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EXPERIMENTAL ARTICLES

Occurrence, Diversity, and Abundance of Methanogenic Archaea in Terrestrial Hot Springs of Kamchatka and SãoMiguel Island

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Abstract—Detection and analysis of the *mcrA* gene encoding methyl-coenzyme M reductase, the key enzyme of methanogenesis, was used to assess occurrence and diversity of methanogenic archaea in terrestrial hot springs of Kamchatka and São Miguel Island (the Azores). For this analysis, phylogeny of methanogens was initially reconstructed based on available sequences of the *mcrA* gene, which is a common functional and phylogenetic marker for this physiological group of prokaryotes. Methanogens were revealed in most of the studied terrestrial hot springs with temperatures from 51 to 89°C, although they constituted an insignificant portion of the microbial population. The *mcrA* gene sequences revealed in the samples belonged to members of the genera *Methanothermobacter, Methanothermus*, and *Methanothrix*, previously detected in hot springs, as well as to methanogens not found earlier in these environments. The latter belonged to *Methanomassiliicoccales, Methanocellales*, and *Methanomethylovorans*, as well as to MCR-2a, the new deep phylogenetic cluster of uncultured methanogenic archaea; its phylotypes were present in all springs where the *mcrA* gene was detected. Our results indicate high diversity of the thermophilic methanogens inhabiting terrestrial hot springs and the presence among them of new groups with yet unknown substrate specificity.

Keywords: methanogenic archaea, terrestrial hot springs, the *mcrA* gene, phylogenetic reconstruction, MCR-2a cluster of uncultured methanogens

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The processes of methanogenesis and methane oxidation are quantitatively important components of the global carbon cycle on Earth, and they have most probably played this role over the whole period of existence of the biosphere on our planet [1]. Methanogenic microorganisms transform to methane about 2% of the total carbon fixed by phototrophs [1]. From 500 to 600 Tg of methane enters the atmosphere annually [2, 3], and about 70% of this amount is a result of the activity of modern microbial communities [4].

Detection and identification of methanogenic archaea in complex microbial communities using their 16S rRNA gene sequences as a marker is hindered by the fact that this physiological group is not monophyletic [5, 6]. Therefore, the attention of several researchers was attracted by the gene encoding the α subunit of methyl-coenzyme M reductase (*mcrA*) [7, 8]. The results of numerous studies confirmed the efficiency of using this functional and phylogenetic marker for assessment of the diversity and distribution of methanogens [9, 10].

For certain thermal ecotopes, namely for oilfields, hydrothermal sediments, and black smokers, a number of studies of the diversity and distribution of methanogenic archaea have already been carried out [11–15]. Terrestrial hot springs remain, however, poorly studied in this respect.

The first evidence of the presence of methanogens in terrestrial hot springs was obtained in the course of studies of these habitats in the Yellowstone National Park [16]. Subsequent studies of Icelandic hydrothermal vents resulted in the isolation of two hyperthermophilic hydrogenotrophic methanogens representing a new family, *Methanothermaceae: Methanothermus fervidus* and *M. sociabilis* [17, 18]; they were subsequently shown to be endemic to this island [15]. In the hot springs of Kamchatka, intense methanogenesis was recorded by radioisotopic methods [19, 20]. In all of the hydrogen-utilizing enrichment cultures obtained from the Kamchatkan hot springs where methane formation was detected, growth of thin hydrogenotrophic rods phenotypically similar to *Methanothermobacter* representatives was observed [21]. Moreover, in 1982 Nozhevnikova and Yagodina reported the isolation from thermal lake sediment of a new filamentous organism incapable of hydrogen utilization and growing on acetate with methane formation, *Methanothrix thermoacetophila* [22]. Thus, currently, the diversity of methanogens isolated from terrestrial hot springs is restricted to representatives of the genera *Methanothermobacter, Methanothrix*, and *Methanothermus*.

The goal of the present work was molecular detection, identification, and evaluation of the abundance

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of methanogenic archaea in terrestrial hot springs of Kamchatka Peninsula (Russia) and São Miguel Island (Portugal).

