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EXPERIMENTAL ARTICLES

Methanotrophic Bacteria in Cold Seeps of the Floodplains of Northern Rivers

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Abstract—Small mud volcanoes (cold seeps), which are common in the floodplains of northern rivers, are potentially important (although poorly studied) sources of atmospheric methane. Field research on the cold seeps of the Mukhrina River (Khanty-Mansiysk Autonomous okrug, Russia) revealed methane fluxes from these structures to be orders of magnitude higher than from equivalent areas of the mid-taiga bogs. Microbial communities developing around the seeps were formed under conditions of high methane concentrations, low temperatures (3–5°C), and near-neutral pH. Molecular identification of methane-oxidizing bacteria from this community by analysis of the *pmoA* gene encoding particulate methane monooxygenase revealed both type I and type II methanotrophs (classes *Gammaproteobacteria* and *Alphaproteobacteria*, respectively), with prevalence of type I methanotrophs. Among the latter, microorganisms related to *Methylobacter psychrophilus* and *Methylobacter tundripaludum, Crenothrix polyspora* (a stagnant water dweller), and a number of methanotrophs belonging to unknown taxa were detected. Growth characteristics of two methanotrophic isolates were determined. *Methylobacter* sp. CMS7 exhibited active growth at 4–10°C, while *Methylocystis* sp. SB12 grew better at 20°C. Experimental results confirmed the major role of methanotrophic gammaproteobacteria in controlling the methane emission from cold river seeps.

Keywords: cold methane seeps, psychrophilic methanotrops, *Methylobacter, Crenothrix, pmoA* genes
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Methane is an important greenhouse gas affecting the atmospheric photochemistry: its direct potential for global warming (calculated per unit concentration for a period of 20 years) is 39 times higher than that of carbon dioxide [1]. According to current knowledge, the major natural source of methane are wetlands (113 Mt CH_4 $year^{-1}$ of the total 530 Mt CH_4 $year^{-1}$ of the methane flux to the atmosphere) [2].

Recently, geological sources, producing the global flux of 53 ± 11 Mt CH_4 $year^{-1}$, were proposed as the second important natural source of methane [3]. These sources are associated with hydrocarbon-containing sedimentation basins: natural gas difuses to the atmosphere through the above-lying soils both ubiquitously and through separate channels terminating with macroseeps (mud volcanoes and other seep types) and microseeps. Methane flux from a mud volcano may reach 1 kt $year^{-1}$ [4].

In southern regions (Azerbaijan, Burma, Italy, East India, Trinidad island, Turkmenistan, and Java), mud volcanoes not only have long been discovered, but have been sufficiently well studied in terms of the volume and frequency of gas eruption (including CH_4) for about a century; see, for example, [5]. The basic information on methane emission by mud volcanoes has been summarized in a series of publications [6, 7] (see also an important commentary on the latter one [8]). Etiope [9] made a rather detailed analysis of the geological sources of methane, and reviews [3, 4] may be recommended as the most comprehensive ones. Lately, methane seeps were discovered in the northern regions at the sites of thawing permafrost [10].

Given the extensive areas of oil- and gas-bearing sediments in Russia, wide distribution of seeps might be expected here. In particular, we revealed numerous methane-emitting vents or mud microvolcanoes (several centimeters in diameter) in the West Siberian mid-taiga in the floodplains of small rivers, tributaries of the Ob and Irtysh Rivers close to the site of their

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fusion [11]. Microbial communities developing around such mud microvolcanoes are of considerable interest. High concentrations of available methane, low temperatures (3–5°C), and near-neutral pH values are favorable for active development of psychrophilic methanotrophic microorganisms. However, we are not aware of any microbiological studies performed on continental methane seeps.

The goal of the present work was to evaluate the rates of CH_4 emission from methane seeps of the Ob–Irtysh floodplains and to identify the key microbial agents responsible for attenuating methane fluxes from these sources.

