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EXPERIMENTAL ARTICLES

Investigation of the Methanotrophic Communities of the Hot Springs of the Uzon Caldera, Kamchatka, by Molecular Ecological Techniques

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Abstract—The methane-oxidizing microbial communities inhabiting the bottom sediments of 36 hot springs of the Uzon caldera (Kamchatka, Russia) located in the thermal fields Vostochnoe, Oranzhevoe, and Severnoe, as well as near the lakes Fumarol'noe and Khloridnoe and the Izvilistyi stream, were studied. Methanotrophic bacteria were detected by PCR and FISH in only 8 hot springs. The highest numbers of copies of the *pmoA* gene (molecular marker of methanotrophy) (2.8×10^7 and 1.1×10^7 copies/mL sediment) were detected in the Kul'turnyi and Kvadrat springs; however, in other springs, the numbers of the *pmoA* gene copies were significantly lower (5.4×10^3–2.8×10^6 copies/mL sediment). By using the FISH method, only type I methanotrophs were detected in these springs, with their percentage ranging from 0.3 to 20.5% of the total number of eubacteria. PCR–DGGE analysis of the *pmoA* gene showed that the diversity of methanotrophs was extremely low (no more that two components). Analysis of the deduced PmoA amino acid sequences demonstrated that methanotrophic bacteria of the genus *Methylothermus*, closely related to representatives of two valid species, widely occurred in the thermal springs near Lake Fumarol'noe. Other bacteria differing considerably from the detected *Methylothermus* species were detected as well. In the springs with low pH values (2.6–3.8), methanotrophic *Gammaproteobacteria* most closely related to the genera *Methylomonas* and *Methylobacter* were detected for the first time.

Keywords: terrestrial hydrotherms, microbial communities, methanotrophs, methane oxidation, in situ hybridization, PCR, DGGE, *pmoA*

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Methane is one of the most important greenhouse gases. It accumulates thermal radiation 20 times more effectively than CO_2, therefore having an active influence over the global climate. Investigations carried out over the last decade revealed a new natural source of methane, which is released into the atmosphere as a component of magmatic juvenile gases from fractures of the Earth's crust and is actively emitted into the atmosphere from areas of geothermal activity [1]. Methane emission from this "geological" source (which has not been previously considered as part of the global methane pool) comprises, according to a recent assessment, about 10% of the total methane emission, being second only to CH_4 emission from bog soils [2].

The Uzon caldera (Kamchatka, Russia) is a unique area of contemporary volcanism. Hydrothermal activity was detected within a narrow east-west oriented zone along the central line of the caldera. In the Uzon caldera, there are four hydrothermal fields, Zapadnoye, Severnoe, Vostochnoe and Oranzhevoe, as well as Lake Fumarol'noe. In these fields, there are numerous boiling gryphons, mud pools, small volcanoes, and hot platforms with steam and water vents and temperatures varying from 45 to 98°C. About 160 groups of thermal springs with temperatures up to 98°C inhabited by different communities of thermophiles are presently known. Russian microbiologists obtained priority data on the activity and composition of the communities of litho- and organotrophic thermophilic bacteria and archaea inhabiting the high-temperature springs of the Uzon caldera [3]; the majority of microbiological studies were carried out in the Vostochnoe thermal field.

Until recently, high-temperature prokaryotic communities inhabiting the regions of intense volcanic and geothermal activity were considered to be incapable of methane oxidation due to high temperatures and sulfur gas concentrations, as well as to low pH values and oxygen concentrations, which were considered unfa-

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vorable for the known species of methanotrophic bacteria [4]. Recent investigations demonstrated the in situ methane consumption [5, 6] and methanotrophic activity in the hydrothermal fields of the Mediterranean [7]. The extremely acidophilic and thermophilic methanotrophic representatives of the phylum *Verrucomicrobia* were isolated from hot springs in Italy [8] and New Zealand [9]. However, data on the composition of the methanotrophic communities of these ecosystems are scarce.