MATERIALS AND METHODS

Site of sampling and sample collection and storage. Samples were collected in Uzon Caldera and Geyser Valley (Kamchatka Peninsula, Russia) and São Miguel Island (the Azores). Samples of water, sediments, silts, microbial mats, and biofilms from terrestrial hot spring were collected into 15- or 50-mL Falcon-type plastic tubes or 60-mL glass flasks. The tubes and flasks were completely filled with the samples, tightly closed, and transported to the laboratory, where they were stored at 4°C prior to analyses.

Molecular genetic methods. DNA from the samples was isolated by standard methods [23, 24]. For DNA isolation, the sample was resuspended in an equal volume of lysing solution (0.15 M NaCl, 0.1 M Na_2EDTA, pH 8.0) containing 15 mg/mL of lysozyme, and the mixture was processed using glass beads of various diameters and a FastPrep® 24 homogenizer (MP Biomedicals, United States) for sample homogenization and cell disruption. The homogenized mixture was incubated at 37°C for 40 min with thorough shaking every 10 min. After that, the mixture was supplemented with a buffer (0.1 M NaCl and 0.5 M Tris-HCl, pH 8.0) in an amount equal to that of the initial solution and with sodium dodecyl sulfate to a concentration of 0.5% and Proteinase K to a concentration of 100 μg/mL). The resulting mixture was incubated for 40 min at 50°C and then for 10 min at 60°C. The lysis efficiency was estimated by cell counting under a phase contrast microscope. DNA was extracted from the lysate by standard phenol–chloroform extraction and precipitated with 96% ethanol. The precipitate was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To remove the RNA admixtures in the resulting DNA solution, it was incubated with 0.2 mg/mL of RNase A for 2 h at 37°C. If necessary, DNA was also purified with a Wizard® DNA Clean-Up System kit (Promega, United States), intended for large DNA fragments. Qualitative and quantitative assessment of DNA preparations was performed on a DropSense-96® spectrophotometer (Trinean, Belgium).

PCR amplification, gel electrophoresis, cloning, denaturing gradient gel electrophoresis (DGGE), and quantitative PCR were carried out according to commonly accepted methods [23, 25–27]. Primary screening for methanogens was performed using two primer systems specific to the *mcrA* gene: MLf–MLr [9] and mlas–mcrA-rev [28].

Quantitative PCR was carried out using the SYBR Green I® intercalating dye and the ready-to-use qPCRmix-HS SYBR PCR master mix (Evrogen, Russia) on a StepOnePlus® Real-Time PCR System (Life Technologies, United States). The correlation coefficients were not lower than 0.97 for all of the calibration curves, and the reaction efficiencies were not lower than 70%.

Quantitative PCR that was performed in order to estimate the abundance of methanogenic archaea, total archaea, and bacteria in samples from the Thermophilny, Zavarzin, and 2012 hot springs used the primer systems mlas (F)–mcrA-rev, Arch931F–Arch1100R, and Bact338F–Bact907R, respectively [26, 29–31].

Analysis of the newly determined nucleotide sequences. The nucleotide sequences were edited and assembled using BioEdit 7.1.3 [32], translated into amino acid sequences, aligned with ClustalW [33], and combined into representative operational taxonomic units (OTUs) with cd-hit [34]. The OTUs obtained were checked for chimeras using Pintail [35]. Phylogenetic reconstruction was performed with ARB [36] using the Maximum Likelihood algorithm and nonparametric bootstrap analysis of 100 replicates.

RESULTS

At the first stage of this work, we performed phylogenetic reconstruction of methanogenic microorganisms based on the *mcrA* gene. More than six thousand complete and partial translated amino acid sequences corresponding to the *mcrA* gene were retrieved from GenBank and FunGene databases and combined into 366 representative OTUs. Of them, 211 represented uncultured microorganisms and 155 represented cultured methanogenic species (Fig. 1). This made possible reliable determination of the phylogenetic position of the microorganisms detected in the present work by phylogenetic analysis independent of a particular set of reference sequences.

