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EXPERIMENTAL ARTICLES

Diversity of Methanogenic Archaea from the 2012 Terrestrial Hot Spring (Valley of Geysers, Kamchatka)

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Abstract—Archaeal diversity in the 2012 terrestrial hot spring (Valley of Geysers, Kronotsky Nature Reserve, Kamchatka, Russia) was investigated using molecular and cultivation-based approaches. Analysis of the 16S rRNA gene sequences revealed predominance among archaea of uncultured microorganisms of the pSL12 and THSCG clusters. Analysis of the *mcrA* genes revealed that members of the order *Methanomassiliicoccales* were predominant (68%) among methanogens; the latter constituted 0.15% of the total number of archaea. Five stable thermophilic methanogenic associations utilizing hydrogen, formate, acetate, or methanol as substrates were obtained from the sediments of spring 2012. The diversity of cultured methanogens was limited to members of the genera *Methanothermobacter, Methanothrix,* and *Methanomethylovorans*. The association growing at 65°C and producing methane from methanol contained two components, which probably formed a syntrophic relationship: a *Methanothermobacter* methanogenic archaeon and a bacterium representing an separate cluster within the *Firmicutes* phylum, which was phylogenetically related to the genera *Thermacetogenium* and *Syntrophaceticus*. These data indicate high diversity of methanogens, notwithstanding their low abundance among archaea. The group of thermophilic *Methanomassiliicoccales*, which predominated among methanogens, is of special interest.

Keywords: methanogenic archaea, terrestrial hot springs, the *mcrA* gene, deep phylogenetic lineages, uncultured microorganisms

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Methanogenic archaea are a large and diverse group within the phylum *Euryarchaeota*. Since they can be retrieved from a wide range of anaerobic ecotopes, this is one of the truly cosmopolite archaeal groups. Of special interest are the ecotopes where methanogens thrive on the substrates of abiogenic origin, especially the thermal ecotopes (hot springs, marine deep- and shallow-water hydrothermal vents, and subsurface layers of the Earth crust). In such ecosystems, where hydrogen and low-molecular organic compounds produced by abiotic reactions arrive from the lower, high-temperature horizons, thermophilic methanogenic archaea may act as primary producers, a key component of the newly forming microbial communities.

Thermophilic methanogens associated with deep-sea hydrothermal vents are rather well studied (Miroshnichenko and Bonch-Osmolovskaya, 2006; Ollivier and Cayol, 2010). Members of two orders of hydrogenotrophic methanogens, *Methanococcales* and *Methanopyrales,* are usually detected in and isolated from the samples from such ecotopes (Jones et al., 1983; Kurr et al., 1991; Takai et al., 2004; Flores et al., 2012; Stewart et al., 2015). These deep-sea thermophilic methanogens are lithoautotrophs that utilize hydrogen as the sole energy source and thus act as producers of organic matter in deep-sea hydrothermal ecosystems. Wide occurrence of lithotrophic methanogens in deep-sea hydrothermal vents is probably associated with higher hydrogen solubility under high hydrostatic pressure.

Although the investigation of methanogenic communities from terrestrial hot springs began earlier, they are less studied than those from marine hydrothermal vents. The first thermophilic methanogen, *Methanobacterium thermoautotrophicus* (now *Methanothermobacter thermautotrophicus*), which was initially isolated in 1972 from sewage sludge, was subsequently detected in hot springs of the Yellowstone National Park (Zeikus and Wolfe, 1972; Zeikus, 1977). Two hydrogenotrophic hyperthermophilic methanogens of the new family *Methanothermaceae*, *Methanothermus fervidus* and *M. sociabilis,* which were isolated from the hydrothermal vents of Iceland (Stetter et al., 1981; Lauerer et al., 1986), were subsequently shown to be endemic to this island (Ollivier and Cayol, 2010). A thermophilic filamentous microorganism using acetate for methanogenesis was originally isolated in 1982 from the Uzon caldera hydrothermal vent (Nozhevnikova and Yagodina, 1982). It was subsequently described as a new spe-

cies, *Methanothrix thermoacetophila* (Nozhevnikova and Chudina, 1984). Intense methane production was revealed in Kamchatka hot springs by radioisotope techniques (Bonch-Osmolovskaya and Karpov, 1987, 1999).