Microorganisms inhabiting the hot springs of Kamchatka and utilizing methane and/or other C_1 compounds are poorly studied. From one of the Kamchatka hot springs with an acidic pH (3.5), methane-oxidizing microorganisms of the phylum *Verrucomicrobia* were isolated [10]; however, any other data on the methanotrophic bacteria inhabiting the hot springs of Kamchatka are lacking in literature.

In the course of our 2008 expedition to the Uzon caldera, molecular biological investigation revealed the presence of methanotrophs in water and mat samples collected from five hot springs near Lake Fumarol'noe [11]. In 2010, molecular biological analysis of the samples collected from 36 hydrothermal springs located in different zones of the Uzon caldera was carried out in order to determine the numbers of methanotrophs and the composition of methanotrophic communities.

MATERIALS AND METHODS

Subject of study. In the course of our expedition to the Uzon caldera (July, 2010), samples of sedimentary material were collected from the vents of 36 hot springs with temperatures and pH ranging from 37°C to 86.6°C and from 2.6 to 6.8, respectively, located in the thermal fields Vostochnoe, Oranzhevoe, and Severnoe, as well as near the Izvilistyi stream and the lakes Fumarol'noe and Khloridnoe. The physicochemical parameters (temperature, pH, and Eh) were determined at the moment of sampling using a pH-420 portable pH meter (Aquilon, Russia).

DNA extraction and PCR analysis of methanotrophs. The total DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, United States) according to the manufacturer's protocol with minor modifications. To enhance the yield of microbial DNA from bacterial cells, the cells were treated with the lysing buffer at high temperature (80°C) and agitation. The *pmoA* gene fragments encoding the β subunit of the membrane-bound methane monooxygenase (MMO), the key enzyme of methane oxidation, were amplified using a system consisting of the degenerate oligonucleotide primers A189F (5'-GGN GAC TGG GAC TTC TGG-3') and A682R (5'-GAA SGC NGA GAA GAA SGC-3') [12]. Further separation of the amplicons by DGGE was performed using two-round nested PCR amplification; for the second round of amplification, the direct A189F primer containing a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CGG-3') [13] and the reverse mb661 primer (5'-CCG GMG CAA CGT CYT TAC C-3') [14] were used. The first-round PCR product was used as a template for the second round of amplification. 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), 5 pmol of each dNTP, 25 pmol of each primer, and 0.6 U of Taq DNA polymerase (Helicon Co., Russia). The reaction mixture was supplemented with dimethyl sulfoxide (DMSO, 5%) to facilitate amplification of GC-rich fragments for DGGE. 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 0.5°C every new cycle; 72°C for 45 s; 94°C for 30 s, 52°C for 30 s, 72°C for 45 s, 15 cycles; and final elongation for 5 min at 72°C (first round); 94°C for 2 min; 94°C for 1 min, 55°C for 1.5 min, 72°C for 1 min, 25 cycles; and final elongation for 5 min at 72°C (second round). Amplification was performed on a MyCycler thermal cycler (BioRad, United States).

Analysis of the PCR products was carried out by electrophoresis in 1.2% agarose gel stained with ethidium bromide. The gel slabs were digitalized with a Gel Doc gel imaging and documentation system (BioRad, United States). PCR fragments for further study were extracted and purified using the QiaQuick Gel Extraction Kit (Qiagen, United States) according to the manufacturer's recommendations.

Real-time quantitative PCR. The end-point PCR reaction mixture (25 μL) contained the following: 1× BioTaq DNA polymerase buffer ($(NH_4)_2SO_4$, 17 mM; Tris-HCl, 67 mM; $MgCl_2$, 2 mM; pH 8.8), 12 pmol of each dNTP, 20 pmol of each primer, 1.5 U of BioTaq DNA polymerase (Dialat Ltd., Russia), and 25 ng of DNA template. The PCR cycle parameters were as follows: 94°C for 5 min; 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, 30 cycles; and final elongation for 5 min at 72°C. PCR amplification was performed on a Mastercycler Gradient amplifier (Eppendorf, Germany).

Analysis of PCR products was carried out by electrophoresis in 1% agarose gel at 6 V cm^{-1}. The results of electrophoresis were digitized using a BioDocII documentation system (Biometra, Germany).