MATERIALS AND METHODS

Study site. Field studies of mud microvolcano abundance and methane emission rates were carried out in August–September 2009 in the valley of the Mukhrina River and its tributaries flowing to the Irtysh River floodplain, near the Mukhrino international field stationary located in the West Siberian mid-taiga on the left-bank terrace of the Irtysh River, south-southwest of Khanty-Mansyisk (60°53′ N, 68°42′ E). The Ob–Irtysh floodplain in the studied area is predominantly presented by meadows with multiple stagnant water pools and streams that get filled with water flow from the rivers Ob and Irtysh in the second half of May. The valleys of the incoming small rivers and springs also get flooded. When the water level in Ob and Irtysh falls down, water leaves small rivers and springs, resulting in rapid development of meadows and wetlands dominated by *Carex aquatilis* [12].

Methane emission measurement. The main method to measure methane emission by mud microvolcanoes was the method of water replacement with gas in a graduated cylinder. A funnel was placed on top of a mud microvolcano (so that it was completely submerged under the water), to serve as a collector for emitted gas. On top of the funnel, a graduated cylinder filled with water was placed, so that its lower edge would also be below the water surface; water in the cylinder was displaced by gas emitted from the microvolcano. Dynamics of gas emission was recorded over time. The second method used to measure methane flux was the classic static chamber method [13, 14].

Concentration of CH_4 in the samples was determined using a Kristall-5000 chromatograph (Khromatek, Yoshkar-Ola, Russia).

Sample collection for analysis. To identify methanotrophic bacteria developing at the sites of gas emission, sludge samples were collected from a nameless mud microvolcano (60°53.358′ N, 68°42.486′ E) in the floodplain of the Mukhrina River in September 2009. Conductivity of the surface water covering the microvolcano was determined using an SG-3 (Mettler Toledo, Switzerland) portable conductivity meter. Water pH was measured by an SG-2 portable pH meter (Mettler Toledo, Switzerland).

An aliquot of collected sludge was used as an inoculum to obtain enrichment cultures of methanotrophic bacteria, while the rest of the sample was frozen at –20°C for subsequent molecular analyses.

Molecular identification of methanotrophic bacteria. Extraction of total DNA was performed using the FastDNA SPIN kit for soil (Bio101, United States) according to the manufacturer's recommendations. DNA extraction was performed in triplicate, using three weighed portions of sludge (0.5 g each). DNA samples were stored at –20°C.

Total DNA extracted from the sludge was used as a template in polymerase chain reaction (PCR). PCR amplification was performed with the primers A189f (GGNGACTGGGACTTCTTG) and A682r (GAASGCNGAGAAGAASGC) specific to the *pmoA* gene coding for the active-site polypeptide of particulate methane monooxygenase (pMMO) [15]. DNA from the methanotrophic bacterium *Methylocystis heyeri* $H2^T$ was used as the positive control. PCR was performed in a PE GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems, United States) thermocycler. The reaction mixture contained 0.5–1.0 μL DNA, A189f and A682r primers, 1 μL each, 50 μL MasterMix (Promega), and sterile water to the total volume of 100 μL. The thermal profile of the reaction was as follows: initial denaturation (1 min at 94°C); 33 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 55°C), and elongation (1 min at 72°C); followed by terminal elongation (7 min at 72°C). PCR products were examined by electrophoresis in 1.2% agarose gel followed by ethidium bromide staining and visualization of reaction products under a UV transilluminator. Products of three independent reactions obtained with the template DNA of three extracts were pooled together and used for further cloning. Cloning was performed using the pGem-T Easy Vector System II (Promega) according to the manufacturer's recommendations. Recombinant clones were selected using the blue-white screening. Additional verification of the selected clones for the presence of the cloned fragments of specific length (approximately 530 bp) was achieved by direct amplification of the fragments–inserts using vector-specific primers T7 and Sp6 (Promega). The temperature profile of the reaction was as follows: initial denaturation (1 min at 94°C); 35 cycles of DNA denaturation (1 min at 94°C), primer annealing (1 min at 52°C), and elongation (1.5 min at 72°C); and terminal elongation (7 min at 72°C). The PCR products were then analyzed by electrophoresis and the clones carrying the DNA fragments of specific length were selected. The clones were used to isolate plasmid DNA with a Wizard Plus Minipreps DNA Purification System (Promega). Nucleotide sequences of the cloned DNA fragments were determined with the M13F primer 5'-GTTTTCCCAGTCACGAC-3' on an

ABI 377A (Perkin-Elmer Applied Biosystems) sequencer.