While assessment of the diversity and occurrence of methanogenic archaea in terrestrial hot springs by molecular techniques revealed the presence of methanogens in most of the studied hydrothermal vents in Kamchatka and São Miguel Island (the Azores, Portugal) with temperatures from 51 to 89°C, they constituted a small part of the microbial population (Merkel et al., 2015a). The highest abundance of methanogens (0.09% of the total number of prokaryotes) was detected in the microbial community of the 2012 hot spring (Valley of Geysers, Kamchatka), one more peculiar feature of which was predominance of archaea (58% of the microbial number) over bacteria (42%).

In the present work, further investigation of the diversity of methanogenic archaea in the 2012 spring was carried out using both molecular and cultivation-based approaches.

MATERIALS AND METHODS

Samples and enrichment cultures. The 2012 hot spring is located in the Valley of Geysers, Kronotsky Nature Reserve, Kamchatka, Russia. The temperature and pH of the water at the time of sampling were 58°C and 5.7, respectively.

The composition of the archaeal population and the ratio of various groups of methanogens were determined using total DNA preparations isolated from mixed samples of water and sediment as described previously (Merkel et al., 2015a). Based on these DNA preparations, *mcrA* and 16S rRNA gene libraries were constructed according to standard procedures (Steinberg and Regan, 2008).

Methanogenic archaea were cultivated in anoxically prepared DSM no. 203 medium (*Methanothermus fervidus* medium). The cultivation was carried out in 60-mL vials with a 1 : 2 ratio of the liquid and gas phases. Initial inoculation with 1 mL of the environmental sample of water and sediment was carried out under an N_2 flow. Incubation temperature and pH of the medium varied from 55 to 70°C and from 5.0 to 7.5, respectively. The growth substrates used were formate, acetate, methanol, or propionate (added as sterile stock solutions to a final concentration of 20 mM) or an 80 : 20 H_2/CO_2 gas mixture introduced prior to sterilization. Yeast extract (0.1–0.5 g/L) was added to the medium in all cases to serve as a source of growth factors. Substrate-free medium was inoculated and incubated for controls. The cultivation was carried out under strictly anoxic conditions. Enrichment cultures exhibiting significant difference (at least fivefold) in methane production between the experimental and control vials were considered positive. Enrichment cultures persisting for at least four sequential transfers without a decrease in methane production were considered stably positive.

Analytical techniques. Growth of methanogens was ascertained by methane production as determined on a Khromatek Kristall 5000.1 gas chromatograph (Russia) according to the manufacturer's recommendations. Growth monitoring and cell counts were carried out under an Olympus CX-41 phase contrast microscope (Japan).

Molecular biological and bioinformatics techniques. Methanogenic enrichment cultures were analyzed by PCR with primers specific to *mcrA* and 16S rRNA genes and subsequent separation (and sequencing) of the amplicons by denaturing gradient gel electrophoresis (DGGE) as described previously (Watanabe et al., 2009).

Nucleotide sequences were edited using BioEdit 7.1.3 (Hall, 1999), aligned with ClustalW (Thompson et al., 1994), and grouped into operational taxonomic units (OTUs) using the cd-hit software package (Li and Godzik, 2006). The total OTU set was analyzed using Pintail (Ashelford et al., 2005) in order to detect chimeric sequences. The OTUs were identified using the same phylogenetic reconstruction as applied for identification of the OTUs revealed by DGGE in our previous work (Merkel et al., 2015a). Phylogenetic reconstruction was carried out using the ARB software package (Ludwig et al., 2004), with the maximum-likelihood algorithm and nonparametric bootstrap analysis (100 repeats). The GenBank information on the sites of detection of members of the revealed archaeal clusters containing no cultured representatives was analyzed using the GetIsolationSources software package (https://github.com/allista/GetIsolationSources/releases) (Merkel et al., 2015b).