The obtained PCR fragment was purified using the QIAquick-spin PCR purification kit (Qiagen, Hilden, Germany). The concentration of the PCR product was determined on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, United States). The calibration curve was obtained using a series of replicate dilutions of the PCR product containing from 10^8 to 10^2 copies/μL.

Real-time PCR reaction mixture (25 μL) contained 1× PCR buffer B, Taq DNA polymerase, deoxynucleoside triphosphates, glycerol, Tween 20, the

intercalating dye SYBR Green I and the passive reference dye ROX (Syntol Co., Russia), and 5 pmol of each primer and DNA template. Amplification was performed in a CFX96^{TM} DNA amplifier (Bio Rad, United States). The PCR cycle parameters were as follows: polymerase activation, 95°C for 3 min; 95°C for 30 s, 56°C for 30 s, and 62°C for 1 min, 40 cycles.

The number of gene copies in the studied specimens was calculated on the basis of the calibration curve and the PCR product concentration. All reactions were performed in triplicate.

In situ hybridization with fluorochrome-labeled oligonucleotide probes (FISH). The specimens (2 mL) were sonicated at 22 kHz for 40 s in order to ensure desorption of microbial cells from the sediment particles. Fixation of the samples with a paraformaldehyde solution and hybridization with fluorochrome-labeled oligonucleotide probes were carried out at 46°C according to the protocol described in [15]. For specific detection of type II methanotrophs, the probe M-450 (5'-ATC CAG GTA CCG TCA TTA TC-3') (30% formamide in the hybridization buffer) was used; type I methanotrophs were detected using a mixture of the probes M-84 (5'-CCA CTC GTC AGC GCC CGA-3') and M-705 (5'-CTG GTG TTC CTT CAG ATC-3') (20% formamide) [16]. The mixture of the universal probes EUB 338mix (EUB338 I, 5'-GCT GCC TCC CGT AGG AGT-3' *Eubacteria*; EUB338 II, 5'-GCA GCC ACC CGT AGG TGT-3' *Planctomycetales*; EUB338 III, 5'-GCT GCC ACC CGT AGG TGT-3' *Verrucomicrobiales*) was used for detection of representatives of the domain *Bacteria* [17]. The set of Cy3-labeled oligonucleotide probes applied in this study was synthesized by Syntol Co. (Russia).

Denaturing gradient gel electrophoresis 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 were separated at a constant voltage of 200 V in a denaturing gradient (formamide and urea, 35–60%) for 6 h. The gel slabs were stained with ethidium bromide and digitalized with a Gel Doc gel imaging and documentation system (Bio-Rad, United States). The specific dominant bands were excised; DNA was eluted, purified, and used as a template for reamplification. After reamplification, the obtained PCR product was purified using the QiaQuick Gel Extraction Kit (Qiagen, United States) according to the manufacturer's recommendations, and used for determination of the *pmoA* gene fragments.

Sequencing of the PCR products was performed in the service laboratory using the Big Dye Terminator v3 sequencing kit (Applied Biosystems Inc., United States). After reamplification, the obtained PCR products were sequenced with the primer mb661. Nucleotide sequence analysis was conducted in an ABI Prism 3100 automatic sequencer (Applied Biosystems Inc., United States).

Analysis of nucleotide sequences was performed using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST). Translation of the *pmoA* gene fragments was performed using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide sequences and the deduced amino acid sequences were edited and aligned with the appropriate sequences from the closest relatives using the same software package.

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://bioc-www.uia.ac.be/u/yvdp/treeconw.html).

Deposition of the nucleotide sequences. The obtained nucleotide sequences of the *pmoA* genes were deposited in GenBank under accession numbers JQ513935–JQ513944.

RESULTS

Studied area, hot springs. The studied hot springs were located in different zones of the Uzon caldera; most of them were found in the Vostochnoe field (18 springs), near Lake Fumarol'noe (6 springs), and near the Izvilistyi stream (8 springs). The properties of the studied springs are listed in Table 1.