Editing of the nucleotide sequences was performed using the SeqMan (Lasergene 7.0; DNA Star Package) software package. Phylogenetic trees were constructed using the ARB software package [16].

Nucleotide sequences of the *pmoA* gene fragments determined in this study were deposited in GenBank under accession nos. KC844881–KC844913.

Methanotrophic enrichment cultures and analysis of their composition. To obtain enrichment cultures of methanotrophic bacteria, 1 g of sludge was added to a 500-mL vial with 100 mL liquid medium of the following composition (g/L): $NaNO_3$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; 100 mM phosphate buffer, pH 7.0, 1% (vol/vol); and trace element solution for methanotrophs 0.1% (vol/vol) [17], containing the following (g/L): EDTA, 5; $FeSO_4 \cdot 7H_2O$, 2; $ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; $CoCl_2 \cdot H_2O$, 0.2; $CuCl_2 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.02; and Na_2MoO_4, 0.03. Methane (up to 10–20% vol/vol) was added to the gas phase and the vials were incubated either under static conditions at 4°C or upon shaking (120 rpm) at 20°C during 2–3 weeks.

An aliquot (0.5 mL) of the enrichment culture was fixed with 0.4% formaldehyde solution and used to identify methanotrophic bacteria by fluorescence in situ hybridization (FISH). Probes specific to type I methanotrophs (M84 + M705) and to the *Methylosinus/Methylocystis* group of type II methanotrophs (M450) were used [18]. Synthesis of Cy3-labeled probes was performed by Syntol (Moscow, Russia). The samples were prepared, hybridized with probes, and stained with 1 μM solution of the universal DNA-specific dye 4',6-diamidino-2-phenylindole (DAPI) as was described previously [19]. The samples were analyzed using a Zeiss Axioplan 2 (Jena, Germany) epifluorescence microscope.

Isolation and identification of methanotrophic bacteria. To isolate methanotrophic bacteria, two approaches were used: (1) plating on solid medium and (2) serial dilutions in liquid medium. In the first case, enrichment cultures were plated onto agar medium and incubated for 4–5 weeks at 20°C in desiccators with 20–30% methane in the gas phase. Colonies formed on the plates were examined under a phase-contrast microscope and transferred onto the same medium. To purify the culture, up to ten consecutive transfers of isolates were performed. In the case of serial dilutions, enrichment culture suspension (1 mL) was repeatedly diluted fivefold in 35-mL serum vials containing 5 mL medium. The vials were sealed and methane was added with a syringe to the concentration of 25% in the gas phase. The vials were incubated on a shaker (130 rpm) at 24°C. Culture grown in the vail with the highest dilution was examined under a microscope and subjected to further transfers. The procedure was repeated until a culture of morphologically identical cells was obtained.

Identification of the isolates was performed by the 16S rRNA and *pmoA* gene sequencing. PCR amplification of the 16S rRNA genes was performed using universal bacterial primers [20] and those for the *pmoA* genes, as described above for seep sludge analysis.

Analysis of growth characteristics of the isolates depending on temperature. Comparative analysis of growth characteristics of the isolates was performed by monitoring the dynamics of their growth in liquid mineral medium with methane under static conditions at 4, 10, and 20°C. The experiment was performed in triplicate. Growth dynamics of methanotrophic isolates were determined by measuring OD_{600} of the cultures on an Eppendorf Biophotometr AG spectrophotometer.

