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EXPERIMENTAL ARTICLES

Molecular Analysis of High-Affinity Methane-Oxidizing Enrichment Cultures Isolated from a Forest Biocenosis and Agrocenoses

I. K. Kravchenko[1], A. K. Kizilova, S. A. Bykova, E. V. Men'ko, and V.F. Gal'chenko

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

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Abstract—Methane oxidation by microorganisms inhabiting aerobic soils is a key process involved in the regulation of the concentration of this significant greenhouse gas in the atmosphere; however, the microorganisms responsible for this process remain unknown. Three stable methane-oxidizing cultures were isolated from samples of forest soils (FS) and agricultural soils (AS) of Moscow oblast, as well as from soil samples collected from a Belgian agrocenosis (BS). The obtained enrichment cultures exhibit a high affinity for methane; their k_m values range from 54.2 to 176.8 nM CH_4 and are comparable to those of aerobic soils. Analysis of the fragments of the ribosomal (16S rRNA) and functional (*pmoA*) genes of methanotrophs by PCR–DGGE and cloning demonstrated the presence of bacteria belonging to the genera *Methylocystis* in FS, *Methylosinus* in AS and BS, and *Methylocella* in BS. It was established that *Methylocystis* and *Methylosinus* detected in the enrichment cultures contain the genes encoding the synthesis of the active center of two membrane-bound particulate methane monooxygenases; it is likely that one of these genes (*pmoA2*) is responsible for the capacity of these microorganisms for oxidation of atmospheric methane.

Key words: aerobic soils, methanotrophs, enrichment cultures, in situ hybridization, PCR, DGGE, *pmoA2*.

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Methane is one of the most important greenhouse gases, and its content in the atmosphere is determined by the global balance between its production and oxidation. Despite the fact that the CH_4 concentration in the atmosphere is 200 times lower than the concentration of CO_2, the processes of methane turnover have aroused considerable scientific interest, which can be attributed to its significant greenhouse effect. Calculations suggest that a twofold increase in CH_4 content in the atmosphere may cause a global increase in temperature of 0.2–0.4°C [1]. The activity of methanotrophic communities inhabiting aerobic soils is the only known biological mechanism for the oxidation of atmospheric methane, which determines the key role of these microorganisms in the regulation of the global methane budget [2]. It was established that soils from various ecosystems absorb atmospheric methane [3–6], and the net methane sink is 20–60 Tg CH_4 per year or about 10% of the global methane sink [7].

To assess the functional role of methanotrophs in natural ecosystems, the data on their numbers and diversity are required. Previous investigations of methanotrophic microorganisms inhabiting aerobic soils were based on cultivation [8] and application of immune sera [9], as well as on various molecular techniques for analysis of the fragments of the ribosomal (16S rRNA) and functional (*pmoA, mmoX*, and *mxaF*) marker genes of methanotrophs [5, 10–12].

In all known methanotrophs, except for *Methylocella*, the first stage of methane metabolism (oxidation to methanol) is carried out by the membrane-bound methane monooxygenase (pMMO). Phylogenies based on the sequence analysis of the *pmoA* gene encoding the α subunit of this enzyme are virtually the same as those based on the 16S rRNA gene analysis. Thus, *pmoA* sequences may be efficiently used for determining the taxonomic position of methane-oxidizing organisms to the genus, and sometimes to the species, level [11]. Analysis of the *pmoA* gene fragment is widely used to assess the diversity of methanotrophs in various natural ecosystems, including methane-oxidizing aerobic soils. Methanotrophic bacteria of the genera *Methylocystis, Methylosinus, Methylomonas, Methylobacter, Methylomicrobium, Methylococcus*, and *Methylocaldum* [5, 9, 10, 12], as well as the specific nonculturable methanotrophs that form the clusters USCα and USCγ [5, 10], were detected in the studied aerobic soils by molecular biological techniques. It is assumed that the latter are specialized oligotrophic organisms that are able to grow only due to the oxidation of atmospheric methane. The sequences of the acidophilic methanotroph *Methylocapsa acidiphila* [13] are closest to the *pmoA* sequences of the representatives of the cluster USCα ("forest sequence cluster").

The goal of the present work was to apply molecular biological techniques to characterization of the methane-oxidizing enrichment cultures that were iso-

[1] Corresponding author; e-mail: irinakravchenko@inbox.ru

lated from aerobic soils of a forest biocenosis and two agrocenoses.