RESULTS

Archaeal population of the 2012 spring. Clonal libraries of 16S rRNA genes and *mcrA* genes were constructed using total DNA samples from the 2012 spring in order to analyze the composition of the archaeal population and the relative abundance of various groups of methanogens. A total of 120 partial sequences of archaeal 16S rRNA genes and 108 partial sequences of the *mcrA* genes were obtained. All partial sequences of archaeal 16S rRNA genes were grouped in 12 OTUs, and all *mcrA* sequences were grouped in 6 OTUs. In both cases the sequences were grouped in an OTU based on their similarity above 98%.

Phylogenetic analysis resulted in assignment of all OTUs for the partial 16S rRNA gene sequences to eight phylogenetic clusters according to the SILVA ribosomal RNA database project classification: ANT06-05, DSEG, SAGMEG, THSCG, OPPD003, pSL12, *Desulfurococcales*, and TMCG (Fig. 1). Quan-

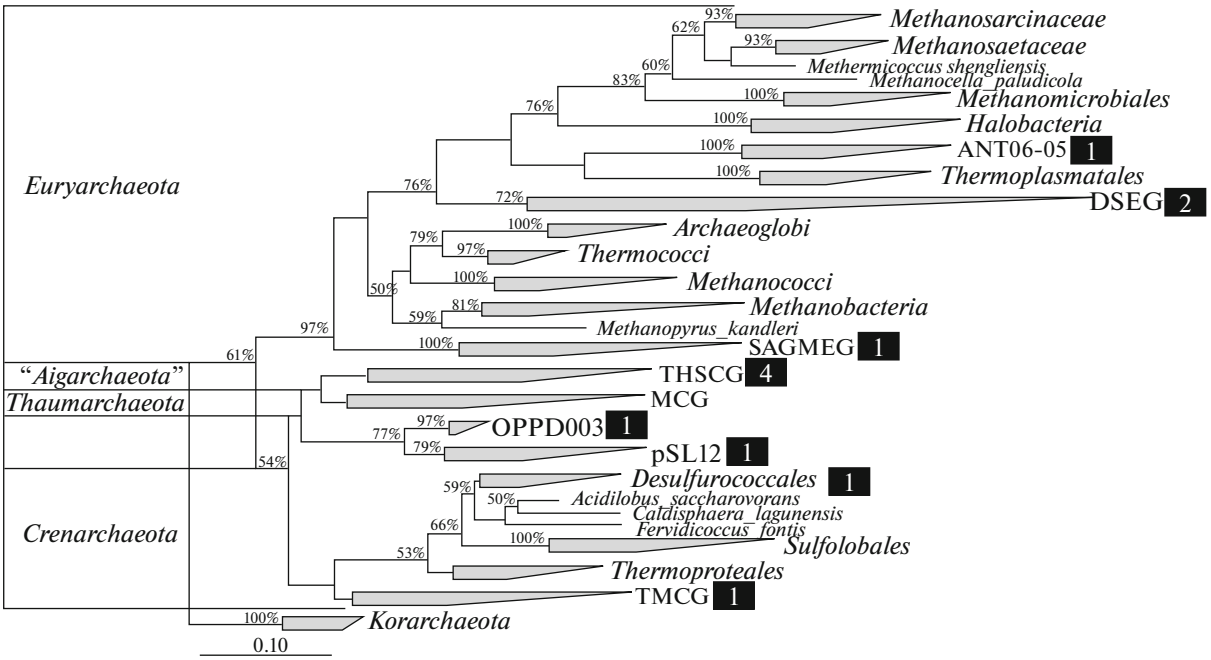

Fig. 1. Phylogenetic tree constructed based on comparative analysis of archaeal 16S rRNA gene sequences using the ARB software package, maximum likelihood algorithm, and bootstrap analysis (100 replicates, values below 50% are not shown). Black squares indicate the phylogenetic clusters to which the revealed OTUs were assigned. The numerals within the squares indicate the OTU number within a given cluster.

titative assessment of the abundance of different OTUs in this clone library (Fig. 2a) revealed predominance of uncultured microorganisms belonging to the clusters pSL12 (34%) and THSCG (28%).

