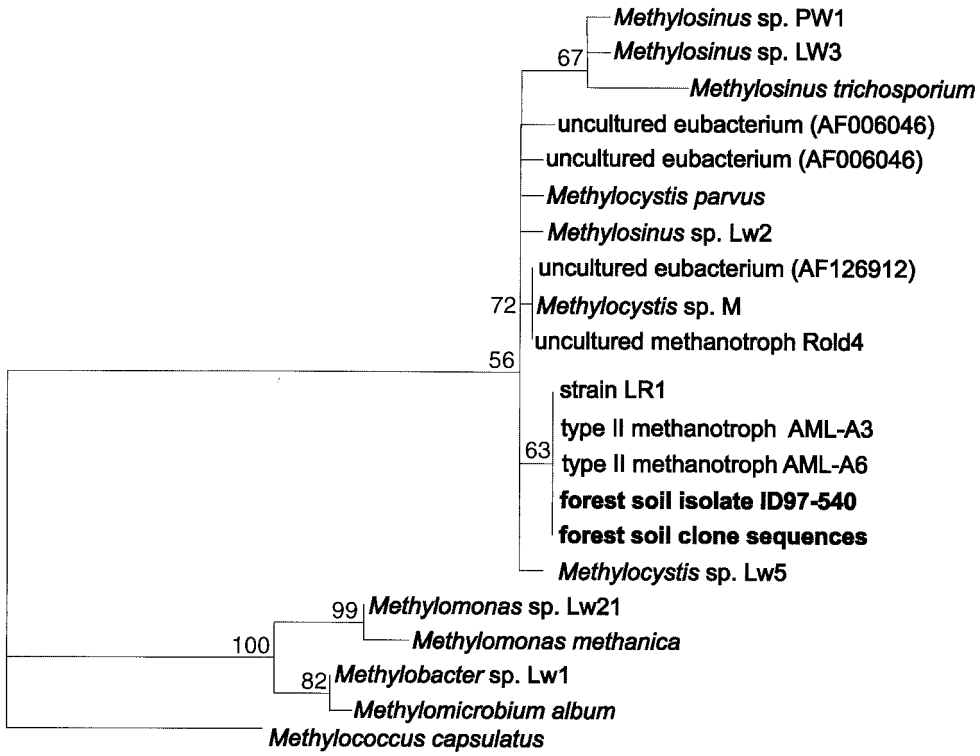

Fig. 5. Phylogenetic tree based on the deduced amino acid sequences of *pmoA* genes. The tree shows the relationship between (a) the *pmoA*-product obtained from isolate ID 97-540 (forest soil isolate ID 97-540) and *pmoA*-products derived from DNA sequences obtained by PCR with template DNA extracted from the Ah soil layer and (b) the *pmoA* products of other methanotrophs. The length of the tree branches is proportional to percentage dissimilarity in amino acid sequences. The numbers on the branches represent the bootstrap values from 100 replications.

suggesting that no or only little inhibition of PCR by agarose or other remaining contaminants took place. To evaluate the efficiency and purity of the established DNA extraction/purification method, we compared it to the yield and purity of a commercially available soil DNA isolation kit (Mo Bio, Solana Beach, CA, USA). Although the application of this kit achieved DNA yields in a comparable range (yield 1.7–3.6 μg DNA g^{-1} Ah-soil layer) as the newly developed method, the DNA extracted from soil with the soil DNA isolation kit was more degraded (Fig. 3B). The band of the high nuclear band was less sharp in the case of the DNA purified by the kit than when using the newly developed extraction method (Fig. 3A). Nevertheless, the DNA extracts obtained with the kit could also be used in PCR and resulted in amplification products of the expected size. Both methods can be completed in less than 3 h including the DNA purification/quality check by electrophoresis. However, since the newly developed extraction method resulted in sharp, high, nuclear DNA bands (Fig. 3A), it was considered to be the more advanced method and, thus, was used for the further investigations.

The application of the new *pmoA* primers resulted always in PCR products of the expected size when DNA from the Ah layer was used as template DNA (Fig. 4). In contrast to the eubacterial primers 27f and 1492r, the *pmoA* primers generate no PCR amplification products from template DNA obtained from the organic layer. This result is in good agreement with the observation that soil from the organic layer showed in general no or only minor CH_4 oxidation activity [24]. That the organic layer is of minor importance for the oxidation of atmospheric CH_4 was also observed by others [4–6, 14, 15, 21].

A number of *pmoA*-derived PCR products (n = 6) were selected for DNA cloning, followed by sequence analysis. The obtained clone sequences were closely related, but with one exception not identical to each other. The level of identity varied between 96.8% and 99.3%. These differences are too large to result from amplification errors by the Taq-polymerase. It is more likely that a number of closely related methanotrophs exist in the Ah soil layer. The partial *pmoA* sequences were 59.5%–98.2% identical to previously described *pmoA* sequences. The highest similarity (97.2–98.9% identity) was found to a *pmoA* sequence of a type II methanotroph designated as ID 97-540, isolated from the

Ah soil layer of the experimental site [24]. This methanotrophic strain, the only one we can isolate in pure culture from the Ah soil layer, is most related (sequence identity 97.9%) to an unidentified type II methanotroph designated as AML-A6 (GenBank accession number AF177299). On the basis of the deduced amino acid sequence, the dendrogram shown in Fig. 5 was constructed. The partial amino acid sequence of the soil clones showed 100% identity to the *pmoA* of the methanotrophic forest isolate ID97-540, as well as to the *pmoA* sequences of the methanotrophs designated as AML-A6, AML-A3, and LR1 (Y18443). Kinetic experiments on CH_4 oxidation by the isolate LR1 have given first evidence of the existence of conventional methanotrophs exhibiting high-affinity methane oxidation [8]. The level of identity to members of the two genera *Methylocystis* and *Methylosinus* varied between 91% and 98%. A distinct weaker similarity (at most 63%) was detected to the *pmoA* gene products of type I methanotrophs. The similarity to *amoA* sequences of ammonia-oxidizing nitrifiers was at best 54%, confirming that the used *pmoA* primers were indeed specific for methanotrophs. The fact that the six *pmoA* sequences were very similar to each other and to the methanotrophic forest soil strain ID97-540 may suggest that the investigated forest soils contained a methanotrophic community of low diversity, dominated by ID97-540-related bacteria in the Ah soil layer. Therefore, it cannot be excluded that ID97-540-related bacteria play a major role in atmospheric CH_4 oxidation in the investigated spruce forest soils. With the new *pmoA* primer set, we provide a valuable tool for further investigations concerning the structure of methanotrophic bacterial communities in environmental samples as well as investigations concerning the contribution of ammonia-oxidizing bacteria in the consumption of atmospheric CH_4 via unspecific CH_4 oxidation by *amoA* [3, 13]. Furthermore, soil sample processing with the newly established method for soil DNA extraction allows simple and rapid isolation and purification of DNA from small quantities of soil and makes it possible to screen a large number of soil samples in less time.

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