MATERIALS AND METHODS

Isolation and maintenance of enrichment cultures. Enrichment cultures of methane-oxidizing microorganisms were isolated from the gray forest soil of a forest biocenosis and the arable soil of the winter wheat agrocenosis (Pushchino, Moscow oblast), as well as from the loamy arable soil of the corn agrocenosis (Melle, Belgium). Detailed characteristics of these sites are presented in [6] and [14], respectively. To obtain primary enrichment cultures, aliquots (0.1 g) of soil were placed in 500-ml serum vials with 50 ml of mineral medium [15] and incubated under static conditions in the air : methane atmosphere (95 : 5) at 22°C for 4–8 weeks. Subsequent transfers of the obtained cultures were carried out under the same conditions; the inoculum dose was 5% (vol/vol). By the beginning of the experiments, three stable (more than 50 transfers, homogeneous growth of mixed colonies on agarized media, microscopic control) enrichment cultures were isolated from the forest (FS) and arable soils of Russia (AS) and Belgium (BS).

Determination of methane-oxidizing activity. The cells of 7-day enrichments were harvested by centrifugation at 4°C and washed three times with fresh medium with agitation in order to remove dissolved methane. Then, the suspension with $OD_{600} = 0.6 \pm 0.02$, equivalent to about 10^8 cells/ml, was prepared using the same medium. The aliquots (10 ml) of the suspension and chloramphenicol solution (to the final concentration of 50 μg/l) were transferred to 120-ml vials and injected with methane at mixing ratios of 10, 20, 50, 100, and 200 ppm (14, 28, 70, 140, and 280 nM CH_4, respectively). Each measurement was performed in five replicates. During 14 days of incubation, CH_4 was measured at 12- to 24-h intervals using a Kristall 2001 gas chromatograph (Russia) equipped with a flame ionization detector. The rate of methane oxidation was estimated by linear regression of methane mixing ratios versus time. The kinetic parameters of methane oxidation (k_m) were calculated using the Michaelis–Menten equation.

Fluorescence in situ hybridization. Detection of methanotrophs in enrichment cultures was carried out by fluorescence in situ hybridization (FISH). Fixation of the samples with a paraformaldehyde solution and hybridization of the samples with fluorescently labeled oligonucleotide probes were carried out at 46°C according to the protocol described in [16]. The mixture of universal probes EUB 338mix was used for detection of representatives of the domain *Bacteria* [17]. For specific detection of type II methanotrophs, the probe M-450 (30% formamide in the hybridization buffer) was used; type I methanotrophs were detected using a mixture of the probes M-84 and M-705 (20% formamide) [18]. The set of Cy3-labeled oligonucleotide probes applied in this study was synthesized by Syntol Co. (Russia). The total number of bacterial cells was determined in slides stained with a solution of DAPI, a DNA-specific fluorescent dye. The cells that responded positively to the probes were enumerated under an AxioImager D1 epifluorescence microscope (Carl Zeiss, Germany) equipped with Zeiss 20 and Zeiss 49 light filters for the Cy3-labeled probes and DAPI, respectively, with subsequent conversion per 1 ml of enrichment culture.

DNA extraction and amplification of the *pmoA* and 16S rRNA gene fragments. DNA was extracted from the cells using a Wizard Genomic DNA Purification Kit (Promega, United States) according to the manufacturer's protocol with minor modifications. For the enhancement of microbial DNA yield from dormant bacterial cells, the cells were treated with the lysing buffer at high temperature (80°C) and agitation.

PCR amplification of the *pmoA* gene was performed using a system consisting of the degenerate oligonucleotide primers A189F (5'-GGNGACTGGGACTTCTGG-3') and A682R (5'-GAASGCNGAGAAGAASGC-3') [19]. Further separation of the amplicons by DGGE was performed using the A189F primer containing a G+C clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CGG-3') [20]. The reaction mixture (25 μl) contained the following: 1× PCR buffer ($(NH_4)_2SO_4$, 17 mM; Tris–HCl, 67 mM; $MgCl_2$, 2 mM; pH 8.8), 50 mmol of each dNTP, 25 pmol of each primer, and 0.6 U of *Taq* DNA-polymerase (Helicon Co., Russia). The PCR cycle parameters were as follows: 94°C for 3 min; 94°C for 30 s and 62°C for 30 s, 20 cycles with a subsequent decrease in temperature by 1°C every new cycle; 72°C for 45 s; and final elongation for 5 min at 72°C. Amplification was performed on a MyCycler thermal cycler (BioRad, United States).

For amplification of the 16S rRNA gene fragments of methanotrophs, two-round nested PCR amplification was carried out in accordance with the protocol described in [21]. Initially, the primers targeting the specific 16S rRNA gene fragments of type I and type II methanotrophs (MB10γ and MB9α, respectively) and the universal primer P518,were used. The PCR products obtained were then used as a template for amplification with the universal primers P338-G+C and P518.

Analysis of the PCR products was carried out by electrophoresis in 1.2% agarose gel stained with ethidium bromide. PCR fragments were isolated and purified using the Wizard PCR Preps kit (Promega, United States) according to the manufacturer's recommendations.

