

Uncultured archaea dominate in the thermal groundwater of Uzon Caldera, Kamchatka

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Abstract The thermoacidophilic microbial community inhabiting the groundwater with pH 4.0 and temperature 50°C at the East Thermal Field of Uzon Caldera, Kamchatka, was examined using pyrosequencing of the V3 region of the 16S rRNA gene. Bacteria comprise about 30% of microorganisms and are represented primarily by aerobic lithoautotrophs using the energy sources of volcanic origin—thermoacidophilic methanotrophs of the phylum *Verrucomicrobia* and *Acidithiobacillus* spp. oxidising metals and reduced sulfur compounds. More than 70% of microbial population in this habitat were represented by archaea, in majority affiliated with “uncultured” lineages. The most numerous group (39% of all archaea) represented a novel division in the phylum *Euryarchaeota* related to the order *Thermoplasmatales*. Another abundant group (33% of all archaea) was related to MCG1 lineage of the phylum *Crenarchaeota*, originally detected in the Yellowstone hot

spring as the environmental clone pJP89. The organisms belonging to these two groups are widely spread in hydrothermal environments worldwide. These data indicate an important environmental role of these two archaeal groups and should stimulate the investigation of their metabolism by cultivation or metagenomic approaches.

Keywords Microbial community · Thermoacidophiles · Pyrosequencing · Thermal groundwater · Uncultured archaea · Uzon Caldera

Introduction

The unique features of the Kamchatka peninsula, Russia, include its pristine conditions and access to a wide variety of recently formed geothermal features. Terrestrial hot springs of Kamchatka form unique niches for thermophilic microorganisms and represent an amazing reservoir of biodiversity. Uzon Caldera is located in the South-Eastern part of Kamchatka and contains numerous hot springs and pools with the temperatures from 40 to 96°C, concentrated, in majority, in five thermal fields. Microbial activity (inorganic carbon assimilation, methanogenesis, CO oxidation, glucose and acetate oxidation, sulfate reduction) was studied using in situ experiments with ¹⁴C- or ³⁵S-labelled substrates (Bonch-Osmolovskaya et al. 1999; Pimenov and Bonch-Osmolovskaya 2006; Prokofeva et al. 2006). A considerable contribution to the knowledge of thermophilic microorganisms in the Uzon Caldera has been made by culture-dependent methods (Bonch-Osmolovskaya 2004; Islam et al. 2008; Miroshnichenko et al. 2008, 2009; Prokofeva et al. 2009; Perevalova et al. 2010). Microbial communities of Uzon thermal environments were also studied by molecular methods—amplification of

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16S rRNA genes or key functional genes with primers of different specificity followed by the division of products by DGGE or the formation of clone libraries (Hohn et al. 2002; Perevalova et al. 2008; Reigstad et al. 2008, 2010; Kublanov et al. 2009). Cultivation, molecular and radioisotopic approaches revealed the presence of diverse phylogenetic and metabolic groups of microorganisms, as well as their active participation in carbon and sulfur cycles in Uzon Caldera thermal environments. However, standard molecular methods due to limited sampling (typically dozens/hundreds of clones) do not allow the deep analysis of community structure and quantitative determination of the relative abundances of different groups of thermophilic microorganisms. Such information could be obtained using pyrosequencing approach targeting variable regions of 16S rRNA gene (Sogin et al. 2006). Pyrosequencing was recently used to analyse the structures of bacterial communities of two alkaline hot springs in Yellowstone park (Miller et al. 2009), but the deep structure of microbial community has never been studied in Kamchatka thermal environments.

In this work, we report the results of pyrosequencing analysis of microbial community inhabiting the groundwater of East Thermal Field of Uzon Caldera.

Materials and methods

Sampling

Sample of the groundwater from Uzon Caldera was obtained in summer 2008. The sampling site designated as 1884 was located at the northern part (3rd region) of East Thermal Field (54°30'056"N, 160°00'107"E), between a mud pool with temperature 91°C and pH 4.0 and cold Komariny Creek flowing on the margin of the thermal area. About 20-cm deep hole (20 × 30 cm) dug at the sampling site was immediately filled with hot groundwater (Fig. S1). The chemical composition of water was determined in the Analytical Center of the Institute of Volcanology and Seismology, Far-East Branch of the Russian Academy of Sciences, Petropavlovsk-Kamchatsky, Russia. The composition of the effluent gas in the neighbouring mud pool was determined by gas chromatography, either on Gasochrom 3101, Russia (H₂), or on LHM-806, Russia (other gases).

