Microbiology, Vol. 71, No. 2, 2002, pp. 227–233. Translated from Mikrobiologiya, Vol. 71, No. 2, 2002, pp. 264–271.
Original Russian Text Copyright © 2002 by Kalyuzhnaya, Makutina, Rusakova, Nikitin, Khmelenina, Dmitriev, Trotsenko.

EXPERIMENTAL ARTICLES

Methanotrophic Communities in the Soils of the Russian Northern Taiga and Subarctic Tundra

M. G. Kalyuzhnaya, V. A. Makutina, T. G. Rusakova, D. V. Nikitin, V. N. Khmelenina, V. V. Dmitriev, and Yu. A. Trotsenko[1]

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

Received April 1, 2001; in final form, June 1, 2001

Abstract—The PCR analysis of DNA extracted from soil samples taken in the Russian northern taiga and subarctic tundra showed that the DNA extracts contain genes specific to methanotrophic bacteria, i.e., the *mmoX* gene encoding the conserved α-subunit of the hydroxylase component of soluble methane monooxygenase, the *pmoA* gene encoding the α-subunit of particulate methane monooxygenase, and the *mxaF* gene encoding the α-subunit of methanol dehydrogenase. PCR analysis with group-specific primers also showed that methanotrophic bacteria in the northern taiga and subarctic tundra soils are essentially represented by the type I genera *Methylobacter, Methylomonas, Methylosphaera*, and *Methylomicrobium* and that some soil samples contain type II methanotrophs close to members of the genera *Methylosinus* and *Methylocystis.* The electron microscopic examination of enrichment cultures obtained from the soil samples confirmed the presence of methanotrophic bacteria in the ecosystems studied and showed that the methanotrophs contain only small amounts of intracytoplasmic membranes.

Key words: methanotrophs, psychrotrophic and psychrophilic communities, group-specific primers.

Methane is one of the most important greenhouse gases [1]. Ecological studies showed that more than 80% of methane formed in ecosystems is oxidized by methanotrophic bacteria present in these ecosystems [2]. In polar regions, methane fluxes are controlled by psychrotrophic and psychrophilic microbial communities, which have been of great interest in recent years [3–6]. Omel'chenko *et al.* described a psychrophilic type I methanotroph, *Methylobacter psychrophilus*, which was isolated from one of the soil samples taken in the northern taiga and tundra [4]. Another such methanotroph, *Methylosphaera hansonii*, was isolated from an Antarctic meromictic lake [5]. Vasil'eva *et al.* obtained enrichment cultures of psychrophilic acidophilic methanotrophs from a permafrost sphagnum peatland [6]. The comprehensive analysis of methanotrophs in low-temperature ecosystems by microbiological methods is difficult because of a considerable complexity of techniques used for the isolation of psychrophilic methanotrophs in pure cultures and their cultivation. On the other hand, such analysis can relatively easily be carried out by immunological and molecular biological methods. For instance, the immunofluorescence investigation of psychrophilic methanotrophic communities in Kolyma sphagnum wetlands made it possible to detect the genera *Methylobacter, Methylomonas, Methylococcus*, and *Methylocystis* [7]. One of the disadvantages of the immunofluorescence approach is that it allows the detection of the taxa that are already known, i.e., those against which antisera are raised. It is for this reason that Vecherskaya *et al.* failed to detect the new genera *Methylomicrobium* and *Methylosphaera* in the methanotrophic communities studied [7].

There is a wide range of group-specific genetic probes f or detecting methanotrophic bacteria in natural samples [8]. However, the probes available so far failed to reliably detect the genera *Methylobacter* and *Methylomonas*. Furthermore, there are no genetic probes for the genus *Methylosphaera*.

The aim of the present study was to study the population of methanotrophic bacteria in low-temperature ecosystems by the PCR technique using new genetic probes for detecting the genera *Methylobacter* and *Methylosphaera.*

MATERIALS AND METHODS

Samples of the northern taiga and subarctic tundra cryozems were kindly donated by D.A. Gilichinskii, Institute of Fundamental Problems of Biology, Russian Academy of Sciences, Pushchino. The samples were collected in places described in Table 1 from depths of 5 to 15 cm, except for sample 2-94, which was taken from a depth of 40 to 50 cm.