The GenBank database was analyzed in order to find out the origin of the 16S rRNA gene sequences related to those revealed in the 2012 spring. Sequences detected in sites associated with geothermal activity were shown to predominate in most clusters: OPPD003 (100% of the sequences), TMCG (68%), THSCG (62%), pSL12 (53%), and DSEG (45%). In two clusters, however, the abundance of such sequences was relatively low: ANT06-05 (15%) and SAGMEG (4%).

Methanogen diversity in the 2012 spring was studied using the *mcrA* gene encoding methyl coenzyme M reductase, the key enzyme of methanogenesis. The results of analysis of the relevant clone libraries were in

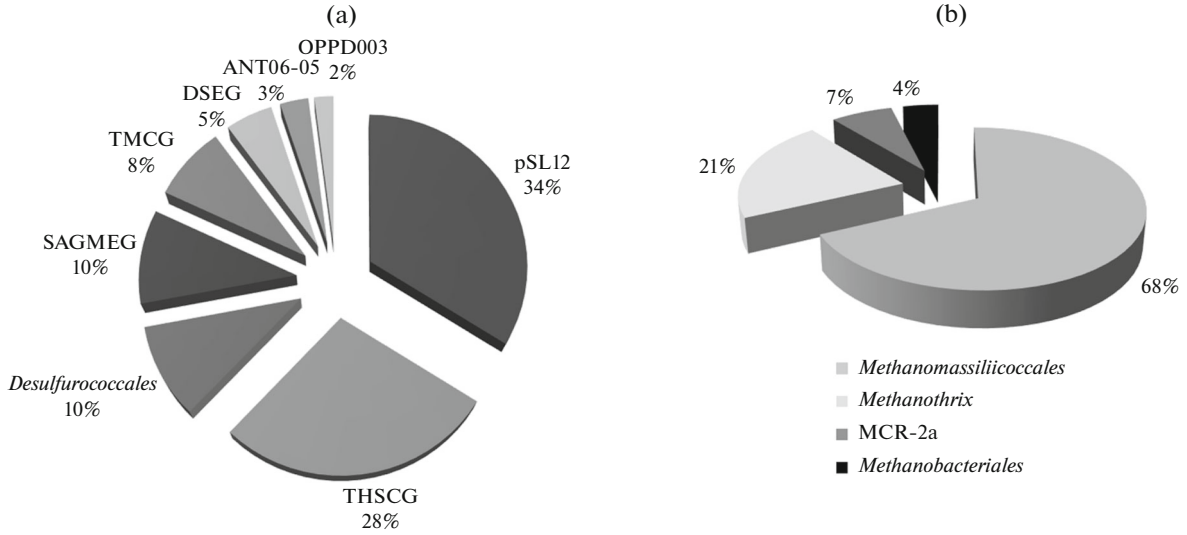

Fig. 2. Ratios of phylogenetic groups in the 16S rRNA gene (a) and *mcrA* gene libraries (b) from the 2012 hot spring.

Methanogenic enrichment cultures obtained using the sample from the 2012 hot spring (Valley of Geysers, Kamchatka)

Name of enrichment	*T*, °C	pH	Substrate*	Closest cultured methanogen	Similarity by the *mcrA* gene, %	Similarity by the 16S rRNA gene, %
2012-HTEC	60	6.5	H_2/CO_2	*Methanothermobacter thermautotrophicus*	97	99
2012-FREC	60	6.5	Formate	*Methanothermobacter thermautotrophicus*	97	99
2012-ACEC	55	6.0	Acetate	*Methanothrix thermoacetophila*	88	98.2
				Methanothermobacter thermautotrophicus	97	99
2012-MDEC55	55	7.0	Methanol	*Methanomethylovorans thermophila*	99	ND**
2012-MDEC65	65	7.0	Methanol	*Methanothermobacter thermautotrophicus*	97	99

* The substrate concentrations were 20 mM for soluble substrates and 70 mmol/L of liquid phase for hydrogen present in the gas phase.
** ND stands for "not determined."