RESULTS AND DISCUSSION

Methane emission from mud microvolcanoes and their physicochemical characteristics. Field studies performed in floodplains of the Mukhrina River revealed over 50 distinct mud microvolcanoes (Fig. 1) over approximately 3 km distance along the river bed. The true number of methane seeps in this region probably exceeded several hundreds, since only those that were covered with water and were easily identified by gas bubbles or the relatively large ones were detected (some examples are presented on Fig. 1b). While similar, although less abundant and smaller, seeps were revealed in the channel near the Shapsha settlement approximately 40 km to the northwest of the study site; no studies were performed there and only the results obtained with the samples collected in the Mukhrina River floodplain are discussed below.

According to single-time measurements performed for the large majority of the microvolcanoes, methane flux did not exceed 0.5 g CH_4 h^{-1} during the study period, although in some sites it reached over 5.5 g CH_4 h^{-1} (Fig. 2). Such high intensities of fluxes suggest that mud microvolcanoes may be considered important methane sources, which are characteristic of the swampy floodplains of northern rivers. Intensity of CH_4 emission from these local sources exceeded the values typical of Western Siberian bogs of the mid-taiga zone by 3–5 orders of magnitude [13, 21, 22], although the total area of these sources was incomparably smaller than the total area of the bogs in the region.

In the water layer above the mud microvolcanoes, pH varied within the range of 6.6–7.1. Water temperature at the surface was 5–6.5°C, and at 45-cm depth it declined to 3–4.5°C. Therefore, the microbial community of this ecological niche was formed under conditions of high methane concentrations, low temperatures, and near-neutral pH values. To identify methanotrophic bacteria developing under these conditions,

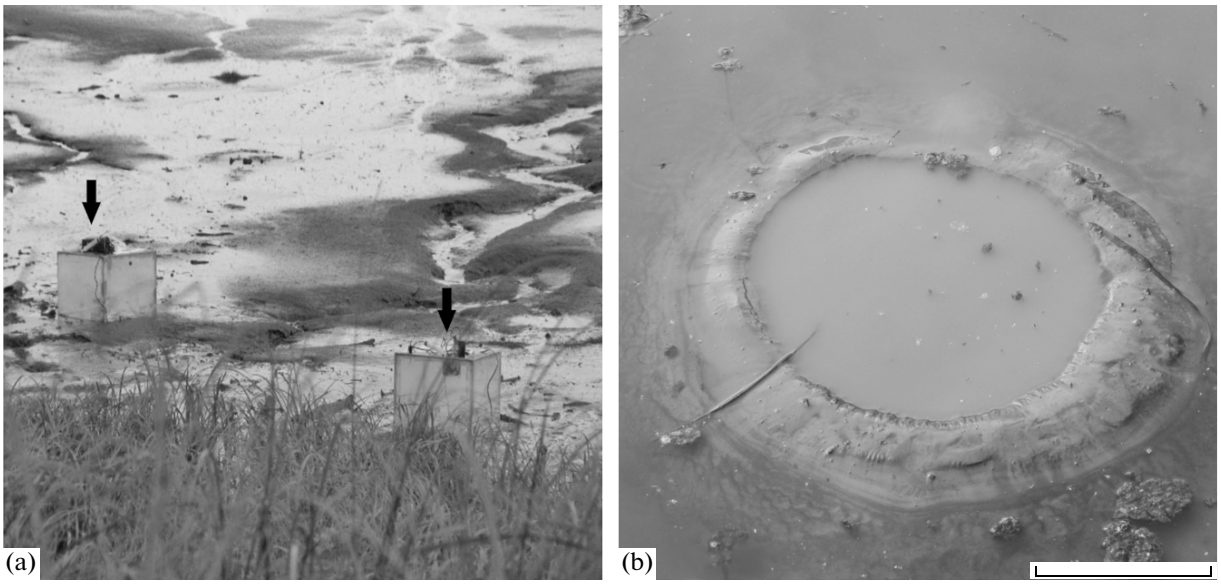

Fig. 1. Field measurements of methane emission in the floodplain of the Mukhrina River. Arrows indicate the chambers installed at the sites of methane seeps (a). A mud microvolcano (b). Bar length 3 cm.

we used the molecular approach based on analysis of the fragments of the *pmoA* gene coding for the key enzyme of methanotrophs.