At the next stage of the work, we analyzed samples from 16 hot springs located on Kamchatka Peninsula (Russia) and São Miguel Island (Portugal) and exhibiting diverse physicochemical characteristics (table). Sampled were microbial mats and biofilms, silts, sediments, and water of the hot springs. Primary screening for the *mcrA* genes by PCR with the *mcrA*-specific primer pairs MLf–MLr and mlas–mcrA-rev yielded amplicons for 12 of the 16 investigated hot springs (table). The amplicons were separated by DGGE, and the resulting bands were sequenced. After translation, the sequences pertaining to particular samples were combined into OTUs with the amino acid sequence identity threshold of 95% (table).

Further phylogenetic analysis, performed by repeated construction of the global McrA tree (including the OTUs revealed in the present study) showed that these OTUs represented several taxonomic groups of cultivated methanogens (*Methanothrix, Methanocellales, Methanobacteriales*, and *Methanomassiliicoc-*

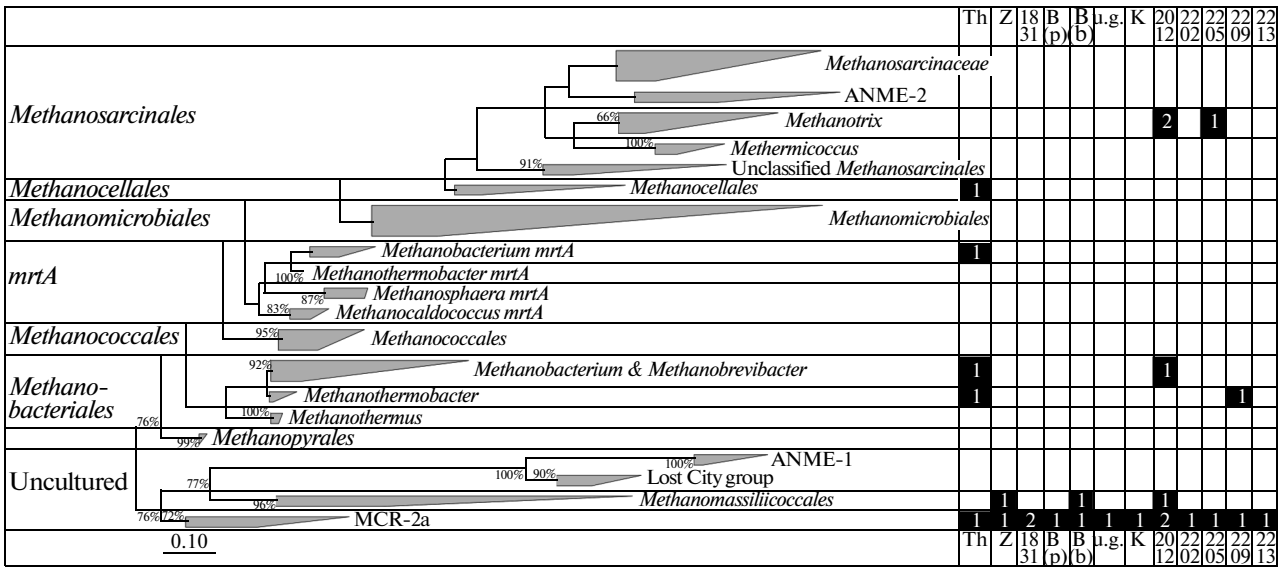

Fig. 1. Phylogenetic tree constructed based on the analysis of amino acid sequences of the α subunit of methyl-coenzyme M reductase, including OTUs revealed in the present work. The tree was constructed with ARB software using the Maximum Likelihood algorithm and bootstrap analysis of 100 alternative trees (values below 50% are not shown). On the right, black squares arranged in a tabular form indicate the detection of particular groups in particular samples. The numerals in the squares show OTU numbers. Designations of the columns are spring designations or abbreviated spring names (Th, Thermophilny; Z, Zavarzin; B(p), Bourlyashchy (pool); B(b), Bourlyashchy (brook); u.g., underwater grotto; K, Kukhonny).

cales) and the MCR-2a cluster of uncultivated microorganisms [37] (Fig. 1).