Denaturing gradient gel electrophoresis, cloning, and sequencing. Amplicon separation by denaturing gradient gel electrophoresis (DGGE) was carried out using the DCode Universal Mutation Detection System (BioRad, United States) at a constant temperature of 60°C. The *pmoA* gene fragments (three repli-

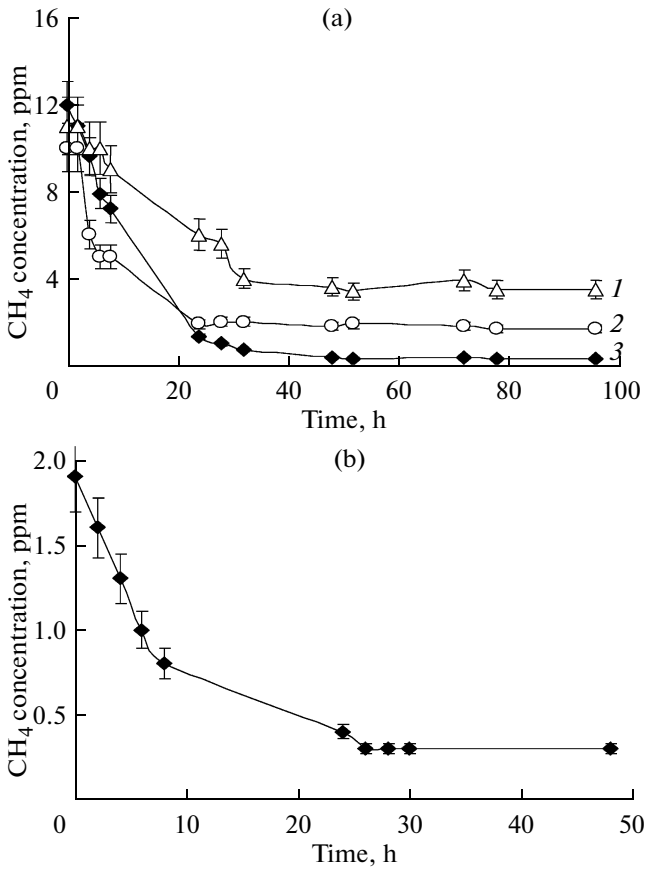

Fig. 1. Dynamics of methane oxidation at the initial concentration in the gas phase of 10–12 ppm (a) by the AS (*1*), BS (*2*), and FS (*3*) enrichment cultures, and (b) of the oxidation of atmospheric methane at a CH_4 concentration of 1.8 ppm by the FS enrichment culture.

cates) were separated at a constant voltage of 200 V in a denaturing gradient (formamide and urea, 35–60%) for 6 h. The 16S rRNA gene fragments (two replicates) were separated at 40 V in a denaturing gradient from 50 to 65% for 16 h. Each repeat of DGGE analysis was an individual amplification product. The gels were stained with ethidium bromide (*pmoA*) or SYBR Gold (16S rRNA genes) and digitalized with a Gel Doc gel imaging and documentation system (BioRad, United States). The similarity coefficients between the samples stained with SYBR Gold were calculated using the TREECONW software package. Dominant bands were eluted; the solutions were purified and used to determine the nucleotide sequences.

Cloning of the *pmoA* gene fragments was performed using the pGEM-T easy vector system I (Promega, United States) in competent *E. coli* DH10B cells. For each clone library, 50 colonies exhibiting a positive reaction (white) were randomly chosen. PCR analysis of the colonies was carried out using the universal plasmid primers M13F and M13R. To determine which colony contained an insert, agarose gel electrophoresis was carried out.

Sequencing of the PCR products was performed in the service laboratory using the Big Dye Terminator v.3 sequencing kit (Applied Biosystems Inc., United States). The clones and DGGE bands with *pmoA* fragments were sequenced using the A682R primer; DGGE bands containing the 16S rRNA fragments were sequenced with the universal primers P338 and P518. Nucleotide sequence analysis was conducted in an ABI Prism 3100 automatic sequencer (Applied Biosystems Inc., United States).

Analysis of nucleotide sequences. Preliminary analysis of the obtained nucleotide sequences of the gene fragments was performed using the NCBI BLAST software package [http://www.ncbi.nlm.nih.gov/BLAST]. The ORF Finder software package [http://www.ncbi.nlm.nih.gov/gorf/gorf/html] was used for translation of the nucleotide sequences of the *pmoA* gene fragments. The nucleotide sequences and the deduced amino acid sequences of the studied genes were edited and aligned with the appropriate sequences from the closest relatives using the BioEdit software package [http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html]. The phylogenetic tree was constructed on the basis of deduced amino acid sequences of the *pmoA* gene fragments by the methods implemented in the TREECONW software package [http://www.uia.ac.be/u/yvdp/treeconw. html].