DNA extraction and pyrosequencing of 16S rRNA genes

For metagenomic DNA extraction, the 50-ml groundwater sample was fixed by addition of 5% formaldehyde. Community DNA was isolated by the method developed previously for extraction of DNA from soils (Zaporozhenko

et al. 2006). Two pairs of PCR primers for amplification of the V3 variable region of the 16S rRNA gene were used: U341F (5'-CCTACGGGSGCAGCAG)/U515R (5'-TTACCGCGGCKG CTGVCAC) for amplification of both bacterial and archaeal sequences, and A333F (5'-TCCA GGCCCTACGGG)/U534Rm (5'-GWATTACCGCGGCK GCTG) for specific detection of archaeal 16S rRNA sequences. The two PCR fragments were pyrosequenced on GS FLX (Roche) in a total of 38516 reads for “universal” and 35479 reads for “archaeal” sample. Most of the reads covered the full length of the PCR fragment.

Data analysis

The data were analysed employing the RDP Classifier program package (Cole et al. 2009). At first the sequences were distributed between bacteria and archaea using online RDP Naive Bayesian rRNA Classifier Version 2.0 (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). Subsequently, bacterial and archaeal data sets were analysed separately using the Pyrosequencing pipeline (<http://pyro.cme.msu.edu/>) of the RDP Classifier (Cole et al. 2009). Cluster analysis and selection of representative sequences for operational taxonomic units (OTUs) were performed by Complete linkage clustering and Dereplicate request functions of the RDP Classifier. Then, we assigned OTUs to taxonomic groups (i.e., bacterial and archaeal divisions) on the basis of BLASTN sequence similarity searches against the NCBI database. The taxonomic assignments were refined following construction of phylogenetic trees consisting of representative sequences of the clusters and a set of 16S rRNA sequences of representatives of different archaeal and bacterial lineages. Sequences were aligned using ClustalX (Thompson et al. 1997) and the neighbour-joining trees were computed by TREECON (Van de Peer and De Wachter 1994). The rarefaction curves and Chao1 (Chao 1984) richness estimates were calculated using the corresponding functions of the RDP Classifier. The representative sequences of the clusters have been deposited in the GenBank database under accession numbers JF450625–JF450697.

Results

Sampling site characteristics

The East Thermal Field is the biggest one in Uzon Caldera; its hot springs and mud pools are most diverse from the points of temperature, pH of the water and its chemical composition. The 3rd region where the sampling site was located is the closest to the line of geologic fault, and is characterised by a high methane concentration in the

gaseous exhalations and by the unique phenomenon of modern oil formation (Bazhenova et al. 1998; Simoneit et al. 2009). The groundwater level at this region is at a depth of about 20–30 cm. The hole dug in the ground was filled with water (temperature 50°C, pH 4.0) with a sulfidic film on the surface (Fig. S1). The water was turbid and blackish because of the presence of pyrite and clay particles. The analysis of its composition showed a high concentration of sulfates (Table S1). Evolving gas from the closest mud pot was gathered and analysed: CO₂ was its major component (91.2%) followed by H₂S (3.2%), N₂ (2.1%), CH₄ (2.0%), and H₂ (1.4%), while oxygen was not detected. Similar methane and hydrogen content was found in the gas samples at several other sites of the 3rd region of the East Thermal Field (data not shown).

Relative abundance and richness of archaea and bacteria

In order to analyse the microbial community of thermal groundwater, both qualitatively and quantitatively, we used the approach based on deep pyrosequencing of V3 regions of 16S rRNA genes (Sogin et al. 2006). The “universal” data set contained 38516 sequences, of which 23037 were assigned to archaeal and 12234 to bacterial 16S rRNA. Remaining 3245 reads had no similarity with 16S rRNA sequences and were excluded from the analysis. Thus, archaea apparently account for about 70% of microorganisms. Since the “universal” primers failed to detect some environmental crenarchaeal lineages identified with “archaeal” primers (data not shown), the actual fraction of archaea might be even higher. The data set generated with “archaeal” primers consisted of 35479 sequences, of which 29837 represented archaeal 16S RNA genes, others being bacterial or non-16S rRNA sequences. Thus, 12234 bacterial sequences extracted from the “universal” data set were used to analyse the composition of bacterial community, and 29837 archaeal sequences from the “archaeal” data set were used for archaeal part of the community.