DNA extraction from soil samples. DNA was extracted from soil samples by the method of Lee *et al.* [9] with minor modifications. A soil sample (1 g) was

[1]Corresponding author. E-mail: trotsenko@ibpm.serpukhov.su

0026-2617/02/7102-0227$27.00 © 2002 MAIK "Nauka / Interperiodica"

Table 1. Description of soil sampling locations

Sample group	Sample serial number	Soil section	Soil sampling location	Soil type
I	27	618-94	The Mal'chukovka River distributary basin	Light gley cryozem in northern taiga
	37			
II	44	619-94	The Mal'chukovka River distributary basin	Light gley cryozem in northern taiga
	45			
III	70	101-94	Mount Rodinka foot	Light hillside gley cryozem in northern taiga
	71	106-94		
IV	76	122-91	Mount Rodinka foot	Bog peaty gley soil in northern taiga alass
V	204		The environs of Lake Akhmela	Podzolic podbur in subarctic tundra
	207			
	211			
VI	40–50	2-94	Northeastern Kolyma Lowland, the right bank of the Chukoch'ya River	Light loam

Table 2. Structural and phylogenetic group–specific primers

Primer	5'–3' sequence	Target microorganisms	Annealing temperature, °C	Ref.
16SrRNA 27f	AGAGTTTGATCMTGGCTCAG	All eubacteria	60	[8]
16SrRNA 1490r	TACGGYTACCTTGTTACGACTT	All eubacteria	60	″
mxaF 1003f	GCGGCACCAACTGGGGCTGGT	All G-negative methylotrophs	59	″
mxaF 1561r	GGGCAGCATGAAGGGCTCCC	All G-negative methylotrophs	59	″
pmoA 189f	GGNGACTGGGACTTCTGG	pMMO/AMO	56	″
pmoA 682r	GAASGCNGAGAAGAASGC	pMMO/AMO	56	″
mmoX 882f	GGCTCCAAGTTCAAGGTCGAGC	sMMO-positive methanotrophs	55	″
mmoX 1403r	TGGCACTCGTAGCGCTCCGGCTCG	sMMO-positive methanotrophs	55	″
mmoX1 2008f	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	sMMO-positive methanotrophs	72	[17]
mmoX2 2376r	GGCTCGACCTTGAACTTGGAGCCATACTCG	sMMO-positive methanotrophs	72	″
Mb 1007r	CACTCTACGATCTCTCACAG	*Methylomicrobium*	60	[8]
Mc 1005r	CCGCATCTCTGCAGGAT	*Methylococcus*	55	″
Ms 1020r	CCCTTGCGGAAGGAAGTC	*Methylosinus*	55	″
Mm 1007r	CACTCCGCTATCTCTAACAG	*Methylomonas* and *Methylobacter*	55	″
Mm 835	GCTCCACYACTAAGTTC	*Methylomonas*	50	[15]
Type 2b	CATACCGGRCATGTCAAAAGC	*Methylosinus* and *Methylocystis*	55	″
MbII 884r	ATGCGTTCTGCGCCACTA	*Methylobacter*	55	This work
Mh 996r	CACTCTACTATCTCTAACGG	*Methylosphaera*	55	″

Note: AMO is ammonium monooxygenase.

extracted twice with 0.2 M phosphate buffer (pH 8.5). The extracts were pooled and centrifuged at 6000 *g* for 20 min. The sediment was suspended in 5 ml of the lysis solution (0.1 M NaCl, 0.1 M EDTA, and 10 mg/ml lysozyme, pH 8.0), incubated with stirring at 37°C for 1 h, supplemented with 20 μl of proteinase K (20 mg/ml), 10 μl of ribonuclease A (10 mg/ml), and 1–2 ml of 10% SDS, and incubated for the next 1 h at the same temperature. The mixture was subjected to three freeze–thaw cycles at –70 and 65°C and centrifuged at 12000 *g* for 15 min. The supernatant was mixed with 5 M NaCl and 10% cetyltrimethylammo-