Molecular identification of methanotrophs developing at the sites of methane emission. Isolation of DNA from sludge samples followed by amplification of the DNA fragments with the primers A189f and A682r and cloning produced a library of 32 *pmoA* and 15 *amoA* clones. The comparative analysis of the nucleotide sequences of the cloned *pmoA* fragments and those from GenBank database showed that the sequences retrieved from the seep sludge fell into four groups (Fig. 3).

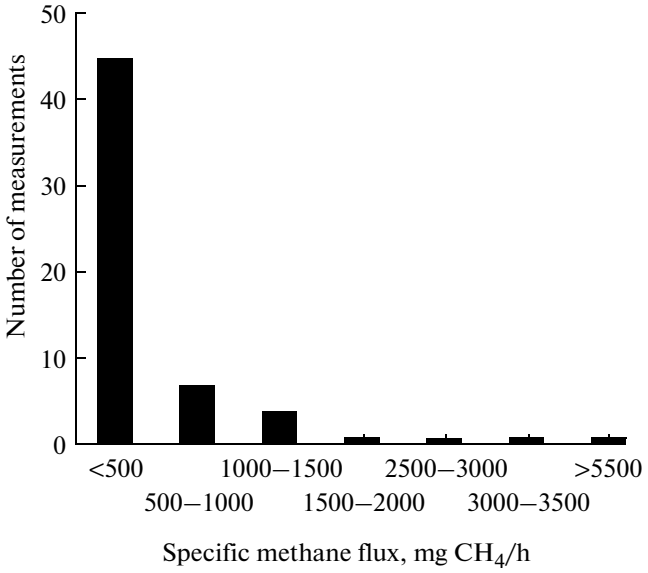

Fig. 2. Distribution of methane flux intensities from mud microvolcanoes. The plot was built using the data of single-time measurements of methane fluxes from mud microvolcanoes revealed in the course of the study in the floodplain of the Mukhrina River.

The first group of sequences comprising 22 clones represented type I methanotrophs (class *Gammaproteobacteria*) of the *Methylobacter* genus group. Six of them (clones V2, V3, V9, V24, V30, and V81) exhibited high similarity (98–99%) with the PmoA sequence of *Methylobacter psychrophilus*, a psychrophilic methanotroph isolated from tundra soil [23]. Nucleotide sequence of the V6 clone was nearly identical to PmoA of another psychrotolerant representative of the genus, *Methylobacter tundripaludum* [24]. The remaining 15 cloned PmoA sequences demonstrated only 92–96% similarity to PmoA fragments of previously characterized methanotrophs, apparently representing unknown species of the genus *Methylobacter*. A series of similar *pmoA* sequences was revealed earlier in cold highland regions of China (deposited in GenBank under accession nos. GQ906794 and GQ906782).

The second group of cloned PmoA sequences represented type II methanotrophs (class *Alphaproteobacteria*). The sequences revealed in seep sludge were 98–99% identical to the respective PmoA fragments of representatives of the genus *Methylocystis*, i.e., *Methylocystis heyeri* isolated from northern peat bogs [25] and *Methylocystis rosea* from arctic tundra soil [26].

The third group of clones comprised five PmoA sequences close to those of the poorly studied filamentous methanotroph *Crenothrix polyspora*, a stagnant water dweller, which has not been isolated in pure culture [27]. Finally, the remaining three clones comprised the fourth group of PmoA sequences exhibiting