Deposition of the nucleotide sequences. The *pmoA1* and *pmoA2* gene fragments obtained in this work were deposited in the GenBank under accession numbers FJ805256, FJ805257, FJ807557, and FJ807558.

RESULTS AND DISCUSSION

Methane-oxidizing activity of enrichment cultures. To obtain enrichment cultures, they were cultivated in an atmosphere of a gas mixture containing methane (5% by volume). The methane concentration was four to ten times lower than that usually required for the isolation of enrichment cultures of methanotrophs. The authors are aware of only one work in which an enrichment culture of methanotrophic bacteria was isolated from aerobic soil at an ultralow (less than 0.03%) methane content in the gas phase [22]. The ability of the obtained cultures to oxidize methane at low methane concentrations (close to the atmospheric ones) was tested. All three cultures were found to have a high-affinity activity for methane oxidation. The rates of methane oxidation (at the initial concentration of 10–12 ppm) by the suspensions with a density of 10^8 cells/ml were 0.12, 0.09, and 0.07 ng CH_4/(ml h) for the FS, BS, and AS cultures, respectively (Fig. 1a). After 48-h cultivation, the residual methane concentration in the gas phase decreased to 0.34, 3.5, and 1.7 ppm for the cultures FS, AS, and BS, respectively. The FS culture oxidized atmospheric methane at a CH_4 concentration of 1.8 ppm (Fig. 1b); after 48-h cultivation, the residual methane concen-

tration decreased to 0.3 ppm, which is probably the threshold level for this culture.

To define the Michaelis constant (k_m), the rates of methane oxidation at various initial methane concentrations were determined; the curves (an example of which for the FS culture is shown on Fig. 2) were plotted using the results obtained. The k_m values were 54.2 ± 21.3, 103.6 ± 34.8, and 176.8 ± 29.4 nM CH_4 for the FS, BS, and AS cultures, respectively. For all known collection cultures of type I and type II methanotrophs, the k_m values were in the micromolar range and varied between 0.8 and 66 μM CH_4 [23]. At the same time, the high-affinity microbial communities of aerobic soils are known to oxidize atmospheric methane; their k_m values are in nanomolar concentrations in the order of 10–280 nM CH_4 [2, 24]. The enrichment cultures under study exhibited high affinity for methane and the ability to oxidize atmospheric methane despite the fact that their isolation and maintenance were carried out at a high (5%) methane concentration. The values of the Michaelis constant for the enrichment cultures were in the nanomolar range and were comparable to those for aerobic soils. However, as the methane content in the gas phase increased, the methane-oxidizing activity increased as well; the k_m values within a methane concentration range of 1000–5000 ppm were comparable to those for pure cultures of methanotrophic bacteria (data not presented). To determine which microorganisms have a high affinity for methane, molecular analysis of methanotrophic bacteria present in the enrichment cultures was carried out.

The results of the detection of methanotrophic bacteria by FISH are shown in Table 1. The number of DAPI-stained cells varied from 3.3×10^7 to 4.25×10^7 cells/ml, whereas the percentage of metabolically active bacteria bound to the eubacterial probe (EUB 338mix) was 52.8–55.7%. Type I methanotrophs (probe M-84 + M-705) were scarce in the enrichment cultures and did not exceed $3–10 \times 10^3$ cells/ml, whereas the number of II type methanotrophs (probe M-450) ranged from 1.6 to 2.4×10^7 cells/ml. The percentages of type II methanotrophs among the metabolically active eubacteria of the FS, AS, and BS cultures were 87.3, 80.9, and 99.5%, respectively. The predominance of methanotrophic bacteria whose immune characteristics were similar to those of *Methylocystis parvus* was detected in the FS culture by the immunofluorescence assay (data not presented).

To study the species composition of methanotrophic bacteria in the enrichment cultures, a comprehensive molecular analysis based on the PCR amplification of the fragments of phylogenetic and functional genes of methanotrophic bacteria was performed with subsequent amplicon separation by DGGE or creation of a clone library.

