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EXPERIMENTAL ARTICLES

Aerobic Methanotrophs from the Coastal Thermal Springs of Lake Baikal

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Abstract—The number, activity, and diversity of aerobic methanotrophic bacteria in the sediments of three coastal thermal springs of Lake Baikal were analyzed. The average number of methanotrophs was 10^3–10^4 cells per 1 cm^3 of sediment. The highest number of methanotrophs (10^8 cells/cm^3 of silt) and the highest potential rate of methane uptake [7.7 nmol CH_4/(cm^3 day)] were revealed in sediments from the Sukhaya thermal spring. The methods of molecular ecology (DGGE, FISH, analysis of *pmoA* gene fragments) showed the predominance in most enrichment cultures of methanotrophs of type II, i.e., of the genera *Methylocystis* and *Methylosinus.* In only one enrichment culture (from the Sukhaya thermal spring), a type I methanotroph was revealed; its similarity to *Methylococcus capsulatus* Bath did not exceed 80%. These results demonstrate a widespread occurrence and high activity of the aerobic methanotrophic community in the coastal thermal springs of Lake Baikal.

Key words: Lake Baikal thermal springs, methane uptake rate, methanotrophs, *pmoA* gene.

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Aerobic methanotrophs are a structurally and functionally specialized group of gram-negative bacteria that utilize methane and methanol as a sole carbon and energy source. Methanotrophs are widespread in nature and play an important role in the global carbon cycle as the basis for the bacterial filter that reduces the emission of methane and other greenhouse gases into the atmosphere [1, 2]. In the past 50 years, the biology of methanotrophs and processes of methane oxidation in freshwater, marsh, soil, and marine ecosystems have been studied rather completely [1, 3]. On the contrary, the data on the diversity and activity of methanotrophic communities from thermal springs are fragmentary [4–8].

Previous studies in the thermal springs Alla, Kuchiger, and Seya located in the rift zone of Lake Baikal have shown an active methane uptake by the soils of these springs and revealed the presence of thermotolerant methanotrophs of type II in enrichment cultures [9]. At the same time, the processes of methane uptake and the composition of methanotrophic communities have not been investigated in some of the thermal springs on the coast of Lake Baikal, which has defined the goal of this work.

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MATERIALS AND METHODS

Determination of the physicochemical parameters of the habitat. The water temperature at sampling points was measured by a Prima sensor electric thermometer (Portugal). The medium pH was determined potentiometrically by a pHep field pH-meter (Portugal). The total mineralization value was determined by a portable TDS-4 tester conductometer (Singapore).

Measurement of the potential rate of methane uptake and the number of methanotrophs. The potential rate of methane uptake in soils was determined by the radioisotope technique. The samples (1 cm^3) were placed into 12-ml sterile vials and covered with 2 ml of twofold diluted P medium [3] (0.5P), followed by addition of $^{14}CH_4$ (0.5–2.0 μCu, Izotop, Russia). The vials were incubated at a temperature close to the in situ temperature for 10 days under stationary conditions. The process was stopped by adding 0.5 ml of 10% NaOH. The samples were then treated according to the known technique [10, 11].

The number of methanotrophs was determined by the method of tenfold dilution with inoculation of soil samples into a 0.5P aerobic liquid medium, followed by incubation with radiolabeled methane for 6–10 days. The presence and activity of methanotrophs were assessed by the $^{14}CH_4$ uptake.

Table 1. Characteristics of the coastal thermal springs of the Lake Baikal

Characteristics of thermal spring	Thermal spring		
	Sukhaya	Zmeiny	Goryachinsk
Coordinates	N 52°32′, E 107°04′	N 53°45′, E 103°00′	N 52°59′, E 108°18′
Temperature, °C	45	45	51
pH	9.1	9.4	9.0
Mineralization, mg/dm^3	0.5	0.3	0.5
Type of water	hydrocarbonate–sodium	hydrocarbonate–sulfate–sodium	sulfate–sodium
Type of spring by CH_4 gas composition, ml/dm^3, dissolved	methane	nitrogen	nitrogen
	7.6	–	0.09

Note: symbol "–" means "no data available"

Isolation of enrichment cultures. Silt samples (about 5% of the medium volume) were introduced into 750-ml flasks with 100 ml of the 0.5P medium. The pH value was adjusted as consistent with the natural samples: 7.2 with a phosphate buffer (final concentration 0.05 M) and pH 9.0 with a carbonate buffer (0.05–0.2M). Incubation was carried out in the atmosphere of methane and oxygen (1:1) for 1–2 weeks at 29 and 37°C on a rotary shaker (140 rpm). The process of accumulation of methanotrophic bacteria at sequential transfers was controlled in a Jenaval light microscope (Germany).