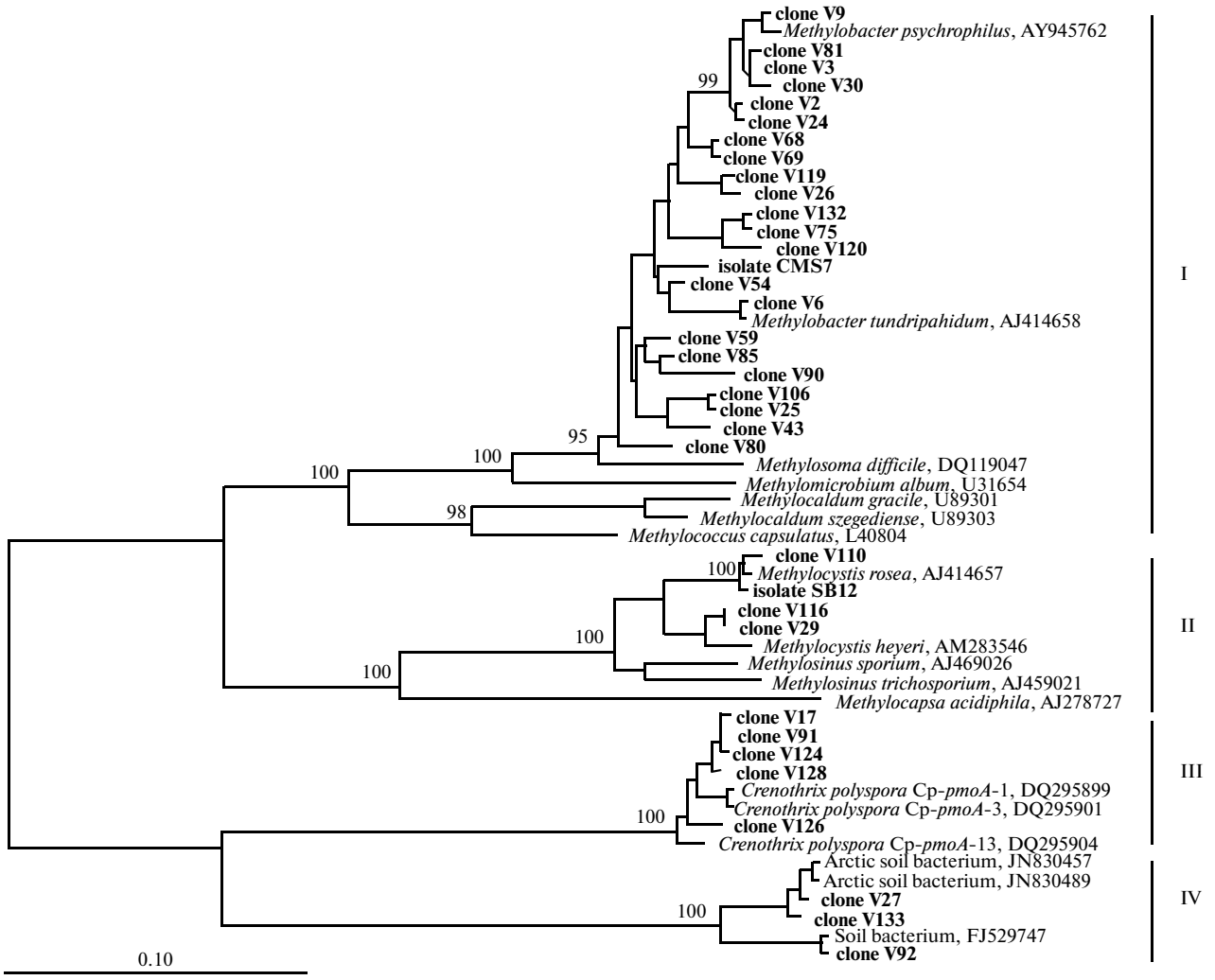

Fig. 3. Dendrogram constructed based on the results of comparative analysis of translated amino acid sequences of the *pmoA* gene fragments retrieved from the seeps, *pmoA* sequences of known type I and type II methanotrophs, and *pmoA* fragments of methanotrophic bacterial isolates obtained in the work. I, type I methanotrophs (*Gammaproteobacteria*), II, type II methanotrophs (*Alphaproteobacteria*), III, *Crenothrix*-like organisms, and IV, group of uncultured methanotrophs. Scale bar, 0.1 substitutions per 1 amino acid position.

only a distant similarity (48–55%) with the sequences of characterized methanotrophic bacteria.