PCR–DGGE analysis of the 16S rRNA gene fragments. Figure 3 shows the results of DGGE separation of amplicons from the ribosomal gene fragments of

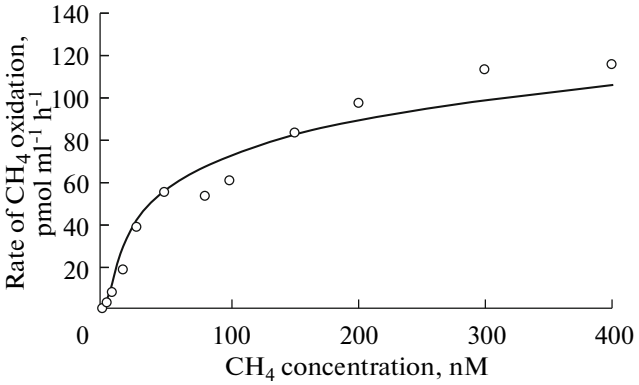

Fig. 2. The curve depicting the relationship between the rate of methane oxidation by the FS enrichment culture and the initial substrate concentration.

methanotrophic bacteria (Figs. 3a, 3b), as well as data obtained by cluster analysis of specific band profiles (fingerprints) (Figs. 3c, 3d). Comparative analysis of the fingerprints generated using the primers targeting types I and II methanotrophs (Figs. 3b and 3d, respectively) revealed a high level of similarity between the AS and BS cultures isolated from the soil samples collected from two agrocenoses (60 and 70%, respectively), but not between these cultures and the FS culture (from forest soil). The sequence analysis of specific DGGE bands revealed the presence of methanotrophic bacteria which are closest to members of the genus *Methylocella* (98% similarity) in the AS culture. All other sequences showed high similarity (97–99%) with nonmethanotrophic representatives of the phyla *Alpha-* and *Gammaproteobacteria* (Table 2). It was found that the AS culture contained sequences that were most closely related to those of *Pseudomonas*, *Kaistobacter*, and *Stenotrophomonas* the sequences detected in the FS culture were closest to those of *Kaistobacter, Brevundimonas, Flexibacter*, and *Stenotrophomonas*; and the sequences detected in the BS culture were closest to those of *Pseudomonas*. This approach turned out to be inefficient for the assessment of the biodiversity of methanotrophs in enrichment cultures. To our opinion, the possible reasons for

Table 1. Cell numbers in enrichment methanotrophic cultures determined by in situ hybridization using fluorescently labeled oligonucleotide probes and DAPI staining

Culture	FS	AS	BS
Staining/hybridization	Cell number, 10^7 cells/ml		
DAPI	3.30 ± 1.5	3.85 ± 1.5	4.25 ± 1.6
EUB338 mix	1.86 ± 0.84	2.03 ± 0.8	2.37 ± 0.9
M-450	1.63 ± 0.9	1.64 ± 0.7	2.35 ± 1.2
	Cell number, 10^3 cells/ml		
M-84 + M-705	3 ± 1.3	8 ± 1	11 ± 2.5