The species richness estimation and rarefaction analysis of both bacterial and archaeal communities revealed that even at present sequencing efforts only a fraction of the entire microbial community was covered and hence some minor groups were missed. When a sequence dissimilarity of 0.05 was used to define OTU, 286 bacterial and 266 archaeal OTU were observed, while Chao1 estimated richnesses of 374 and 343 for the original communities. Moreover, the rarefaction curves computed from the observed OTUs were still far from reaching plateau phase (Fig. 1), suggesting that additional sampling would lead to considerably higher richness estimates. When higher sequence dissimilarity defined OTU (0.1), the rarefaction curves had started to plateau, suggesting that the entire

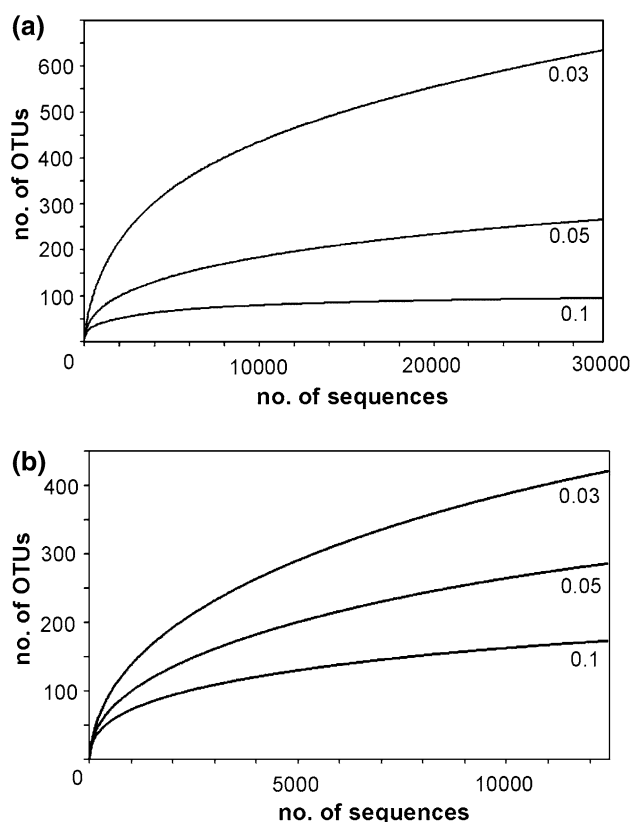


Fig. 1 Rarefaction analysis of archaeal (a) and bacterial (b) communities. Three cluster distances were used to define OTUs: 0.03, 0.05 and 0.1

microbial community was well covered on higher taxonomic ranks. Overall, the results suggest that abundant taxonomic groups were well covered, while a complete coverage of minor groups would require considerable larger sequencing efforts.

The structure of bacterial community inhabiting the groundwater of the East Thermal Field

Taxonomic classification of bacterial community (Fig. 2) was facilitated by an observation that the majority of representative sequences of clusters showed more than 95% homology to 16S rRNAs of validated microorganisms (Table S2), thus allowing us to assign the clusters to particular genera. Several phylotypes, mostly assigned to *Verrucomicrobia* and *Deltaproteobacteria*, were classified only at higher taxonomic levels following construction of phylogenetic trees.

The most abundant group of bacteria (41% of all bacterial clones) phylogenetically belonged to a recently discovered thermoacidophilic methanotrophic lineage within the phylum *Verrucomicrobia*, comprising the genus *Methylacidiphilum* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009; Figure S2,