The Vostochnoe field of the Uzon caldera has been studied in greatest detail. Numerous long-term investigations, including microbiological studies, were carried out. All the studied hot springs are characterized by low acidity (pH 5.3–6.8), high temperatures (48.7–86.6°C), and a mildly reducing environment (Eh from +24 to –107 mV). Near the Izvilistyi stream, six acidic springs (pH 2.6–3.8) with temperatures ranging from 40.6 to 69.2°C were studied. The redox status of the spring may be described as transient with the unstable geochemical regime and variable contents of hydrogen sulfide and oxygen. The third group of hot springs near Lake Fumarol'noe is characterized by low acidity, temperatures ranging from 56.1 to 65°C, and the transient redox status. One of these hot springs, Ryzhik, is characterized by transient conditions (+ 43 mV) and lower temperature (39°C).

Some springs in the Oranzhevoe and Severnoe fields are slightly acidic, high-temperature (65 and 70°C), and with reducing conditions (–37 and –2 mV). Hot springs near Lake Khloridnoe have different temperatures (37 and 64.1°C); however, they are slightly acidic and are characterized by the transient redox status.

Enumeration of methanotrophic bacteria by quantitative PCR and FISH. The presence of bacterial 16S rRNA genes in all the studied hot springs was detected by real-time PCR using the DNA extracted from sed-

iment samples as the template. In hot springs with neutral or slightly acidic pH, their number reached 10^8–10^9 copies/mL irrespective of the temperature. At the same time, in the samples taken from the springs located near the Izvilistyi stream (pH 2.6–2.8), it was significantly lower, not exceeding 10^5 copies/mL sediment.

In 10 out of the 36 studied springs, the number of copies of the *pmoA* gene, the most widely used molecular marker of methane oxidation, exceeded the detection level ($3–6 \times 10^3$ copies/ mL) (Table 2). The highest and the lowest numbers of gene copies differed by three orders of magnitude; the highest values (2.8 and 1.1×10^7 *pmoA* copies/mL) were detected in the Kul'turnyi and Kvadrat springs, respectively. In other springs, these values were considerably lower, ranging from 2.8×10^6 to 4.0×10^4 *pmoA* copies/mL.

The presence of methanotrophic *Gammaproteobacteria* in 8 springs was revealed by FISH using the probe M-84 + M-705 (Table 2). Type II methanotrophs (*Alphaproteobacteria*) were not detected in any of the studied springs. The numbers of methanotrophs varied within a wide range, from 1.3×10^4 cells/mL in the Glaz Drakona spring to 3.5×10^6 cells/mL sediment in the Sbornyi spring. The shares of methanotrophs in the bacterial communities of these springs (probe EUB 338 mix) reached 0.57% and 35%, respectively.

PCR–DGGE analysis of the *pmoA* gene fragment. For sediment samples collected in springs Izvilistyi (Izl5), Kometa (Km17), Sbornyi (Sbl8), Kul'turnyi (Ku19), Terra (T20), Stroma (S21), Kvadrat (Kv22), and Glaz Drakona (DY23), the product of the *pmoA* gene fragment of the expected size was amplified, and the amplicon mixture was separated by DGGE. The number of specific bands for each sample ranged from 1 to 3, indicating the low diversity of methanotrophic bacteria. The only exception was the Stroma spring, for which 8 well-defined separate bands in the polyacrylamide gel slabs were obtained. However, after reamplification and sequence analysis of these bands, all the sequences obtained were found to be identical (100% similarity of the deduced protein sequences) and exhibited high similarity with *Methylothermus subterraneus* sequences.

Phylogenetic analysis revealed the presence of methanotrophs most closely related to *'Methylothermus'* HB (figure), a thermophilic methanotroph which had been lost in pure culture [18], but significantly different from it (less than 90% similarity), in four springs located near Lake Fumarol'noe (Kul'turnyi, Terra, Stroma, and Kvadrat). In the samples collected in the Kul'turnyi and Kvadrat springs, methanotrophs exhibiting high similarity to the thermophilic methanotrophic HB strain were detected [18]. One of the two methane-oxidizing microorganisms identified in the Terra spring was found to be a quite distant relative of the HB strain and of the validly described methanotro-