nium bromide (CTAB) in 0.7 M NaCl to final concentrations of 1 M NaCl and 1% CTAB and incubated at 65°C for 10 min. DNA present in the lysate was extracted with an equal volume of a chloroform–isoamyl alcohol (24 : 1) mixture. The extract was thoroughly mixed and centrifuged at 5000 *g* for 15 min to separate phases. DNA from the upper phase was precipitated by adding two volumes of ethanol. The precipitate was washed with 80 and 70% ethanol, slightly dried, and dissolved in 0.5 ml of TE buffer (10 mM Tris–HCl and 0.1 mM EDTA, pH 8.0). Further purification involved phenol extraction. Finally, DNA was precipitated and washed as described above and dissolved in 50 μl of TE buffer. The isolated DNA was tested by the PCR amplification of eubacterial 16S rRNA genes using universal primers [8].

PCR amplification. Primers specific for functional genes and various phylogenetic groups of methanotrophic bacteria are listed in Table 2. PCR amplifications were run in a Perkin Elmer 2400 thermal cycler (United States) with 10 cycles of DNA denaturation at 94°C for 40 s, primer annealing at 53°C for 40 s, and DNA synthesis at 72°C for 40 s and 20 cycles of DNA denaturation at 94°C for 40 s, primer annealing at 58°C for 40 s, and DNA synthesis at 72°C for 40 s. The last step was DNA synthesis at 72°C for 5 min. Each reaction tube contained 30 μl of the following mixture: 10 mM Tris–HCl buffer, 68 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.1% Tween-80, 5 pmol of the respective primer, and 0.5 μl of template DNA. The reaction tubes were incubated at 95°C for 5 min, rapidly cooled in an ice bath, and then 0.2 μM of each dNTP and 1 unit of *Taq* DNA polymerase were added to the tubes. Along with the total DNA, the amplification products of eubacterial 16S rRNA were used for the qualitative analysis of methanotrophic populations.

The amplification products were analyzed by electrophoresis in 1% agarose gel. For the control, we used DNA isolated from pure cultures of the following methanotrophic bacteria: *Methylobacter bovis* 98, *Methylococcus capsulatus* Bath, *Methylomicrobium album* BG8, *Methylomonas methanica* S1, and *Methylosinus trichosporium* OB3b. The DNA was isolated as described earlier [10].

16S rDNA sequencing and phylogenetic analysis. The 16S rDNA genes of the total DNA were amplified with 16S rRNA 27f and Mb1007r primers. Oligonucleotides were sequenced using the natural conserved pCr primer [11] and a FemtoMol kit (Promega) according to the manufacturer's instructions. The phylogenetic analysis of 16S rDNA sequences was conducted with the aid of PHYLIP software (version 3.5).

Enrichment cultures. Soil samples (1 g) were suspended in 50 ml of fivefold diluted P medium [12] and incubated in a methane–air (1 : 1) atmosphere at 4 or 6°C using a Clim-O-Shaker (Switzerland). The growth

Table 3. The PCR amplification of the group-specific structural genes of the total DNA extracted from soil samples collected in northern taiga and subarctic tundra

DNA sample	*mxaF*-gene of MDH	*pmoA*-gene of pMMO	*mmoX*-gene of sMMO	*mmoSX*-gene of sMMO
I	+	+	–	+
II	+	+	+	+
III	+	+	–	–
IV	+	+	–	+
V	+	+	–	+
VI	+	+	+	+