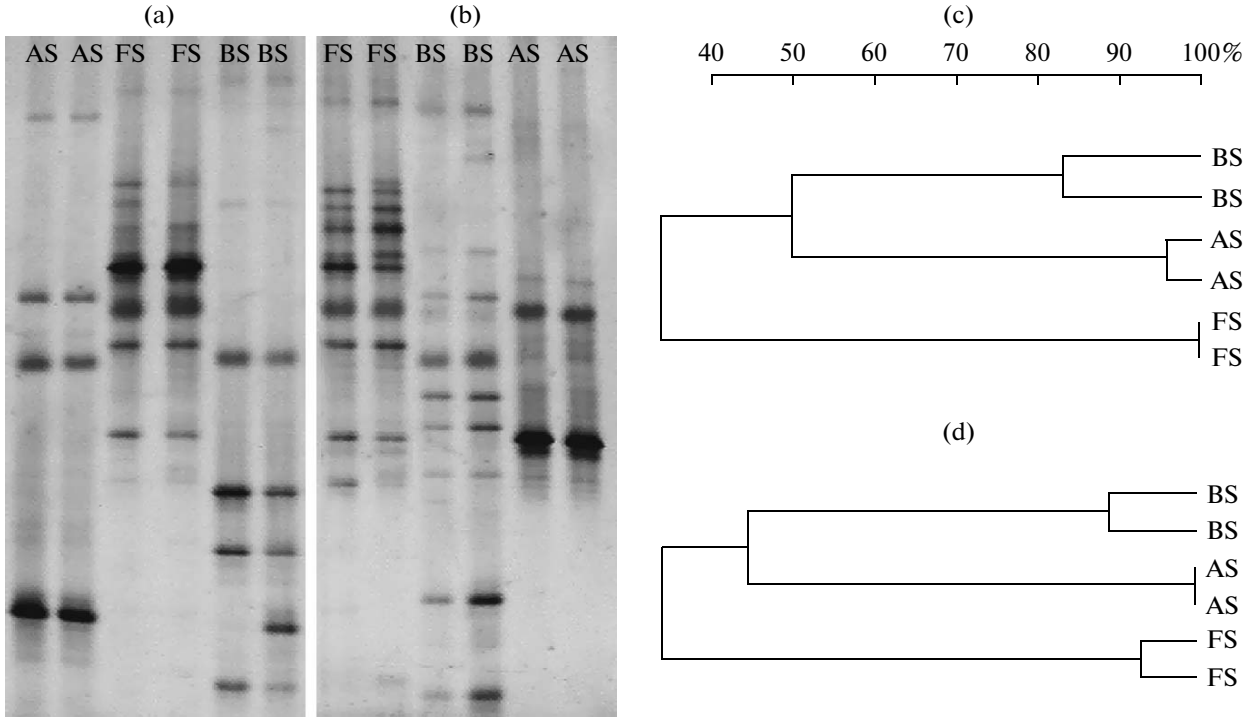

Fig. 3. Profiles of the 16S rRNA gene fragments of the enrichment cultures AS, BS, and FS obtained by DGGE separation using the MB10γ (a) and MB9α (b) primer systems and the results of the cluster analysis of the *Alpha*- (c) and *Gammaproteobacteria* (d) fingerprints.

this may include the small size (approximately 200 bp) of the fragment and low specificity of the primers that were used for the first round of amplification. At the same time, this technique was effectively used for analysis of the community composition of methanotrophic bacteria in the soils of Belgium when applying various fertilizer [25] and herbicide systems [26].

PCR–DGGE analysis and analysis of *pmoA* clone libraries. Another approach is based on the amplification of the gene fragment coding for the membrane-bound methane monooxygenase. Figure 4 shows the results of amplicon separation by DGGE. The number of specific DGGE bands was from four to six for each culture, which may indicate a low diversity of

Table 2. Results of the BLAST analysis of the nucleotide sequences of DGGE bands

Enrichment culture	Primer system	Closest relative (GenBank accession number)	Similarity, %	Taxonomic position
FS	MB10γ	*Stenotrophomonas maltophilia* strain KNUC391 (EU239104)	99	*Gammaproteobacteria*
	MB9α	*Brevundimonas* sp. (AM421787)	97	*Alphaproteobacteria*
		Ochrobactrum sp. (DQ486960)	98	
		Flexibacter sp. (EU910887)	92	
AS	MB10γ	*Stenotrophomonas maltophilia* strain KNUC391(EU239104)	99	*Gammaproteobacteria*
		Pseudomonas sp. (DQ357694)	99	
	MB9α	*Methylocella tundrae* (AJ563929)	98	*Alphaproteobacteria*
		Kaistobacter sp. (FJ436424)	98	
BS	MB10γ	*Pseudomonas* sp. (AM886097)	99	*Gammaproteobacteria*
		Pseudomonas sp. (FJ424487)	97	

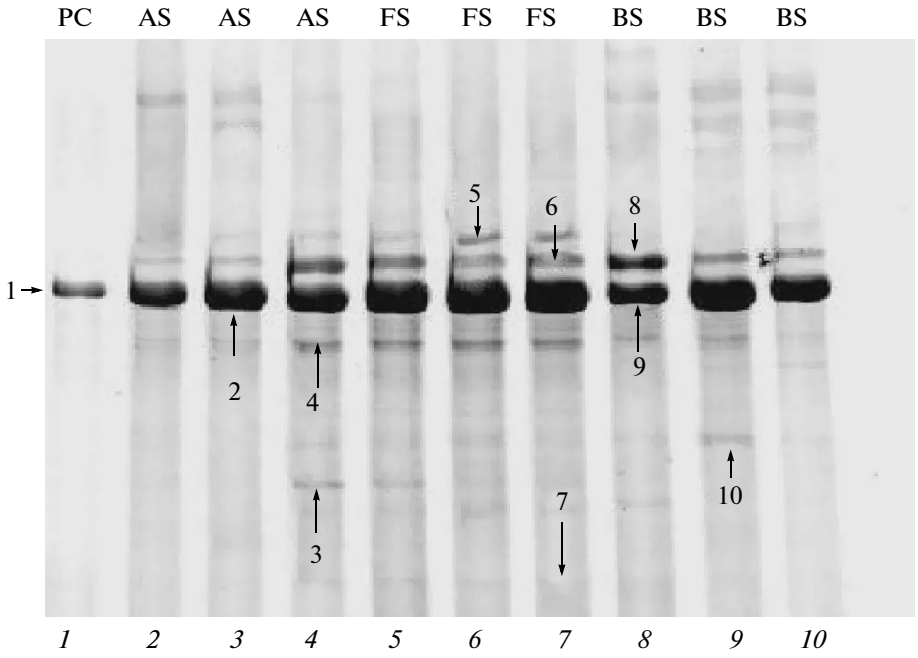

Fig. 4. Profiles of the *pmoA* gene fragments of the three enrichment cultures obtained by DGGE separation. The bands that were used for further sequence analysis are designated with arrows and numerals. Lane designations: *1*, DGGE marker for *Methylosinus trichosporium* OB5b (INMI 40 = UNIQEM 77); *2, 3*, enrichment culture AS; *5, 6, 7*, enrichment culture FS; *8, 9, 10*, enrichment culture BS.