Table 1. Properties of the studied hot springs

Sample no.	Site, spring name	pH	*T*, °C	Eh, mV
	Vostochnoe field			
1	Treshchinnyi	5.6	86.6	–90
2	Zavarzina	5.6	56.7	–30
3	Tsyklop	6.3	59.3	–41
4	Termofil'nyi	5.9	73.2	–107
5	Feniks	6.8	63.0	–8
6	Molochnyi	6.3	67.4	+14
7	Entselad	5.8	48.7	–56
8	Fobos	5.9	50.3	–60
9	Venera	6.1	59.6	–8
10	Vertoletnyi	5.9	64.5	–56
11	Deimos-1	6.5	49.6	+24
12	Zheltyi	5.2	66.8	–64
31	Deimos-2	6.2	65.3	ND
32	Yupiter	5.7	50.8	–13
33	Mars	5.9	63.1	–7
34	Zatsepina	6.2	78.8	–40
36	Saturn	5.2	76.3	ND
37	Prem'er	5.3	70.6	–93
	Izvilistyi stream			
13	Rodzher	5.0	69.7	+98
14	Geizeritovyi	5.1	85.7	–42
15	Izvilistyi stream (30 m from the vent)	2.6	40.6	+242
16	Mutnyi	3.8	69.1	+123
17	Kometa	2.8	51.1	+165
18	Sbornyi	2.7	54.2	+286
	Lake Fumarol'noe			
19	Kul'turnyi	5.2	63.0	+280
20	Terra	6.2	65.0	+273
21	Stroma	5.3	56.1	+205
22	Kvadrat	6.3	64.5	+178
23	Glaz Drakona	6.2	59.8	ND
24	Ryzhyk	6.6	39.0	+43
	Oranzhevoe field			
25	Atsyclik	6.0	65.0	–37
	Severnoe thermal field			
26	Lokon	6.0	70.0	–2
	Thermal site "Razvilka" near Lake Khloridnoe			
27	Tsytron	3.4	64.1	+60
28	Izvilistyi stream (near the boardwalk)	3.1	46.7	+203
	Thermal field near Lake Khloridnoe			
29	Kholodnyi	6.2	37.0	+153
30	Tretiy	5.3	64.1	+150

Note: ND, not determined.

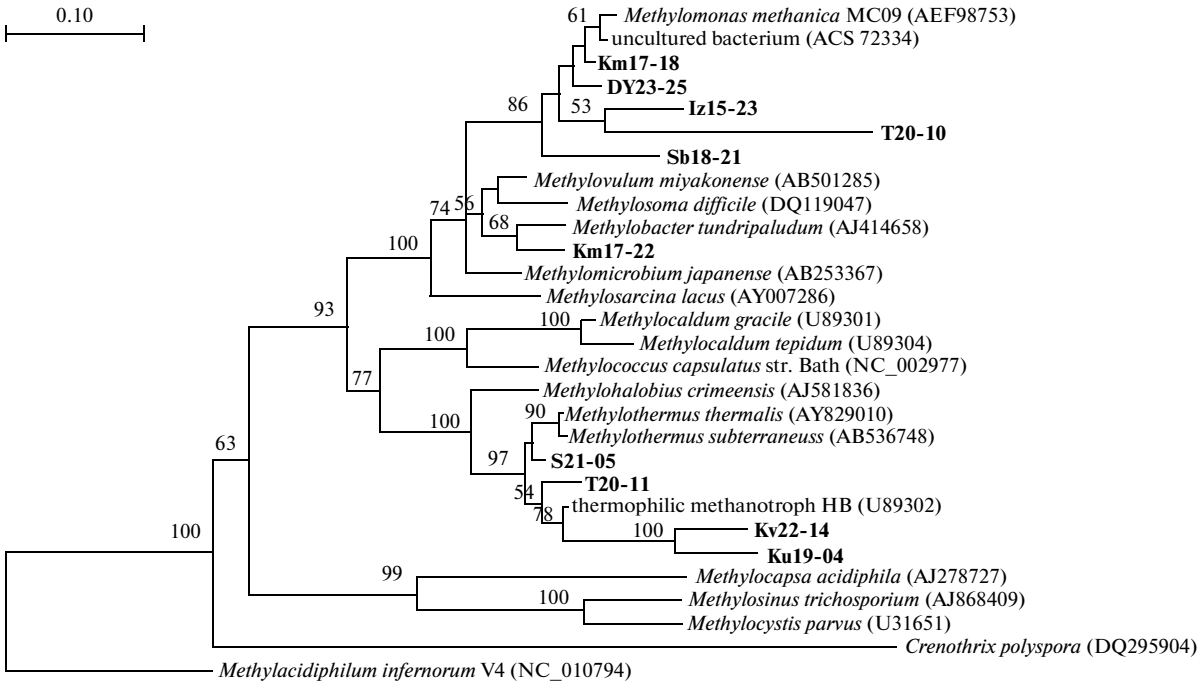
Table 2. Quantitative characteristics of the microbial community obtained by direct microscopic counts and real-time PCR