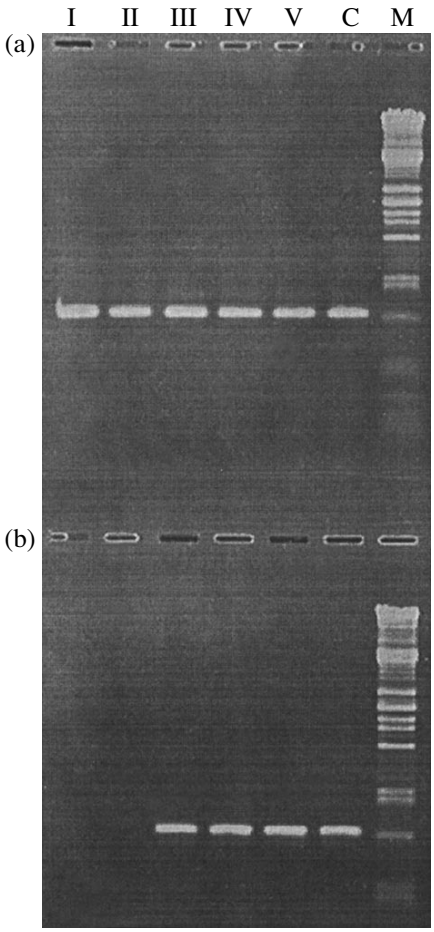

Fig. 1. The PCR amplification of the 16S rRNA genes of the total DNA extracted from soil samples collected in (I–III) northern taiga and (IV–V) subarctic tundra: (a) amplification with the Mb2 primer and (b) amplification with the Mm 835 primer. The letter C indicates the lane for (a) *Methylobacter vinelandii* 30 and (b) *Methylomonas methanica* S1, and M indicates the lane for DNA molecular mass standards (the *Pst*I restriction fragments of bacteriophage λ).

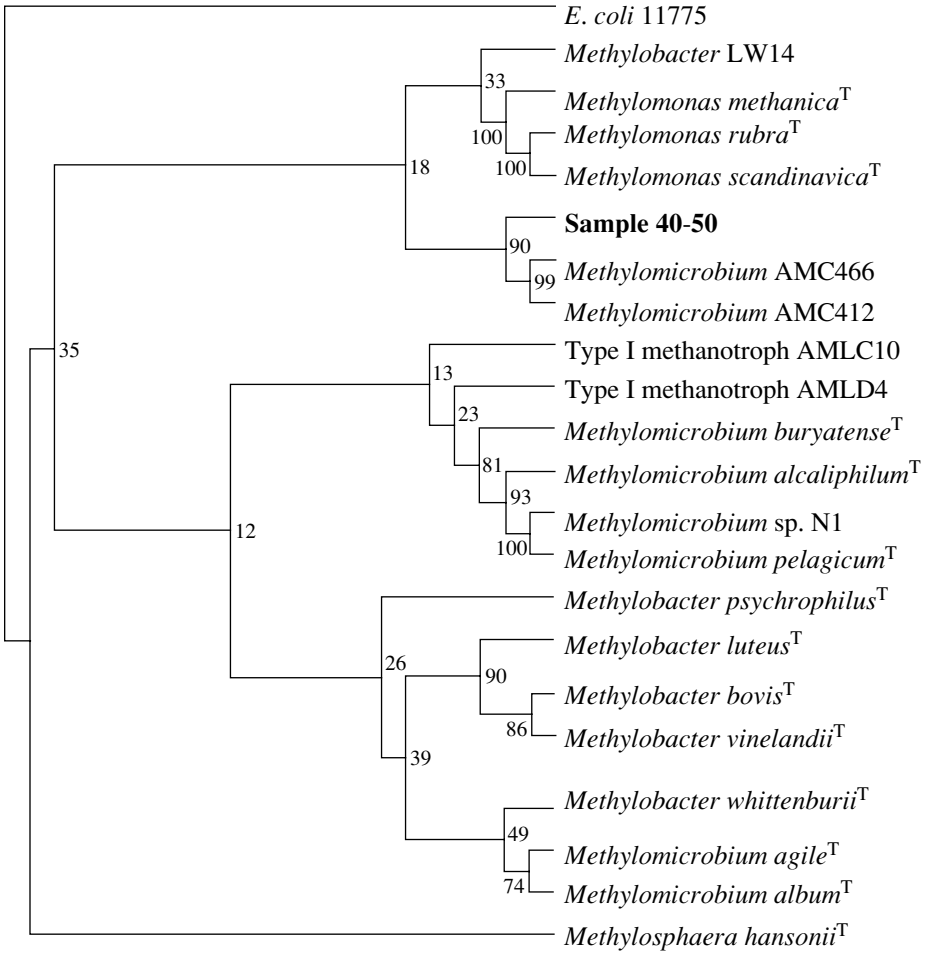

Fig. 2. The phylogenetic analysis of the nucleotide sequences of the total DNA extracted from soil sample 40-50. DNA fragments were amplified using the Mb1007r/16S rRNA 27f primer set.

of methanotrophic cultures became observable after 7–10 weeks of incubation at these temperatures.