agreement with the results obtained previously using PCR–DGGE (Merkel et al., 2015a). Four phylogenetic clusters of methanogens were detected in the spring: MCR-2a, *Methanomassiliicoccales*, *Methanobacteriales,* and *Methanothrix*. Quantitative assessment of the abundance of different OTUs in the *mcrA* clone library from the 2012 spring revealed predominance of representatives of the order *Methanomassiliicoccales* (68% of the total number of methanogens (Fig. 2b)). Members of the genus *Methanothrix* and of the order *Methanobacteriales* accounted for 21 and 4% of the total number of methanogens, respectively. Phylotypes of the cluster MCR-2a, for which no cultured representatives are presently known, constituted 7% of the methanogen population.

Enrichment cultures of methanogenic archaea. On all of the tested substrates, stable methanogenic associations (a total of five) growing at 55–65°C and pH 6.0–7.0 were obtained from the water and sediment sample from the 2012 spring (table). PCR–DGGE analysis of the *mcrA* and 16S rRNA gene sequences revealed the presence in the enrichments of methanogens of the genera *Methanothermobacter, Methanothrix,* and *Methanomethylovorans* (table, Fig. 3). Comparison of the detected 16S rRNA gene sequences with those of the type strains revealed high similarity (>98%) (table); for the *mcrA* gene sequences the similarity varied from 88 to 99%.

The 2012-MDEC65 methanogenic association growing on methanol at 65°C and pH 7.0 was studied in more detail. The only methanogenic microorganism was identified as a member of the genus *Methanothermobacter,* with 16S rRNA gene sequences exhibiting 99% similarity to those of *Methanothermobacter thermautotrophicus*. Microscopy of this association revealed, apart from long, thin rods typical of the genus *Methanothermobacter,* numerous short, thick rods (Fig. 4c). Definition of the bacterial component by amplification, DGGE, and sequencing of the 16S rRNA gene resulted in identification of the major bacterial phylotype as a member of the phylum *Firmicutes,* with 90% similarity of the 16S rRNA gene sequences to *Thermacetogenium phaeum* and 89% similarity to *Syntrophaceticus schinkii* (Fig. 5).

DISCUSSION

In hot springs with temperatures above 80°C and in low-pH springs, archaea constitute a significant part of microbial populations, as was confirmed by the works on microbial communities of the Uzon caldera (Mardanov et al., 2010; Chernyh et al., 2015). Bacteria form an absolute majority of microorganisms in neutral springs with a moderately high temperature from 50 to 60°C (Gumerov et al., 2011; Rozanov et al., 2014). The microbial population of the 2012 spring with the temperature of 58°C and pH 5.7, in which 58% is represented by archaea (Merkel et al., 2015a), is an exception deserving special attention.

Analysis of the 16S rRNA gene sequences showed a vast majority of archaea found in the spring (90%) to belong to the phylogenetic clusters with no cultured representatives: pSL12 and OPPD003 (phylum *Thaumarchaeota*), the THSCG group belonging to the recently proposed phylum *Aigarchaeota*, the TMCG group (phylum *Crenarchaeota*), and the groups SAGMEG, DSEG, and ANT06-05 (phylum *Euryarchaeota*). *Desulfurococcales,* an order of hyperthermophilic archaea of the phylum *Crenarchaeota,* was the only archaeal phylogenetic cluster with cultured representatives revealed in the 16S rRNA gene clone library (10% of archaea). The predominance of uncultured organisms prevents reconstruction of the community structure; however, it indicates high activity of these microorganisms in the 2012 spring, the more so that five of the seven clusters listed above exhibit high levels of phylotypes detected in sites associated with geothermal activity.