Spring	Number of cells/mL sediment*			Number of gene copies/mL sediment*	
	DAPI	EUB338 mix	M-84 + M-705	16S rRNA	*pmoA*
Kul'turnyi	$(2.40 \pm 0.22) \times 10^7$	$(9.70 \pm 0.39) \times 10^6$ (40.4%)**	$(1.50 \pm 0.46) \times 10^6$ (15.5%)***	$(10.57 \pm 1.26) \times 10^8$	$(2.83 \pm 0.25) \times 10^7$
Terra	$(1.10 \pm 0.39) \times 10^7$	$(4.20 \pm 1.40) \times 10^6$ (38.2%)	$(5.0 \pm 1.9) \times 10^5$ (11.9%)	$(30.43 \pm 0.08) \times 10^7$	$(1.12 \pm 0.28) \times 10^5$
Stroma	$(3.30 \pm 0.56) \times 10^7$	$(1.00 \pm 0.16) \times 10^7$ (30.3%)	$(1.90 \pm 0.61) \times 10^6$ (19.0%)	$(8.47 \pm 0.71) \times 10^8$	$(2.76 \pm 0.25) \times 10^6$
Kvadrat	$(3.60 \pm 0.45) \times 10^7$	$(2.10 \pm 0.33) \times 10^7$ (58.3%)	$(2.00 \pm 0.49) \times 10^6$ (9.5%)	$(2.42 \pm 0.02) \times 10^8$	$(1.13 \pm 0.20) \times 10^7$
Izvilistyi	$(3.30 \pm 0.12) \times 10^7$	$(2.00 \pm 0.07) \times 10^7$ (60.6%)	$(7.00 \pm 0.34) \times 10^5$ (3.5%)	$(9.50 \pm 2.40) \times 10^5$	$(4.67 \pm 0.19) \times 10^4$
Kometa	$(3.30 \pm 0.34) \times 10^7$	$(1.40 \pm 0.16) \times 10^7$ (42.4%)	$(1.50 \pm 0.61) \times 10^6$ (10.7%)	$(2.00 \pm 1.13) \times 10^5$	$(2.17 \pm 0.08) \times 10^4$
Sbornyi	$(1.70 \pm 0.22) \times 10^7$	$(1.00 \pm 0.15) \times 10^7$ (58.8%)	$(3.50 \pm 1.10) \times 10^5$ (35.0%)	$(5.00 \pm 0.98) \times 10^4$	$(0.20 \pm 0.00) \times 10^4$

Note: *Average ± standard deviation; ** % of the total number of microorganisms; *** % of the number of metabolically active eubacteria.

phs of the genus *Methylothermus*. *Methylothermus subterraneus* isolated from a subsurface geothermal water stream in a Japanese gold mine [19] was the closest relative of the methanotroph detected in the Stroma spring (S21). In the samples collected in the Terra and Glaz Drakona springs, the sequences T20-10 and DY23-25 most closely related to uncultured bacteria of the genus *Methylomonas* (less than 65% similarity) were detected, which has not been previously demonstrated for high-temperature springs.