Electron microscopic studies. To prepare thin cell sections, bacteria were fixed with a 1.5% solution of glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4°C for 1 h, washed thrice with the buffer, and refixed with a 1% solution of OsO_4 in the same buffer at 20°C for 3 h. After dehydration, the preparations were embedded in Spurr epoxy resin. Thin sections were mounted onto support grids and contrasted with a 3% solution of uranyl acetate in 70% ethanol for 30 min and then with lead citrate by Reynolds [13]. Freeze-fracturing was carried out in a JEE-4X vacuum evaporator equipped with a device providing a rapid quenching of microbial cells at a rate of about 10^{-6} °C/s [8]. The cells were frozen in liquid propane overcooled to –196°C with liquid nitrogen and freeze-fractured at –100°C at a pressure of 3×10^{-4} Pa. Replicas of freeze-fractured cells were prepared by the vacuum deposition of a platinum–carbon mixture at an angle of 30° and then of carbon alone at an angle of 90° [14]. The replicas thus prepared were analyzed in a JEM 100B electron microscope (Japan).

RESULTS

PCR analysis. To create a detection system for the genera *Methylobacter* and *Methylosphaera*, we analyzed nucleotide sequences available from the GenBank database, National Center for Biotechnology Information, and chose the following conserved oligonucleotide sequences of the 16S rRNA genes of these two genera: 5'-ATGCGTTCTGCGCCACTA (r) for the genus *Methylobacter* (the PCR product contains 1005 nucleotides) and 5'-CACTCTACTATCTCTAACGG (r) for the genus *Methylosphaera* (the PCR product contains 990 nucleotides). The universal eubacterial primer 16S rRNA 27f was used for the positive control (Table 2), and the DNA of *E. coli* and other methanotrophic bacteria was used for the negative control. The competence of each of the primer pairs was tested with the aid of the Oligo program. Synthetic primers were tested using the DNA that was isolated from pure cultures.