Thus, only a small fraction of the *pmoA* gene diversity revealed in methane seep sludge could be attributed to the cultured and taxonomically characterized representatives of methanotrophic bacteria. Notably, all these methanotrophs were isolated from cold ecosystems. Those *pmoA* gene sequences that could not be attributed to known methanotrophs exhibited high similarity with the *pmoA* gene fragments retrieved from various cold habitats, such as arctic soils, *Sphagnum* peat bogs, benthic methane seeps, and lake sediments.

Composition of enrichment cultures of methane-oxidizing microorganisms. In the samples of methane-oxidizing enrichments obtained by 2-week incubation at 4°C, methanotrophs constituted up to 40% of the total number of microbial cells. The major component of the communities formed under these conditions were type I methanotrophs, with the cells clearly detectable by hybridization with Cy3-labeled probes M84 + M705 (Fig. 4). The proportion of the cells of type II methanotrophs, detected by the M450 probe, was insignificant. Thus, the composition of the obtained enrichment cultures correlated well with the results of molecular analysis of methanotrophs in the native sludge.

Isolates of methanotrophic bacteria and their growth characteristics. By means of multiple serial dilutions in liquid medium and incubation under static conditions at 4°C, a pure culture of a methanotrophic bacterium, strain CMS7, was obtained. The cells of this strain were large (1–1.5 × 2–4 μm motile, non-pigmented rods growing homogeneously in liquid medium, but forming no colonies on the same

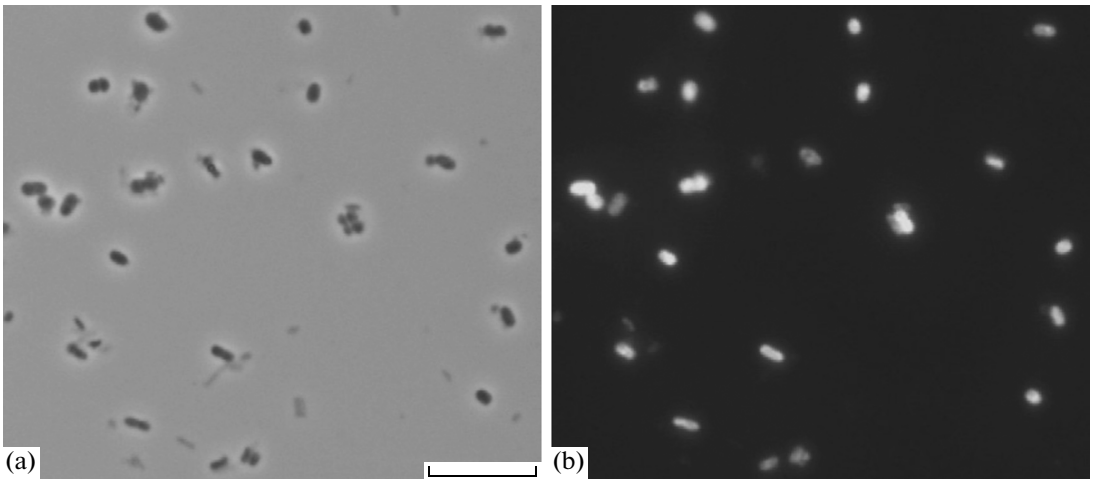

Fig. 4. Composition of methanotrophic enrichment cultures obtained from the sludge seeps upon incubation at 4°C. Phase contrast microscopy image (a) and fluorescent microphotograph of hybridization with Cy3-labeled probes M84 + M705 specific for type I methanotrophs (b). Scale bar, 10 μm.