Our experiments with the primers A189F and A682R and a low annealing temperature (in order to reduce the primer specificity) [8] did not reveal the



Phylogenetic tree constructed on the basis of the deduced amino acid sequences of the *pmoA* gene fragments. The sequences determined in the present work are in boldface. The GenBank accession numbers of the gene fragment sequences obtained are given in parentheses. Scale bar: ten amino acid substitutions per 100 amino acid residues. The numerals show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees (only bootstrap values above 50% are shown).

presence of methane-oxidizing *Verrucomicrobia* in the studied high-temperature springs with high acidity (Izvilistyi, Kometa, and Sbornyi). At the same time, the presence of methane-oxidizing *Gammaproteobacteria* was detected in these three hot springs. In all of these springs, the gammaproteobacterium *Methylomonas methanica* was found to be the closest cultivable relative of the isolated methanotrophs (sequences Izl5-23, Kml7-18, and SM8-21) (figure). In the Kometa spring, another methane-oxidizing microorganism (Km 17-22) exhibiting the highest similarity with *Methylobacter tundripaludum* was detected [20] (figure).

DISCUSSION

On Earth, biogenic methane is produced mainly by methanogenic archaea at the final stages of decomposition of organic matter under anaerobic conditions. In natural high-temperature ecosystems, other mechanisms of methane production are possible, such as abiogenous, based on the reaction of H_2 with CO, and thermogenous, when methane is the result of the physical processes of organic matter decomposition [21]. Usually, methane content in geothermal gases is 0.01–1% [22]; however, sometimes it may be much higher. For instance, as was reported earlier, the content of methane in the geothermal systems of New Zealand is 1–11% and sometimes as high as 27% [23].

Methane influx into the atmosphere from different terrestrial ecosystems depends on the activity of methane-oxidizing microorganisms, which form the so-called "methane filter". Before the beginning of our studies, data on methane oxidation and methanotrophic microorganisms in the Uzon caldera were scarce. The presence of methane-oxidizing *Gammaproteobacteria* in neutral hot springs near Lake Fumarol'noe [11] and of the methane-oxidizing bacterium *Methylacidiphilum* of the phylum *Verrucomicrobia* in the acidic hot spring [10] was revealed. At the same time, various thermal phenomena occur in the Uzon caldera, differing in the temperature and the mineral composition, which makes it reasonable to assume that the methanotrophic communities inhabiting the caldera may vary in composition and activity.

Our studies revealed the presence of dissolved methane in all the hot springs studied. All samples were therefore analyzed by real-time PCR for the presence of the *pmoA* gene, the molecular marker of methane oxidation. Despite the presence of methane, methanotrophs were detected only in 8 hot springs. The springs where methanotrophic bacteria were detected fall into two groups, namely neutral high-temperature hydrotherms near Lake Fumarol'noe and acidic springs with moderate temperatures located along the Izvilistyi stream. The results obtained correlate with the data on the methane-oxidizing activity determined by the radioisotope method (V. Galchenko, unpublished data).

The hydrotherms near Lake Fumarol'noe in which methanotrophic bacteria were detected are characterized by a slight variability in temperature and pH (56–65°C; pH 5.2–6.3). Both real-time PCR and direct microscopic counting confirmed that the cell count was high. The total number of bacterial cells (DAPI staining) was $1.1–3.6 \times 10$ cells/mL. The number of the 16S rRNA gene copies was 3–30 times greater, which may result from the high number of gene copies. On average, bacteria have four 16S rRNA gene copies per cell [24]; however, it is possible that thermophilic microorganisms may have more 16S rRNA gene copies per cell. For all springs, high numbers of the *pmoA* gene copies (up to 2.8×10^7 mL) were demonstrated.