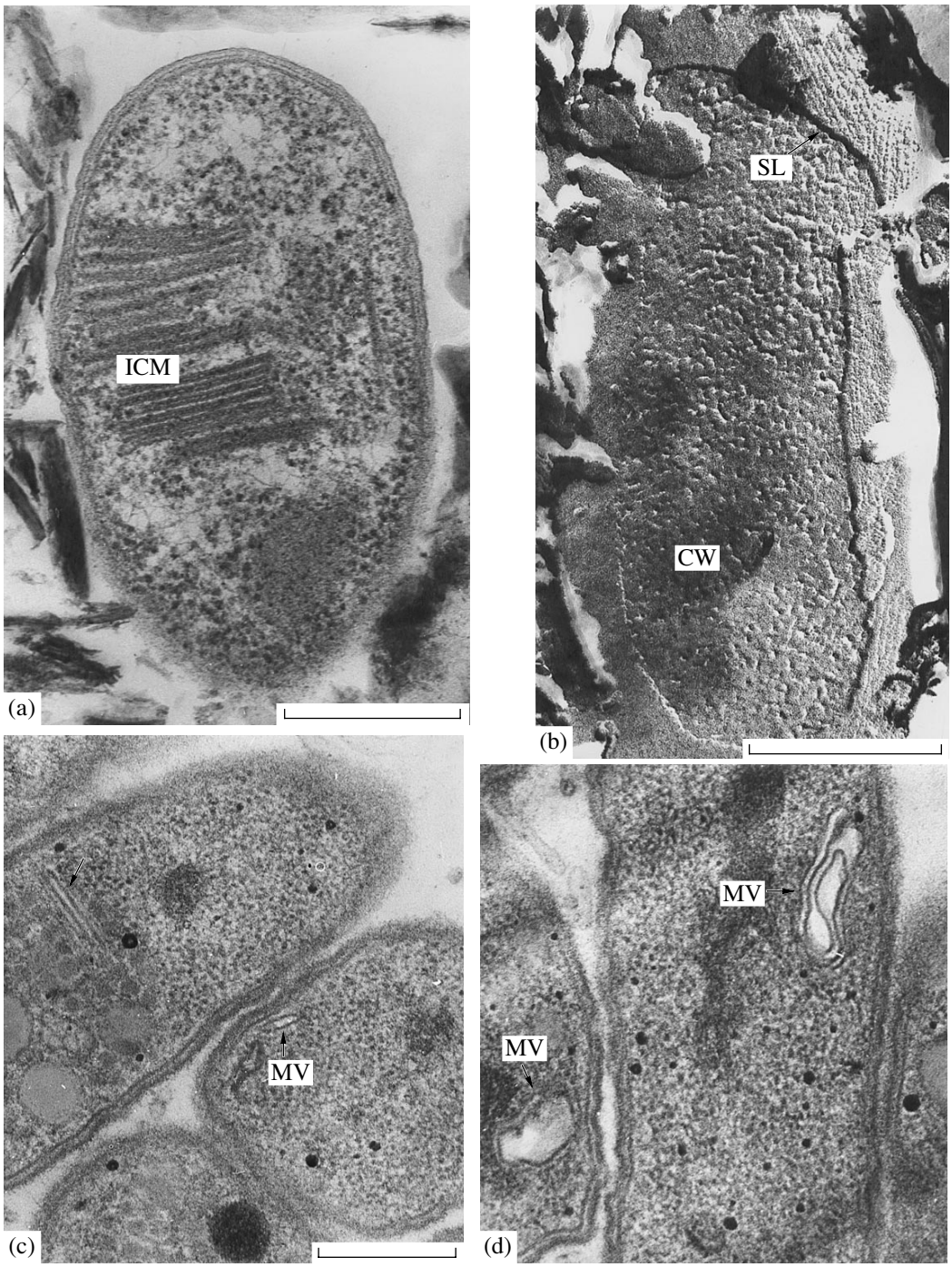

Fig. 3. The electron microscopy of methanotrophs isolated in enrichment cultures from (a and b) soil sample 40-50, (c) soil sample 46, and (d) soil sample 211. Micrographs a, c, and d show thin sections of bacterial cells, and micrograph b shows a freeze-fracture replica coated with a platinum–carbon mixture. CW, cell wall; SL, S layer; ICM, intracytoplasmic membrane; and MV, membrane vesicle (or reduced membrane). Bars represent 0.3 μm.

All of the total DNA samples extracted from the northern taiga and subarctic tundra soils showed the presence of the *mxaF* gene encoding the α-subunit of methanol dehydrogenase (MDH). Most of the DNA samples also gave a positive reaction for the *pmoA* gene encoding the α-subunit of particulate methane monooxygenase (pMMO) and for the *mmoX* and *mmoSX* genes encoding soluble methane monooxygenase (sMMO) subunits (Table 3).

The taxonomic structure of methanotrophic populations was studied using a wide range of primer sets that offered the possibility of detecting virtually all known methanotrophic genera, including *Methylobacter* and *Methylosphaera*. Analysis showed the presence of bac-

Table 4. The PCR amplification of the group-specific 16S rRNA genes of the total DNA extracted from soil samples collected in northern taiga and subarctic tundra

DNA sample	*Methylomicrobium*	*Methylobacter*	*Methylomonas*	*Methylosphaera*	Type II methanotrophs	*Methylosinus*/*Methylocystis*
I	+	+	–	–	+	–
II	+	+	–	–	–	–
III	+	+	+	–	+	–
IV	+	+	+	+	+	–
V	+	+	+	+	+	+
VI	+	+	+	–	+	+

teria of the genera *Methylobacter, Methylomonas*, and *Methylomicrobium* in most of the soil samples (Fig. 1 and Table 4). Gene fragments from soil sample 40-50, which were amplified using the 16S rRNA 27f/Mb1007r primer set, were isolated and partially sequenced. The phylogenetic analysis of these gene fragments showed that they belong to bacteria from the genus *Methylomicrobium* (Fig. 2) and that they are very close to the nucleotide sequences detected in mud samples taken from some lakes in North America [15]. None of the samples showed the presence of bacteria of the genus *Methylococcus*, while soil samples taken in subarctic tundra were found to contain bacteria of the genus *Methylosphaera*, whose representatives have been isolated recently from Antarctic lakes and molluscan gills [5].

