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SHORT COMMUNICATIONS

# Molecular Detection of Methanotrophic Bacteria in the Hot Springs of the Uzon Caldera, Kamchatka

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Microbial communities inhabiting such extreme ecosystems as continental volcanogenic hot springs are of considerable interest for both basic and applied biotechnological studies. The currently available data on methanotrophs from high-temperature habitats are scarce [1]. The validly described species of thermophilic and thermotolerant methanotrophic bacteria include members of the genera *Methylothermus* [2, 3], *Methylococcus* [4], and *Methylocaldum* [5, 6]. The microbial communities inhabiting high-temperature springs of Kamchatka are of great interest to Russian scientists [7]; however, the data on the microorganisms utilizing methane and/or other $C_1$ compounds are fragmentary. Acidothermophilic methane-oxidizing representatives of the phylum *Verrucomicrobia* were isolated from the acidic hot springs of Kamchatka (pH 3.5) [8]. Methane-oxidizing activity was detected by the radioisotope method in the silt samples collected from the springs of the Vostochnoe Field of the Uzon Caldera, with the temperature not exceeding 60°C (Pimenov, unpublished data). There is, however, no data on the presence of methanotrophs in other hot springs of Kamchatka.

Thus, the goal of the present work was to detect aerobic methanotrophic bacteria in the sediments of the hot springs of the Uzon caldera using two molecular biological techniques: fluorescent in situ hybridization (FISH) and analysis of the *pmoA* gene encoding the β subunit of the particulate methane monooxygenase.

In July 2008, silt and cyanobacterial mat were sampled at 18 hot springs near Lake Fumarol'noe (Karbonatnoe Field, Kamchatka), and the composition of microbial communities was analyzed. In July, 2010, silt and cyanobacterial mat samples were collected from 35 hot springs located in the thermal fields Vostochnoe, Oranzhevoe, and Severnoe, as well as near the lakes Fumarol'noe and Khloridnoe and the Izvilistyi Spring. The studied hot springs differed in the temperature (40.6–85.2°C), acidity (pH 2.6–6.8) and redox potential (Eh from –307 to + 80 mV) values.

At the sampling site, aliquots (2 ml) of the samples were transferred to 120-ml vials with 20 ml of fivefold diluted P medium [9] (for the samples collected from springs with the near-neutral water pH) or with 20 ml of M medium [8] (for the samples collected from the springs with low pH values) and incubated under the air : methane atmosphere (1 : 1) in the Zavarzin Spring at 56.7°C for one week. The obtained enriched samples (primary enrichment cultures) were used for FISH and PCR, as well as for the isolation of enrichment cultures of methanotrophic bacteria. Enrichment cultures were obtained from enriched samples by sequential monthly transfers. The samples were incubated under static conditions at 60°C. The obtained cultures were analyzed after three months of incubation (3–4 transfers). Enumeration of bacterial cells and hybridization with oligonucleotide probes in enriched samples and enrichment cultures were carried out as described in [11]. For specific detection of type II methanotrophs, the probe M-450 was used; type I methanotrophs were detected using a mixture of the probes M-84 and M-705 [11]. The mixture of universal probes EUB 338mix was used for detection of representatives of the domain *Bacteria*; for detection of representatives of the phylum *Verrucomicrobia*, the probe EUB338 III was used [12]. DNA was extracted from enriched samples and enrichment cultures using the Ultra Clean Soil DNA Isolation Kit (MoBio, United States) and Wizard Genomic DNA Purification Kit (Promega, United States), respectively, according to the manufacturers' protocols. PCR amplification of the *pmoA* gene was performed using the system of degenerate oligonucleotide primers A189F and A682R. Cloning, sequencing, and analysis of the obtained sequences were carried out as previously described in [13]. Detection of methanotrophic *Verrucomicrobia* strains was performed by amplification of the *pmoA* gene fragments with the primers A189F and A682R at a low annealing temperature [14]. The obtained *pmoA* gene sequences were deposited in the GenBank under accession numbers JF496703–JF496706.

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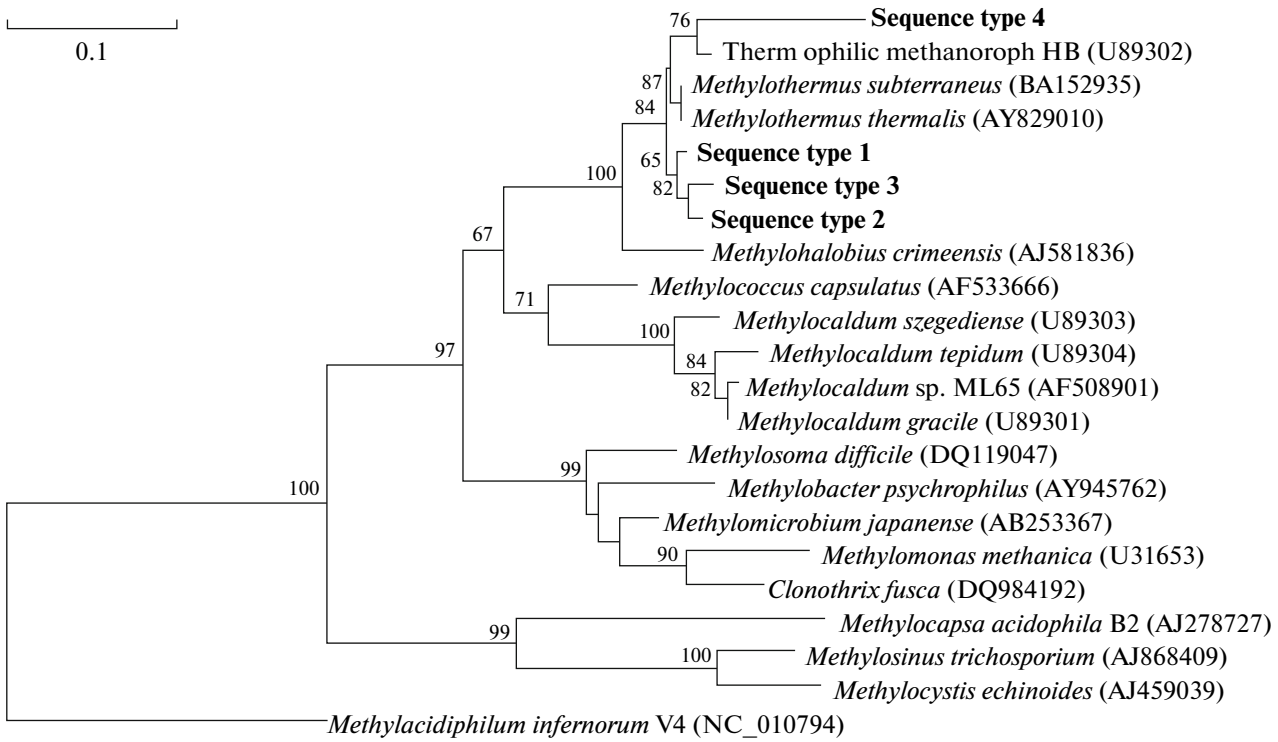