methanotrophs in the enrichment cultures. Separation of the PCR product from the pure culture of the methanotrophic bacterium *Methylosinus trichosporium* 40 (PC variant) produced a single band (band 1). Similar bands were obtained in all variants of analysis of the enrichment cultures, which may indicate the inevitable presence of representatives of the genus *Methylosinus*. The sequence analysis of these bands from the AS (band 2) and BS (bands 8 and 9) cultures confirmed this suggestion. In the FS culture (band 5), the presence of methanotrophic bacteria most closely related to *Methylocystis* was detected. We failed to reamplify band 10 of the BS culture, while the results of sequence analysis indicated that band 6 of the FS culture was chimerical. Interestingly, amplification replicates differed in the presence of minor components. For instance, the sequence (band 7) closest to that of the *pmoA2* gene fragment of *Methylocystis parvus* was detected in the FS culture for only one replicate (lane *7*). In one replicate of the AS culture (lane *4*), the *pmoA2* gene sequence was detected (band 4) and the sequence of band 3 exhibited similarity to the *pmoA1* sequences of *"Methylosinus acidophilus"*.

Denaturing gradient gel electrophoresis allows rapid and efficient identification of dominant microorganisms within microbial communities [20]; however, minor components may be overlooked. To gain more detailed insight into the species composition of methanotrophic bacteria, molecular cloning of *pmoA* fragments was applied. The nucleotide sequences obtained were divided into four sequence types (95–100% similarity). Analysis of the obtained clone libraries demonstrated that the majority of clones in the FS enrichment culture (95% clones in the clone library) contained inserts with sequences that showed high similarity to the *pmoA1* and *pmoA2* gene fragments of the methanotrophic bacterium *Methylocystis* sp. SC2 (98 and 100%, respectively) (Table 3). In the clone libraries of the AS and BS cultures, all identified sequences were closest to those of methanotrophic bacteria belonging to the genus *Methylosinus*. The similarity to the known organisms was low (92% for clones of sequence type 1 and the *pmoA1* of *Methylosinus trichosporium* KS21 and 96% for clones of sequence type 4 and the *pmoA2* of *Methylosinus sporium*). All clones of sequence type 1 were minor components of the FS culture clone library.

The results of phylogenetic analysis are shown in Figure 5. The deduced sequences of the type 3 clones and the DGGE band 7 obtained for the FS culture from the forest soil revealed the greatest similarity to the PmoA2 sequences of *Methylocystis* sp. SC2. This culture isolated from a contaminated water-bearing stratum was used as a model object in which the presence of two significantly different genes, *pmoA1* and *pmoA2* (59.3–65.6% similarity with respect to the protein sequences) [27], forming a single gene cluster was detected for the first time. It was recently established that the *pmoA2* gene is responsible for the synthesis of the active center of the enzyme with a high affinity for methane [28]. Hence, in the case of the FS culture,

Table 3. The percent ratio between methanotrophic microorganisms in the enrichment cultures calculated on the basis of clone library analysis

Sequence type (gene)	Closest relative (GenBank accession number)	Similarity, %	Clone number (%) in the clone library		
			BS	AS	FS
1 (*pmoA1*)	*Methylosinus trichosporium*, KS21 (AJ431388)	92	37(95)	37(95)	2(5)
2 (*pmoA1*)	*Methylocystis* sp. SC2 (CAE47800)	100	0	0	19(53)
3 *(pmoA2*)	*Methylocystis* sp. SC2 (CAE48352)	98	0	0	15(42)
4 (*pmoA2*)	*Methylosinus sporium* (CAD66446)	96	2(5)	2(5)	0

methane oxidation at ultralow atmospheric CH_4 concentrations occurs due to the activity of a methanotrophic bacterium of the genus *Methylocystis* with the *pmoA2* gene. Moreover, type 2 sequences and those of the DGGE band 4 displayed the highest similarity to the *pmoA1* gene fragments of *"Methylocystis aldrichii," Methylocystis* sp. SC2, and *Methylocystis parvus*. It is likely that *Methylocystis parvus* is the closest validly described relative of strain SC2 and the dominant methanotrophic bacterium from the FS culture, as demonstrated by the results of molecular and serological analyses of the enrichment cultures.