The genetic probe specific for type II methanotrophs, 16S rRNA f/type2r, enabled us to detect bacteria of this type in almost all of the soil samples under study. However, a signature of the *Methylosinus/Methylocystis* genome was found only in one soil sample. Presumably, the methanotrophic communities of the two ecosystems studied contain bacteria which are close, but not identical, to members of the genera *Methylosinus* and *Methylocystis*.

Enrichment cultures obtained from soil samples were dominated by cells with a morphology specific to methanotrophic bacteria. The electron microscopic studies of cells from the enrichment cultures obtained from the Kolyma soil samples showed that these cells contain intracytoplasmic membrane (ICM) stacks common to type I methanotrophs (Fig. 3a). The cell exhibited the presence of S layers with linear *p*2 symmetry (Fig. 3b). Most of the methanotrophs detected in the other soil samples lacked common ICM structures but contained a small number of particular membrane lamellae (reduced ICMs) or vesicles (Figs. 3c and 3d).

DISCUSSION

In spite of the wide range of genetic probes appropriate to the detection of methanotrophic genera, no suitable primer has been designed so far to detect one of the most widespread methanotrophic genus, *Methylobacter.* The primer set designed by Murrell *et al.* for detecting this genus was constructed on the basis of the 16S rRNA of *M. album* [8]. A comparative analysis of the nucleotide sequences of this primer set and the 16S rRNA genes (and their amplified fragments) of the type species of the genera *Methylobacter* and *Methylomicrobium* showed that this primer set can reliably detect only the genus *Methylomicrobium.* The second genetic probe (Mm1007r), which was developed by Murrell *et al.* [8] to detect *Methylomonas,* is imperfect and fails to distinguish this genus from *Methylobacter.* At the same time, the primer sets designed by us allow the genus *Methylobacter* to be reliably distinguished from the other dominant methanotrophic genera, i.e., *Methylomonas* and *Methylomicrobium*.

We also designed and tested a genetic probe for detecting the genus *Methylosphaera*, whose species *M. hansonii* was the second psychrophilic methanotroph to be discovered. Some other representatives of this genus were detected in Antarctic aquatic ecosystems [5] and in the subarctic tundra soils (data of the present work).

The methanotrophic bacteria isolated from the northern taiga and subarctic tundra soils were found to contain small amounts of ICMs, which is considered to be a specific feature of methanotrophs from aquatic ecosystems with almost pure water [16]. In general, our observations indicating the prevalence of type I methanotrophs in psychrophilic microbial communities are in agreement with the data of other researchers [3, 4, 7]. On the other hand, PCR analysis showed that the methanotrophic communities of the two ecosystems studied also include type II methanotrophs that are close, but not identical, to bacteria of the genera *Methylosinus* and *Methylocystis.* The investigation of these new methanotrophs is in progress in our laboratory.

ACKNOWLEDGMENT

This work was supported by grants nos. 99-04-49145 and 01-04-48511 from the Russian Foundation

for Basic Research and grant no. 193 from the Russian Academy of Sciences.