The use of the FISH method allowed us to carry out a more differentiating analysis of methanotrophs from the sampled sediments. It was demonstrated that methanotrophs were represented only by the *Gammaproteobacteria* (probe M-84 + M-705), while the number of type II methanotrophs was below the detection limit. These results correlate with the published data on the predominance of type I methanotrophs in high-temperature habitats. *Methylococcus capsulatus* was the first thermotolerant methanotroph isolated in pure culture [25]. Later, other thermotolerant and moderately thermophilic microorganisms classified into the genus *Methylocaldum* were isolated [26]. The thermophilic HB strain growing in a temperature range from 40 to 72°C with a growth optimum at 62–65°C was isolated in 1999 from a hot spring in Hungary [27]. The authors suggested that it should be described as a new genus *Methylothermus*, but, unfortunately, the strain was lost during description. The first microorganism of the genus *Methylothermus* was validly described in 2005. At present, this genus consists of two species, *Methylothermus thermalis* [28] and *Methylothermus subterraneus* [19].

Despite the similar values of the hydrothermal parameters and numbers of microorganisms, assessment of the diversity of methanotrophs using the *pmoA* gene revealed substantial differences. To explain the possible reasons for this diversity, three hot springs—Stroma, Glaz Drakona, and Kul'turnyi—were compared (Table 3). In two of them, Stroma and Kul'turnyi, members of the genus *Methylothermus* were detected. However, these microorganisms exhibited high similarity with different representatives of this genus. For instance, a methanotrophic bacteria most closely related to *'Methylothermus'* HB isolated from a hot spring in Hungary was detected in the Kul'turnyi spring characterized by a high temperature and a low mineralization of water. At the same time, another methanotroph most closely related to *Methylothermus subterraneus* was detected in the Stroma spring with a high sulfate content. In the Glaz Drakona spring, only methanotrophs displaying the highest similarity to uncultured representatives of *Methylomonas* were detected. This spring differs from the other two by the high level of mineralization, especially by the contents

Table 3. Physicochemical properties, mineral composition of the water, and methanotrophs detected in the springs near Lake Fumarol'noe

Spring/coordinate position	*T*, °C	pH	Ion content, mg/L								Methanotrophs
			Cl^-	NO_3^-	SO_4^{2-}	Mg^{2+}	Ca^{2+}	Na^+	K^+	NH_4^+	
Stroma N 54°29.854′ E 159°59.477′	56.1	5.3	36.6	0	223	27	15	16.1	1.8	0	*Methylothermus subterraneus*
Kul'turnyi N 54°30.115′ E 159°59.279′	63.0	5.2	35	0	28	1.2	7.4	4	0.9	0	*'Methylothermus'* HB
Glaz Drakona N 54°29.885′ E 159°59.468′	59.8	6.2	249	0.9	51	5.7	14.8	385	28.3	2.2	*Methylomonas* sp.

of chloride, sodium, and potassium ions, as well as by the presence of mineral nitrogen compounds (ammonium and nitrate).

In the three springs with low pH values (pH 2.6–2.8; 40.6–54.2°C), the total number of microorganisms and the proportion of methanotrophs determined by means of FISH were as high as those detected in the hot springs located near Lake Fumarol'noe. Low numbers of gene copies (especially of the 16S rRNA gene copies) are noteworthy. This phenomenon may be due to the mineral composition of the studied springs. It is quite possible that in the course of DNA extraction from the sediment samples some components (fluorescent bacterial proteins or salts) were not completely removed and probably had an adverse effect on the real-time PCR background.

We failed to detect any methanotrophs of the phylum *Verrucomicrobia* in the studied acidic springs. It is possible that either their numbers were lower than the PCR detection limit, or the *pmoA* gene sequence differed from that of the *Proteobacteria* to the extent that the existing primer systems could not be used even when the procedure for decreasing the primer specificity was applied.

The presence of methanotrophic *Gammaproteobacteria* in the ecosystems with extremely low pH has not been previously reported. According to the published data, there is only one type I methanotroph isolated from an acidic habitat [29]. This microorganism inhabiting *Sphagnum* peat bogs with pH below 4.3 has not been validly described yet; however, its affiliation to the genus *Methylomonas* was demonstrated.

The results obtained made it possible to expand the range of data on the diversity of methanotrophs inhabiting extreme ecosystems, including the hot springs of the Uzon caldera (Kamchatka). The low level of similarity between the microorganisms detected and the previously described methanotrophs suggests that they belong to new species and genera. Further studies will include description of the new thermophilic methane-oxidizing microorganisms, as well as analysis of their physiological and biochemical properties.

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