The highest methanogen diversity was revealed in two Kamchatkan hot springs: 2012 (Geyser Valley) and Thermophilny (Uzon Caldera). In these springs, the water had pH ~6 and temperatures of 60 and 70°C, respectively. For samples from these springs, as well as for the sample from Zavarzin spring, quantitative PCR was carried out to evaluate the abundance of bacteria, total archaea, and methanogenic archaea (Fig. 2). This analysis showed methanogens to constitute a minor portion of the microbial populations. Their highest abundance, which was recorded in the 2012 spring, did not exceed 0.1% of the total prokaryotic population.

DISCUSSION

Our large-scale reconstruction of the phylogeny of methanogenic archaea, which used over 6000 translated nucleotide sequences of the *mcrA* genes, allowed us to bring in order the currently available voluminous data on the *mcrA*-based detection of uncultured microorganisms and to carry out phylogenetic analysis independent of a particular set of reference sequences. No discrepancies were revealed between the phylogenetic positions of cultured methanogens determined using the *mcrA*-based and 16S rRNA gene-based approaches, which demonstrates lack *mcrA* horizontal transfer events and confirms the high efficiency of using this functional and phylogenetic marker in studies of the diversity and distribution of methanogenic and methanotrophic archaea.

Previous radioisotopic studies revealed processes of lithotrophic and aceticlastic methanogenesis in hot springs of the Uzon Caldera with temperatures of 55–96°C and pH 4.2–7.0 [19–21]. Enrichment cultures that produced methane from hydrogen, acetate, and methanol were obtained in wide ranges of temperature and pH [19]. Representatives of the genus *Methanothermobacter* were assumed to be the main agents of lithotrophic methanogenesis at 60–70°C and pH 7.0–8.5 [21]. These methanogens are also components of thermophilic syntrophic associations that utilize methanol [38, 39, and unpublished data of A.Y. Merkel].

The results of our present work with samples taken from terrestrial hot springs of Kamchatka and São Miguel Island show that the gene encoding the α subunit of methyl-coenzyme M reductase is present in the microbial communities of the springs with temperatures from 51 to 89°C and neutral or slightly acidic pH. The *mcrA* amplicons were obtained for 12 of the 16 hot springs investigated. Apart from members of the genera *Methanothermobacter* and *Methanothrix*, found earlier in Kamchatkan hot springs, we revealed the *mcrA* genes of methanogenic archaea of the orders *Methanocellales* and *Methanomassiliicoccales* and of uncultured microorganisms of the order *Methanobacteriales*. However, the results of quantitative PCR show low abundance of methanogens in the springs studied.

In our study, representatives of *Methanocellales* were detected only in a sample from the Thermophilny

Characteristics of the terrestrial hot springs studied in this work

Spring name or designation	Sample type	Geographical location			*T*, °C	pH	*mcrA**
Thermophilny	Gray films	Kamchatka Peninsula	Uzon Caldera	East thermal field	72	6.3	+ (5)
Zavarzin	Mat				57	6.2	+ (2)
Treshchinny	Black silt				74	6.5	–
1831	White films				77	6.5	+ (2)
Bourlyashchy (pool)	Silt			Central thermal field	89	6.0	+ (1)
Bourlyashchy (brook)	Mat				52	7.7	+ (2)
Underwater grotto	Silt				79	5.7	+ (1)
Kukhonny	Silt			Lake Vosmerka	51	5.9	+ (1)
2012	Films		Geyser Valley		58	5.7	+ (6)
2009	Silt				91	7.9	–
2202	Silt	São Miguel Island, Portugal			80	7.5	+ (1)
2203	Silt				49	2	–
2205	Black silt				66	6.5	+ (2)
2209	Films				74	5.7	+ (2)
2210	Grayish-green mat				61	6.5	–
2213	White films				75	5.5	+ (1)

* The positive or negative PCR result is shown; in parentheses is the number of OTUs detected.

spring, where they shared the econiche with representatives of the genera *Methanobacterium* and *Methanothermobacter* and with uncultured methanogens of the MCR-2a cluster. The sample was taken in the hottest site of the spring: near the vent, where the temperature was 72°C. Inflow of surface waters carrying mesophilic organisms to this site is hardly possible; therefore, it may be assumed that the order *Methanocellales* includes thermophilic species. Earlier, representatives of this order were detected in rice fields and were then isolated therefrom; these isolates are mesophilic hydrogenotrophic methanogens [40–42]. In hot springs, *Methanocellales* representatives have never before been detected.