Electron microscopy. Cell fixation, preparation of ultrathin sections on a Reichert ULTRACUT System ultramicrotome (Austria), and their analysis in a Jeol JEM 100B electron microscope (Japan) were carried out as described [12].

DNA isolation and PCR amplification. Isolation of DNA from the enrichment cultures and PCR amplification were carried out as described previously, using oligonucleotide primers A189f and mb661r for the functional *pmoA* gene [13].

Denaturing gradient gel electrophoresis (DGGE). PCR products obtained for the DGGE analysis were amplified with the primer A189f possessing a GC clamp. The resultant PCR products were separated in polyacrylamide gel with a 35–80% gradient. Gradient gel electrophoresis was carried out at 200 V for 5 h in Dcode, Bio-Rad (United States). Individual bands were excised and eluted. Repeated PCR analysis was performed with the eluted product.

The nucleotide sequences of the PCR products were determined in a CEQ 2000XL Beckman Coulter (United States) automatic sequencer using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer, United States) in accordance with the manufacturer's instructions.

Phylogenetic analysis. Translated amino acid sequences of the region of the *pmoA* gene (460 bp) were compared with the GenBank sequences using the NCBI BLAST software package (http//www/ncbi.nlm.nih.gov/Blast). The translated amino acid sequences of the region of the *pmoA* gene were aligned with the Clustal W 1.6 software package. The phylogenetic tree was constructed using the Treecon W 1.3 software package.

Hybridization of the enrichment cultures with fluorescence-labeled oligonucleotide probes (FISH). For the analysis, the enrichment cultures were fixed with 4% formaldehyde solution in a phosphate buffer (g/l): NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44; NaH_2PO_4, 0.2; pH 7.0. The fixed samples were mixed with 100% ethanol, 1 : 1 (vol/vol), and stored at –20°C. Hybridization of the samples with oligonucleotide probes and the counting of the target microbial cells were carried out by the methods described previously [14]. The cells of type I methanotrophs were counted using the M-84 probe, and the cells of type II methanotrophs (*Methylosinus trichosporium, Methylocystis, Methylocapsa, Methylocella*) were counted using the following probes: Msint-1268, Mcyst-1432, Mcaps-1032, and Mcell-1026, respectively. These probes labeled with the Cy3 fluorescent dye were synthesized by Sintol (Moscow, Russia).

RESULTS AND DISCUSSION

Characteristics of thermal springs. Soil samples were collected from the thermal springs Zmeinyi, Goryachinsk, and Sukhaya located on the eastern coast of Lake Baikal. The thermal springs under study are characterized by mineral streams flowing out of spring outlets and falling into Lake Baikal. The samples were taken along the flow of each spring. Water temperature was 45°C at outlets of the Zmeiny and Sukhaya thermal springs and 51°C in the Goryachinsk spring. The pH values varied from 9.0 to 9.4. The physicochemical characteristics of the studied thermal springs are presented in Table 1.

Methane uptake rate. Aerobic methanotrophs were found in all soil samples; in most of them, their

Table 2. Number of methanotrophic bacteria and potential rates of methane uptake in the soils of the Lake Baikal coastal thermal springs

Thermal spring	Sampling site	*T*, °C	Number of methanotrophs, cells/cm^3	Potential rate of $^{14}CH_4$ uptake, nmol/(day cm^3 of silt)	Oxidation to CO_2, %	Assimilation, %
Sukhaya	C3 (spring outlet)	45	10^4	0.8	63	37
	C4 (2 m from the outlet)	36	10^8	7.7	44	56
	C-5 (pond)	1.5	10^5	0.7	14	86
Zmeiny	Z1 (spring outlet)	45	10^3	0.8	75	25
	Z3 (5 m from the outlet)	40	10^3	0.8	50	50
	Z6 (zone of mixing with the lake water)	19	10^4	1.0	40	60
Goryachinsk	G1(spring outlet)	51	10^3	0.6	67	33
	G4 (50 m from the outlet)	4.5	10^3	0.7	57	43
	G6 (zone of mixing with the lake water)	2.5	10^4	0.8	63	37

number was 10^3–10^4 cells/cm^3. The highest number of methanotrophs (10^8 cells/cm^3) was revealed in the Sukhaya spring (Table 2). The obtained values for methanotrophs were much higher than their numbers in the deepwater sediments of Lake Baikal: 10^2–10^3 cells/cm^3 [15].