A phylogenetic tree constructed on the basis of deduced amino acid sequences of the *pmoA* gene fragments. The sequences determined in the present work are in boldface. The GenBank accession numbers of the gene fragment sequences used in this work are given in parentheses. Scale bar: ten amino acid substitutions per 100 amino acid residues. The numerals show the significance of the branching order as determined by bootstrap analysis of 500 alternative trees (only bootstrap values above 50% are shown).

Analysis of the enrichment samples by means of FISH revealed the presence of type I methanotrophs (*Gammaproteobacteria*) in the following springs: Kul'turnyi (N 54°30.115', E 159°59.279'; 63°C; pH 5.2), Kvadrat (N 54°29.862', E 159°C 59.485'; 64.5°C; pH 6.3), Molochnyi (N 54°29.962', E 160°00.653'; 67.4°C, pH 6.3), and Entselad (N 54° 29. 966', E 160°00.670'; 48.7°C, pH 5.8). However, the number of bacterial cells was low and did not exceed $10^3$ cells /ml sediment. No type II methanotrophs (*Alphaproteobacteria*) were detected in the studied springs; no *Verrucomicrobia* cells were detected in the samples collected from the following acidic springs: Izvilistyi (pH 2.6), Mutnyi (pH 3.8), Kometa (pH 2.8), Sbornyi (pH 2.7), and Tsitron (pH 3.4). PCR analysis of the *pmoA* gene fragment revealed the presence of methanotrophic bacteria in the sediments of only two hot springs, Kul'turnyi and Kvadrat. No methanotrophic *Verrucomicrobia* were detected by means of PCR amplification of the *pmoA* gene with a special protocol.

Five enrichment cultures of methanotrophic bacteria were isolated from the silt samples from the Kul'turnyi Spring, as well as from the hot springs Glaz Drakona (N 54°29.885', E 159°59.468'; 59.8°C; pH 6.2) and Stroma (N 54°29.854', E 159°59.477; 56.1°C; pH 5.3). We failed to detect methanotrophic bacteria in the enriched samples obtained from these hot springs. The enrichment cultures consisted of close associations of methane-oxidizing bacteria with heterotrophic satellites. The amount of type I methanotrophs was 6.0–7.8. × $10^6$ cells/ml medium or 37–70% of the total number of eubacteria (FISH).

Analysis of the *pmoA* clone libraries constructed for each enrichment culture revealed a low diversity of thermophilic methanotrophs. The enrichment culture K8 isolated from the hot spring Glaz Drakona contained two strains of methanotrophic bacteria, whereas all other cultures contained only one strain each. Phylogenetic analysis of the deduced nucleotide sequences (figure) revealed that all these sequences, together with the sequences of *Methylothermus* strains, formed a compact cluster. The culture K14 isolated from the Kul'turnyi Spring (type 4 sequence) showed high similarity to the thermophilic methanotroph HB [15]. Other sequences formed three sequence types and exhibited similarity to the *Methylothermus thermalis* isolated from a hot spring in Japan [2] and the *M. subterraneus* isolated from hot subsurface water-bearing strata of gold mines in Japan [3]. The sequence type 1 consisted of the *pmoA* gene sequences of the enrichment culture K8 (Glaz Drakona). The sequence type 2 included the sequences of the enrichment cultures obtained from silt sediments of the hot springs Glaz Drakona (K7) and Stroma (K12), as well as the *pmoA* gene sequences of the enrichment culture

K8. The sequence type 3 included the *pmoA* gene sequences of the enrichment culture K11 isolated from the silt samples collected in the Stroma spring.

Hence, molecular detection of methanotrophic bacteria in the representative samples from the hot springs of the Uzon caldera, Kamchatka, was carried out for the first time. Both the amount and diversity of methane-oxidizing bacteria in the studied hot springs were low, which probably can be attributed to the selective extreme conditions, i.e., to the low contents of dissolved methane and oxygen due to high temperatures and mineralization. All obtained methane-oxidizing enrichment cultures can be described as thermophilic, since no growth was detected at temperatures below 55°C. All detected methanotrophic bacteria were represented by the phylotypes most closely related to representatives of the genus *Methylothermus*, but were phylogenetically distant from the two validly described species of this genus.

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