Although methanogenic archaea represented a quantitatively small part of the microbial community, they exhibited phylogenetic and metabolic diversity. Predominance of microorganisms of the order *Metha-*

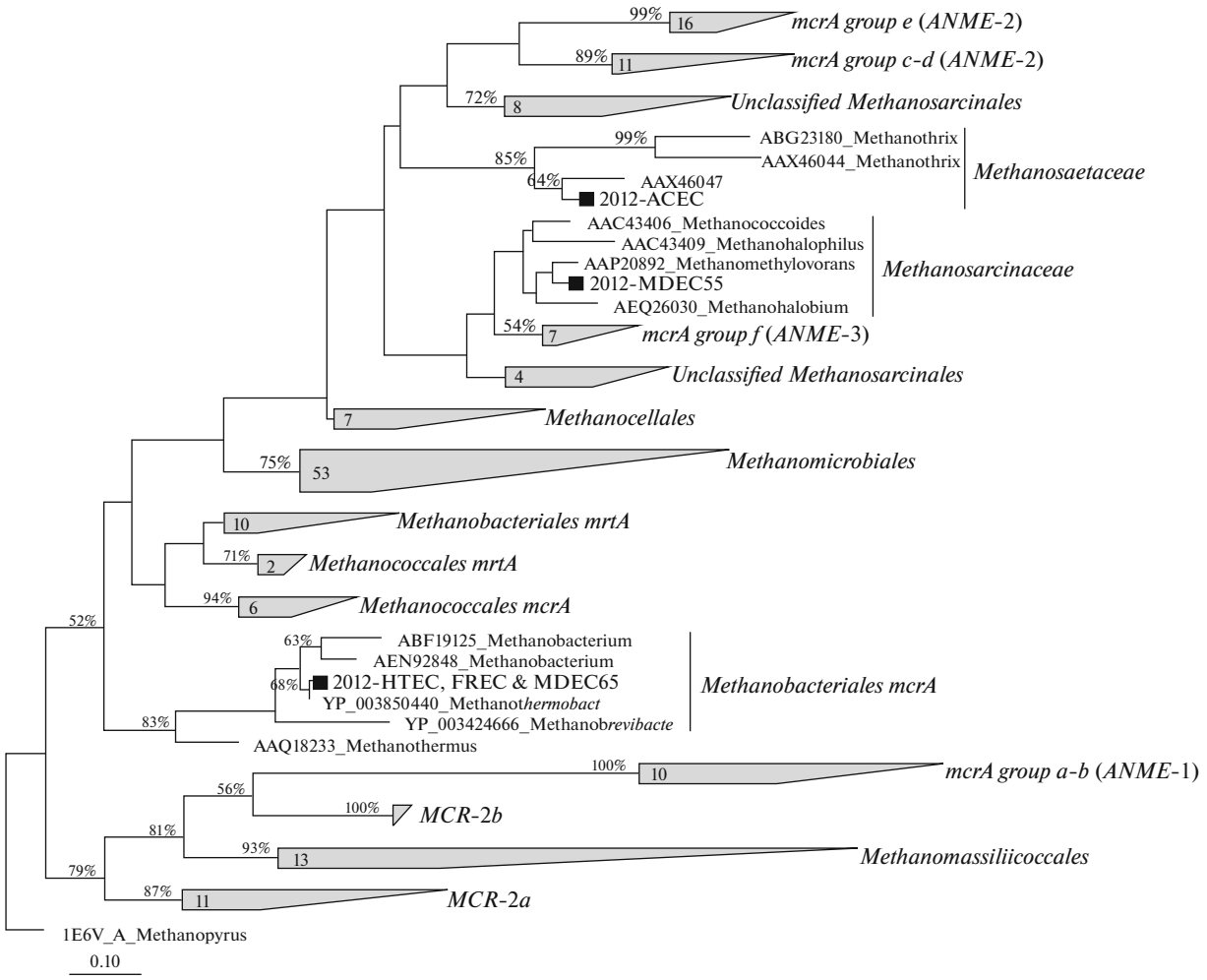

Fig. 3. Phylogenetic tree constructed based on comparative analysis of amino acid sequences of methyl-coenzyme M reductase α-subunit retrieved from the methanogenic enrichment cultures obtained in this work. The tree was constructed using the ARB software package, maximum likelihood algorithm, and bootstrap analysis (100 replicates, values below 50% are not shown). Black squares indicate the sequences of methanogenic microorganisms detected in the enrichment cultures.