The rate of methane uptake in the soils from the springs under study varied from 0.2 to 7.7 nmole CH_4/(cm^3 day). The highest rate of methane uptake was noted in the Sukhaya thermal spring. Methane uptake had the same order of magnitude as the rate of this process in other thermal springs of Buryatia (Alla, Kuchiger, and Seya) but exceeded methane oxidation rates in the deepwater's bottom sediments of Lake Baikal [9, 15].

When exposed with $^{14}CH_4$, a considerable part of radiocarbon (25–86%) was incorporated into the cell biomass and extracellular metabolites, as compared with the deepwater's bottom sediments of Lake Baikal, where methane was mostly oxidized to CO_2. However, the portion of radiocarbon incorporated in the biomass and metabolites was higher in the Baikal sediments where an additional methane discharge and inflow of mineralized waters occurred [15]. These facts suggest more favorable conditions for methanotrophs in the thermal springs under study.

At the outlets of the thermal springs, where the highest temperature was registered, the values of the rate of methane uptake were rather low. This finding can probably be explained by low solubility of methane at high temperatures. Further downstream, where the temperature was lower, the rate of methane uptake increased.

In all the springs, the rate of methane uptake correlated with the number of methanotrophs. The highest number of methanotrophs and rate of methane uptake were noted in the Sukhaya spring, probably due to the high concentration of dissolved methane.

Diversity of methanotrophs in enrichment cultures from the soils of the thermal springs. Nine primary enrichment cultures were obtained to characterize methanotrophic communities in the samples from coastal thermal springs. PCR amplification of the region of the *pmoA* gene encoding the *a*-subunit of membrane-bound methane monooxygenase gave positive results in the DNA of all of the enrichment cultures. The obtained amplicons were analyzed by the DGGE method. Comparison of the sequenced products with the GenBank sequences revealed the predominance of type II methanotrophs. The phylogenetic tree of translated amino acid sequences of the *pmoA* gene demonstrates the closeness of methanotrophs in enrichment cultures to the genera *Methylocystis* and *Methylosinus* (Fig 1). On the contrary, the methanotroph found in the enrichment culture isolated from the Sukhaya spring exhibited up to an 80% similarity to *Methylococcus capsulatus* Bath (140 amino acids were compared), which is indirect evidence of an affiliation with a novel taxon of methanotrophs.

The dominance of type II methanotrophs is confirmed by electron microscopy of ultrathin sections, where peripheral intracytoplasmic membranes (ICM) were found. The enrichment cultures isolated from the Goryachinsk and Zmeiny springs also showed the dominance of type II methanotrophs with the ultrastructural organization close to that of *Methylocystis echinoides* 2 (Fig. 2).

FISH hybridization of the cells from enrichment cultures showed that the methanotrophic communities from the Zmeiny, Sukhaya, and Goryachinsk thermal springs were characterized by the dominance of representatives of three genera of type II methanotrophs: *Methylocystis, Methylosinus,* and *Methylocapsa.* Members of the genus *Methylocystis* constituted the majority (up to 99%) of the total number of type II methanotrophs. Methanotrophs of type I were found only in the