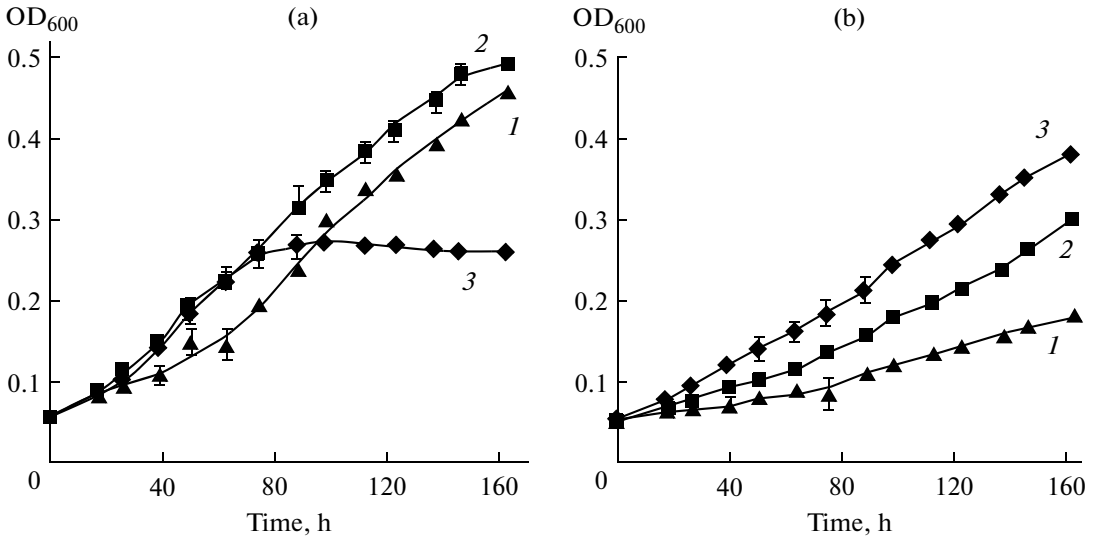

Fig. 5. Growth dynamics of *Methylobacter* sp. CMS7 (a) and *Methylocystis* sp. SB12 (b) on liquid mineral medium with methane upon incubation at 4 (*1*), 10 (*2*), and 20°C (*3*).

medium with agar. Comparative analysis of the *pmoA* gene sequence of strain CMS7 revealed that it belonged to the genus *Methylobacter* and, in particular, to the most abundant *pmoA* gene clone group detected in the seep sludge (Fig. 3). Nucleotide sequence of the 16S rRNA gene of strain CMS7 exhibited 98% similarity to that of *Methylobacter tundripaludum*.

The second isolate of methanotrophic bacteria, strain SB12, was obtained by plating the enrichment culture suspension onto agar medium and incubating it at 20°C. The cells of this isolate were nonmotile bent rods or coccoids (0.8–1.5 × 1–2.5 μm) forming small (1–2 mm in diameter) pink-pigmented colonies. The sequence of the PmoA fragment of strain SB12 exhibited 99.4% similarity with the cloned sequence V110 and was identical to that of *Methylocystis rosea* (Fig. 3). Sequences of the 16S rRNA genes of these two methanotrophs were also highly similar (99%).

The isolates were used for comparison of the growth characteristics of methanotrophs of two phylogenetic groups isolated from a single habitat depending on temperature. For this purpose, dynamics of *Methylobacter* sp. CMS7 and *Methylocystis* sp. SB12

growth in liquid mineral medium with methane were followed at three different temperatures of incubation, 4, 10, and 20°C (Fig. 5). The most active growth of *Methylobacter* sp. CMS7 occurred at 4 and 10°C, with specific growth rates at these two temperatures being almost the same. *Methylocystis* sp. SB12, although it was able to grow at 4°C, exhibited preference for growth at 20°C, which is typical of other representatives of the genus *Methylocystis*.

In summary, we demonstrated that mud microvolcanoes or cold seeps, which are widespread in the floodplains of northern rivers, are potentially important sources of methane emission to the atmosphere. Methane-oxidizing microbial communities developing around the seeps are dominated by type I methanotrophs, phylogenetically close to the genus *Methylobacter*, which are able to develop actively at low temperatures. Unknown organisms, whose *pmoA* gene sequences are only distantly related to those of characterized methanotroph species, are also present in methane-oxidizing communities. Exploration of the nature of these organisms is of considerable interest for further studies.

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