Molecular analysis of the enrichment cultures isolated from two arable soils revealed the predominance of methanotrophic bacteria of the genus *Methylosinus*, most closely related to *Ms. sporium, Ms. trichosporium*, and *"Ms. acidophilus."* DGGE analysis of the *pmoA* gene fragments (band 4) from the AS enrichment culture revealed sequences that fall into the *pmoA2* cluster (Fig. 5) but are distant from the known sequences. It was previously demonstrated that some *Ms. sporium* and *Ms. trichosporium* strains possess the *pmoA2* gene [29]; hence, the obtained sequences may belong to a *Methylosinus* species from the enrichment culture.

Our studies demonstrated that the enrichment cultures isolated from aerobic soils have a high affinity to methane. Bacteria of the genera *Methylocystis* (mixed forest soil) and *Methylosinus* (soil from the two agrocenoses) carrying the genes encoding the synthesis of the active center of two membrane-bound methane monooxygenases were the main methanotrophic components of these cultures. The presence of two enzymes with different levels of affinity for methane explains the fact that these microorganisms are able to

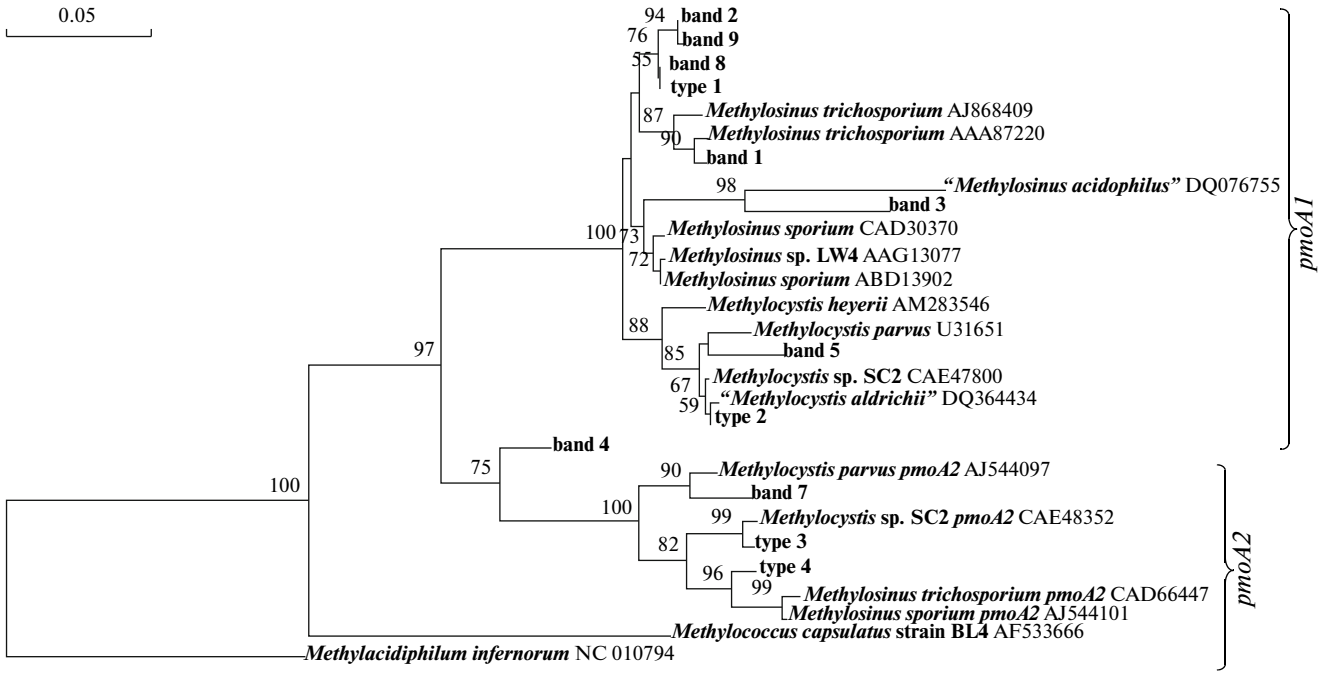

Fig. 5. Phylogenetic tree constructed on the basis of the deduced amino acid sequences of the *pmoA* gene fragments. The GenBank accession numbers of gene fragment sequences are given. Scale bar, five amino acid substitutions for each 100 amino acid residues. The numerals show the significance of the branching order as determined by bootstrap analysis of 500 alternative trees (only bootstrap values above 50 were considered as significant).

adapt to various methane concentrations. This gives them an advantage in aerobic soils over other methanotrophs that do not have *pmoA2* and, as a consequence, are not able to oxidize atmospheric methane. The predominance of bacteria belonging to the genus *Methylocystis* in the methane-oxidizing microbial communities of forest soils had been previously revealed by immunoassay methods [9], which allowed us to assume that the methanotrophic bacteria of the studied enrichment cultures play a key role in this process. Further studies will include molecular analysis of the composition of the methanotrophic community in the gray forest soil from which the enrichment cultures were isolated.

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