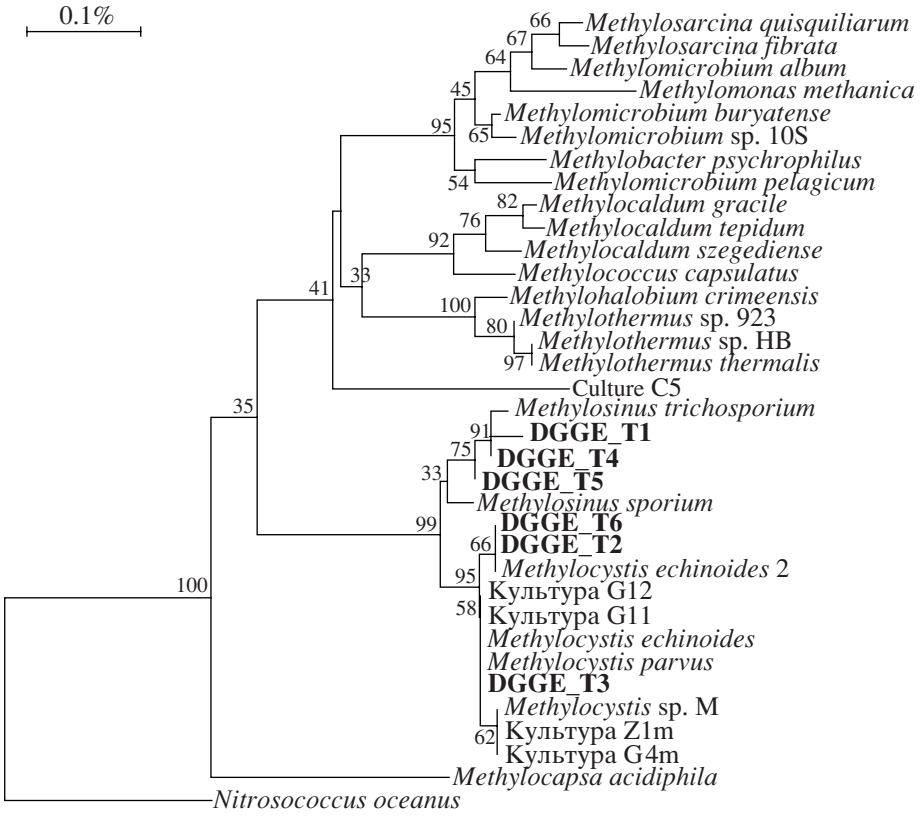

Fig. 1. Phylogenetic tree constructed on the basis of translated amino acid sequences of the *pmoA* gene fragment amplified in the DNA of associative methanotrophic cultures and the sequences obtained for the primary enrichment cultures by the DGGE method.

cultures from the springs Goryachinsk and Sukhaya, where their number did not exceed 1% of the total number of methanotrophic bacteria revealed in the enrichments.

Five "associative" cultures were subsequently isolated from the primary enrichment cultures, and each consisted of one methanotrophic species associated with heterotrophic satellites (Table 3). Phylogenetic analysis of the *pmoA* gene in the DNA of four cultures showed their affiliation with the genus *Methylocystis*. One "associative" culture, C5, isolated from the Sukhaya thermal spring, belonged to type I methanotrophs.

Since different methods of molecular ecology (DGGE, FISH, and *pmoA* gene analysis) were used in the study of the enrichment and "associative" methanotrophic cultures, it was possible to compare the results obtained by these methods. All of the methods showed the dominance of type II methanotrophs in all the cultures. A Type I methanotroph was revealed in the primary enrichment cultures by the DGGE method and was later identified in an "associative" culture by the *pmoA* gene analysis. The composition of methanotrophic communities determined by DGGE included bacteria of the genera *Methylosinus* and *Methylocystis*, whereas the FISH method showed that, apart from these organisms, the members of the genus *Methylocapsa* were present in enrichment cultures. Only the genus *Methylocystis* was detected by the analysis of diversity of "associative" methanotrophic cultures using the *pmoA* gene. These findings suggest the effi-

Table 3. Characteristics of "associative" methanotrophic cultures from the soils of the Lake Baikal coastal thermal springs

Culture	Source of isolation	Color	Predominant morphotyp	Type of methanotroph
C5	Sukhaya	Yellow	Vibrioids	I
G11	Goryachinsk	Pink	Bean-shaped cells	II
G12	Goryachinsk	Pink	Rounded cells	II
G4m	Goryachinsk	Yellow	Vibrioids	II
Z1m	Zmeiny	Yellow	Vibrioids	II