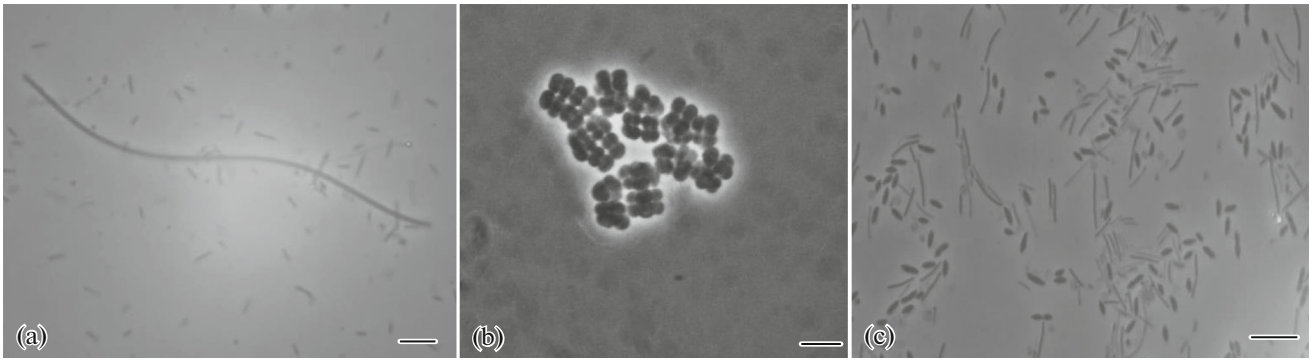

Fig. 4. Methanogenic enrichment cultures: 2012-ACEC ((a) acetate, 55°C, pH 6.0); 2012-MDEC55 ((b) methanol, 55°C, pH 7.0); and 2012-MDEC65 ((c) methanol, 65°C, pH 7.0). Light microscopy. Scale bar, 10 μm.

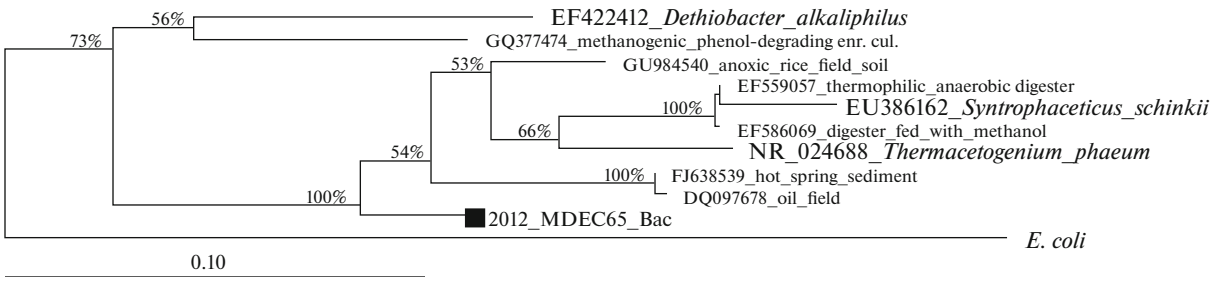

Fig. 5. Phylogenetic tree constructed based on comparative analysis of bacterial 16S rRNA gene sequences using the ARB software package, maximum likelihood algorithm, and bootstrap analysis (100 replicates, values below 50% are not shown). The bacterial phylotype from the methanol-utilizing methanogenic enrichment culture 2012-MDEC65 is indicated by a black square.

nomassiliicoccales (68% of all methanogens) in the 2012 spring is of special interest. Members of this order were not, however, detected in any of the enrichment culture variants. The reason for this may be the absence of an enrichment medium variant that would contain both methanol (or methylamines) and hydrogen, i.e., the medium which provided for the growth of all known *Methanomassiliicoccales* isolates: *Methanomassiliicoccus luminyensis*, "*Candidatus* M. intestinalis," "*Candidatus* Methanogranum caenicola," "*Candidatus* Methanomethylophilus alvus," and "*Candidatus* Methanoplasma termitum" (Dridi et al., 2012; Borrel et al., 2012, 2013; Iino et al., 2013; Lang et al., 2015). All the isolates listed above are mesophiles, and four out of five have been isolated from intestines of humans, termites, or cockroaches. Methanol required for their growth is probably produced in the course of decomposition of pectin (a plant polysaccharide) (Schink and Zeikus, 1980), while methylamines are a product of anaerobic degradation of glycine (and probably of other amino acids) of proteins or peptidoglycan (Chojnacka et al., 2015; Tveit et al., 2015).