REFERENCES

1. Dickinson, R.E. and Cicerone, R.J., Future Global Warming from Atmospheric Trace Gases, *Nature* (London), 1986, vol. 319, no. 60499, pp. 109–115.
2. King, G.M., Regulation by Light of Methane Emissions from a Wetland, *Nature* (London), 1990, vol. 345, no. 6275, pp. 513–515.
3. Zavarzin, G.A. and Vasil'eva, L.V., The Methane Cycle in Russia, *Krugovorot ugleroda na territorii Rossii* (The Carbon Cycle in Russia), Zavarzin, G.A, Ed., Moscow, 1999, pp. 202–230.
4. Omel'chenko, M.V., Vasil'eva, L.V., Zavarzin, G.A., Savel'eva, N.D., Lysenko, A.M., Mityushina, L.L., Khmelenina, V.N., and Trotsenko, Yu.A., A Novel Psychrophilic Methanotroph of the Genus *Methylobacter*, *Mikrobiologiya*, 1996, vol. 65, no. 3, pp. 339–343.
5. Bowman, J.P., McCammon, S.A., and Skeratt, J.H., *Methylosphaera hansonii* gen. nov., sp. nov., a Psychrophilic, Group I Methylotroph from Antarctic Marine-Salinity, Meromictic Lakes, *Microbiology* (UK), 1997, vol. 143, pp. 1451–1459.
6. Vasil'eva, L.V., Berestovskaya, Yu.Y., and Zavarzin, G.A., Psychrophilic Acidophilic Methanotrophs from a Permafrost Sphagnum Peatland, *Dokl. Akad. Nauk*, 1999, vol. 368, no. 1, pp. 125–128.
7. Vecherskaya, M.S., Galchenko, V.F., Sokolova, E.N., and Samarkin, V.A., Activity and Species Composition of Aerobic Methanotrophic Communities in Tundra Soil, *Curr. Microbiol.*, 1993, vol. 27, no. 3, pp. 181–185.
8. Murrell, J.C., McDonald, I.R., and Bourne, D.G., Molecular Methods for the Study of Methanotroph Ecology, *FEMS Microbiol. Ecol.*, 1998, vol. 27, pp. 103–114.
9. Lee, S.Y., Bollinger, J., Bezdicek, D., and Ogram, A., Estimation of the Abundance of Uncultured Soil Bacterial Strain by a Competitive Quantitative PCR Method, *Appl. Environ. Microbiol.*, 1996, vol. 62, no. 10, pp. 3787–3793.
10. Kalyuzhnaya, M.G., Khmelenina, V.N., Starostina, N.G., Baranova, S.V., Suzina, N.G., and Trotsenko, Yu.A., A New Moderately Halophilic Methanotroph of the Genus *Methylobacter*, *Mikrobiologiya*, 1998, vol. 67, no. 4, pp. 527–534.
11. Edwards, U., Rogall, T., Blocker, H., Emde, M., and Bottger, E.C., Isolation and Direct Complete Nucleotide Determination of Entire Genes: Characterization of a Gene Coding for 16S Ribosomal RNA, *Nucleic Acids Res.*, 1989, vol. 17, pp. 7843–7853.
12. Gal'chenko, V.F., Shishkina, V.N., Suzina, N.G., and Trotsenko, Yu.A., Isolation and Properties of New Obligate Methanotrophs, *Mikrobiologiya*, 1977, vol. 46, no. 5, pp. 890–897.
13. Reynolds, E.S., The Use of Lead Citrate at High pH as an Electron-Opaque Stain in Electron Microscopy, *J. Cell Biol.*, 1963, no. 17, pp. 208–213.
14. Fikhte, B.A., Zaichkin, E.I., and Ratner, E.N., *Novye metody fizicheskogo preparirovaniya biologicheskikh ob"ektov dlya elektronno-mikroskopicheskikh issledovanii* (New Physical Methods for Preparing Biological Objects for Electron Microscopic Studies), Moscow: Nauka, 1973.
15. Costello, A.M. and Lidstrom, M.E., Molecular Characterization of Functional and Phylogenetic Genes from Natural Populations of Methanotrophs in Lake Sediments, *Appl. Environ. Microbiol.*, 1999, vol. 65, no. 11, pp. 5066–5074.
16. Dedysh, S.N., Liesack, W., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Abing, A.M., Panikov, N.S., and Tiedje, J.M., *Methylocella palustris* gen. nov., sp. nov., a New Methane-oxidizing Acidophilic Bacterium from Peat Bogs Representing a Novel Subtype of Serine Pathway Methanotrophs, *Int. J. Syst. Evol. Microbiol.*, 2000, vol. 50, no. 1, pp. 955–969.
17. Miguez, C.B., Bourque, D., Green, C.W., and Groleau, D., Detection and Isolation of Methanotrophic Bacteria Possessing Soluble Methane Monooxygenase (sMMO) Genes Using the Polymerase Chain Reaction (PCR), *Microbial. Ecol.*, 1997, vol. 33, pp. 21–31.