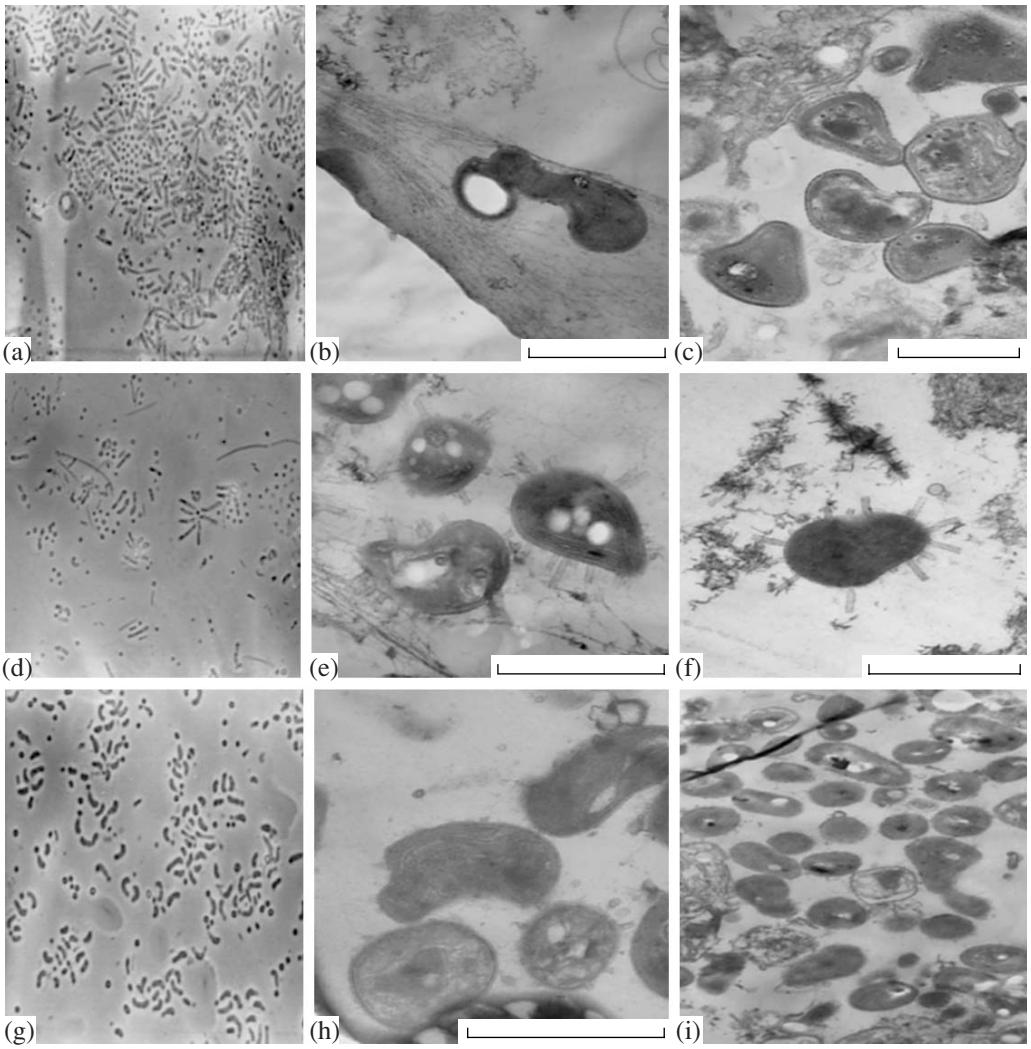

Fig. 2. Morphology and ultrastructure of the cells of some methanotrophic enrichment cultures: a, Z1; b, C5; c, C4; d, G1; e, Z3; f, G4; g, Z6; h, C3; i, G6. Scale bar: 1 μm.

ciency of the DGGE method for assessment of the diversity of methanotrophic communities in enrichment cultures without time-consuming isolation of pure cultures. Application of several methods gives more reliable information about the diversity of methanotrophic communities, which has also been shown by comparison of serological and molecular methods [16].

Thus, the wide distribution and activity of aerobic methanotrophs was revealed in the soils of coastal thermal springs of Lake Baikal. The diversity of methanotrophic communities in enrichment cultures obtained from the soils of the coastal thermal springs of Lake Baikal was confirmed by molecular techniques. Representatives of type II methanotrophs were shown to dominate; among them, the following species were identified: *Methylosinus trichosporium, Methylocystis* sp. M, *Methylocystis echinoides* 2, *Methylocystis parvus*, and *Methylocapsa* sp. Only one culture contained a representative of type I methanotrophs, probably affiliated with a new taxon. Previously, representatives of type II methanotrophs have been shown to predominate in enrichment cultures from the soils of other thermal springs of Buryatia and in the bottom sediments of deepwater regions of Lake Baikal [9, 15], though methanotrophic communities of different water bodies are known to be based on type I methanotrophs [17]. The reason for these differences may be preferential accumulation of type II methanotrophs in the enrichments under standard cultivation methods. The diversity of methanotrophs should be studied in situ by molecular methods to find out whether type II methanotrophs actually predominate in the silts of the Baikal thermal springs.

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