Members of the genus *Methanothrix* were the second most abundant group of methanogens. In the *mcrA* clone library, the phylotypes of this genus were represented by a considerable number of sequences (21%). In the case of this group, cultivation-based and molecular approaches yielded similar results. The same *Methanothrix* phylotype was detected in the *mcrA* clone library and in the enrichment 2012-ACEC, obtained on acetate at 55°C and pH 6.0 (Fig. 4a). The new representative of the genus *Methanothrix* differs from *M. thermoacetophila* (88 and 98% similarity between the *mcrA* and 16S rRNA gene sequences) and probably belongs to a novel species (Yarza et al., 2014; Lever and Teske, 2015). Together with CO_2 and H_2, acetate is one of the central metabolites in the anaerobic decomposition of organic substrates. In hot springs, it may be produced by primary anaerobes or as a result of homoacetic fermentation. Earlier works (Ward and Olson, 1980; Sandbeck and Ward, 1981; Bonch-Osmolovskaya et al., 1987) carried out at a number of hot springs in Yellowstone Park (United States) and Uzon caldera (Kamchatka) with temperatures ranging from 40 to 60°C showed, however, that the activity of aceticlastic methanogens was low compared to that of hydrogenotrophic ones or could not be detected at all. These results were obtained for cyanobacterial communities, where methanogens are involved in decomposition of organic matter of cyanobacterial origin. Our results obtained at the 2012 spring indicate considerable contribution of aceticlastic methanogens to methanogenesis. Since no phototrophic microbial communities are present in this spring, it is organic matter of chemosynthetic or allochthonous origin that is mineralized by organotrophic archaea of the genus *Desulfurococcus* and aceticlastic methanogens.

Two methanol-utilizing methanogenic cultures were also isolated from the 2012 spring. This process occurs at two different temperatures. In the first case (at 55°C), methanogenesis was carried out by a *Methanomethylovorans* representative (Fig. 4b). Phylotypes belonging to this genus have not been detected in geothermal ecosystems previously. The organism from the 2012 spring exhibited 99% similarity of its *mcrA* sequence to the strain isolated in the Netherlands from a methanol-utilizing anaerobic reactor. Since this organism was not detected in the samples from the spring 2012 by molecular techniques, it may be an r-strategist with low abundance in the microbial community. The same assumption applies to microorganisms of the genus *Methanothermobacter,* which were detected in four out of five enrichments, including the enrichment with methanol growing at 65°C (the second temperature). According to the literature data, members of the genus *Methanothermobacter* can use only H_2/CO_2 and, in some cases, formate as the substrates for methanogenesis. In the 2012-MDEC65 enrichment, a syntrophic association consisting of a single methanogenic microorganism, a hydrogenotrophic archaeon of the genus *Methanothermobacter,* and a bacterium belonging to a new phylogenetic lineage within the order *Thermoanaerobacterales,* may be responsible for methane formation from methanol. The closest relatives of the bacterial component of the association were members of the genera *Thermacetogenium* and *Syntrophaceticus*, for which acetate oxida-

tion in syntrophic cultures with hydrogenotrophic methanogens was reported (Hattori et al., 2000; Westerholm et al., 2010). In our enrichment, the bacterium probably oxidized methanol to hydrogen and CO_2, while the methanotrophic archaeon of the genus *Methanothermobacter* consumed hydrogen, maintaining its low concentration and thus enabling anaerobic methanol oxidation.

Our results showed high diversity of methanogens, in spite of their low share in the total archaeal population. The group of thermophilic *Methanomassiliicoccales*, which was predominant among methanogens in the community of the 2012 spring, is of special interest. Laboratory cultivation of these microorganisms is the goal of our further work.

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