Representatives of the order *Methanomassiliicoccales* were detected by us in three Kamchatkan hot springs with temperatures of 52–58°C. Earlier, *Methanomassiliicoccales* phylotypes were detected in termite and cockroach guts and mammal intestinal tracts [43]. *Methanomassiliicoccus luminyensis*, a representative of this order isolated in a pure culture, was obtained from a human feces sample; it produces methane in the course of growth on methanol in the presence of hydrogen [44]. An analogous type of metabolism is featured by another representative of this order isolated from the activated sludge of a thermophilic (55°C) methanogenic reactor and described as *Candidatus* "Methanogranum caenicola" [45]. Thus, the order *Methanomassiliicoccales* includes thermophilic methanogens, which suggests that occurrence of the representatives of this order in hot springs is not accidental. However, the substrate utilized by these organisms in their natural thermal habitats remains unclear.

The MCR-2a cluster of uncultured archaea is among the deepest phylogenetic lineages of microorganisms possessing methyl-coenzyme M reductase (Fig. 1). Unlike other groups of methanogens, which we detected only in particular springs, members of the MCR-2a cluster were found in all of the springs where we detected *mcrA* genes (Fig. 1); these springs had temperatures of 49–80°C and pH 5.5–7.7 (table). Earlier, such *mcrA* genes were also found in the Bourlyashchy pool, the temperature of which was 87°C at the moment of sampling [46]. These results suggest that representatives of this phylogenetic cluster are

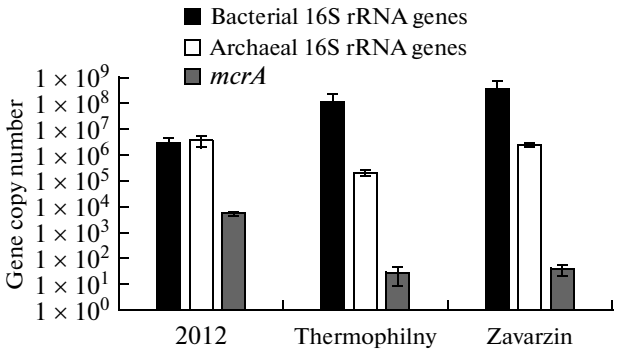

Fig. 2. The copy number of bacterial 16S rRNA genes (*1*), archaeal 16S rRNA genes (*2*), and *mcrA* genes (*3*) calculated per 1 ng of the isolated DNA. Standard deviation intervals are indicated. The calibration curves were characterized by $R^2 > 0.997$ and efficiencies from 70 to 99%.

widespread in ecosystems associated with geothermal activity. Moreover, analysis of the GenBank database showed that representatives of the MCR-2 cluster have been detected in diffuse fluids of deep-sea hydrothermal vents [47], subsurface ecosystems [48], mud volcanos [49], and acidic peat and anaerobic digesters [37], i.e., both in thermal and moderate-temperature environments [47].

Thus, our study of the occurrence and diversity of methanogenic archaea in terrestrial hot springs showed the presence of members of this group in most of the springs studied. In these habitats, diverse electron acceptors are available, which favors other physiological groups of microorganisms competing for the substrate. This fact most probably explains the low cell number of methanogens in thermal environments of volcanic origin. Methanogens, however, are a permanent component of the microbial population. This conclusion is valid, first of all, for representatives of the uncultured MCR-2a cluster. Metagenomic analysis of environmental samples and enrichment cultures is to shed light on the metabolism of these organisms, promoting further attempts of their laboratory cultivation.

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