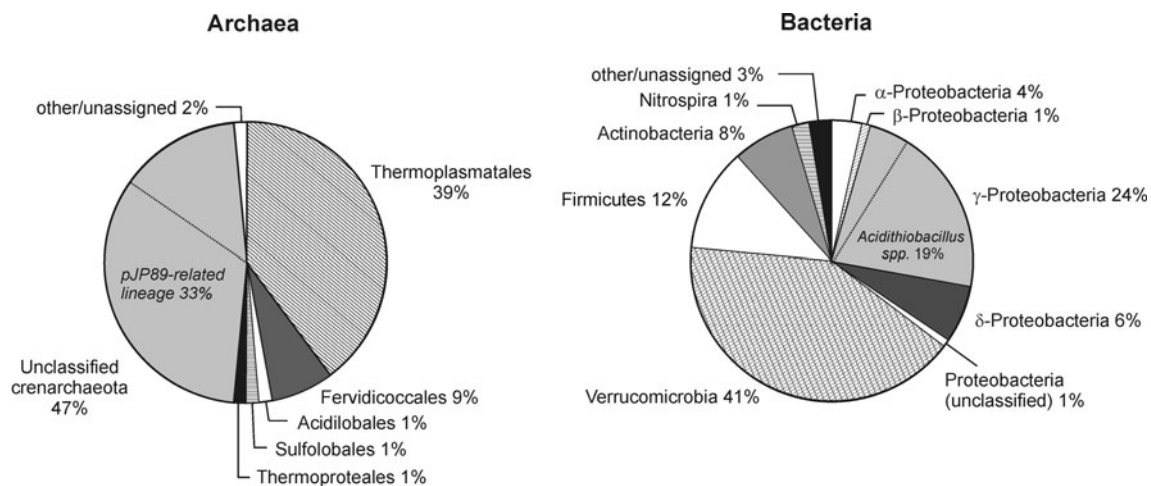


Fig. 2 Relative abundances of archaeal and bacterial taxa

Table S1). *Proteobacteria* represented about a third of bacterial community and included the second most abundant group of bacteria—*Gammaproteobacteria* of the genus *Acidithiobacillus* (19% of all bacterial clones). These organisms are acidophilic aerobic chemolithoautotrophs able to gain energy by oxidation of metals and inorganic sulfur compounds and to fix atmospheric carbon via the Calvin–Benson–Bassham cycle (Valdes et al. 2008). About 12% of bacterial 16S rRNA sequences were affiliated with *Firmicutes*. The majority of them (9% of all bacteria) were closely related to the genus *Geobacillus*, the members of which were involved in aerobic degradation of hydrocarbons (Nazina et al. 2001). About 6% of bacterial sequences were affiliated with *Deltaproteobacteria*. However, most of them were only distantly related to cultured genera, the only exception being sulfur-reducing genus *Desulfurella*, originally isolated in Uzon hot springs (Bonch-Osmolovskaya et al. 1990), but its fraction was only 0.7% of all bacterial sequences.

The structure of archaeal community inhabiting the groundwater of the East Thermal Field

The detailed structure of archaeal community was determined based on the taxonomic assignment of 29837 sequences obtained with archaeal primers (Fig. 2; Table S2). In striking contrast to bacterial community, less than 5% of archaeal sequences exhibited more than 95% homology to 16S rRNAs of validated microorganisms and thus may be assigned to a particular genus; thus, 95% of the archaea inhabiting the groundwater at the site studied belonged to novel taxonomic divisions. About 39% of archaea formed distinct lineages distantly related to the euryarchaeal order *Thermoplasmatales* (Fig. 3), comprising acidophilic aerobic, microaerophilic and facultatively anaerobic organisms oxidising organic matter in the course

of aerobic or anaerobic respiration, using oxygen or elemental sulfur as electron acceptors (Kletzin 2007). Closely related clones (97–99% sequence identity) were found in acidic hot springs and hydrothermally modified soil samples worldwide (Table S3).

Archaea of the order *Fervidicoccales* (Perevalova et al. 2010) comprised about 9.1% of all archaea (Fig. 4). Among them 0.5% were closely related to the only cultivated organism of this order, *Fervidicoccus fontis*, while 8.65% represented a novel genus in this group.

About 47% of archaeal rRNA sequences were affiliated with different crenarchaeal lineages lacking cultured representatives (Fig. 4; Table S2). The majority of them (33% of all archaeal sequences) belonged to a miscellaneous crenarchaeal group I (MCG1) and clustered with pJP89 environmental clone from Yellowstone hot springs (Barns et al. 1996).

Discussion

Hot springs of Uzon Caldera were the objects of many microbiological investigations, while the surrounding ground was never studied in detail. The sampling site analysed in this work, the groundwater of the 3rd region of the East Thermal Field, appeared to harbour very unusual extremophilic microbial community with the evident dominance of uncultured archaea. High temperature and composition of the groundwater indicate its volcanic origin—in the areas surrounding the volcanic fracture the overheated thermal fluid moves to the surface not only along a large cracks opening as a hot pools and springs, but also along small cracks filling the surrounding ground and supplying inorganic substrates for the growth of thermophilic prokaryotes. Since the gas evolving in the neighbouring mud pool is enriched with hydrogen sulfide and

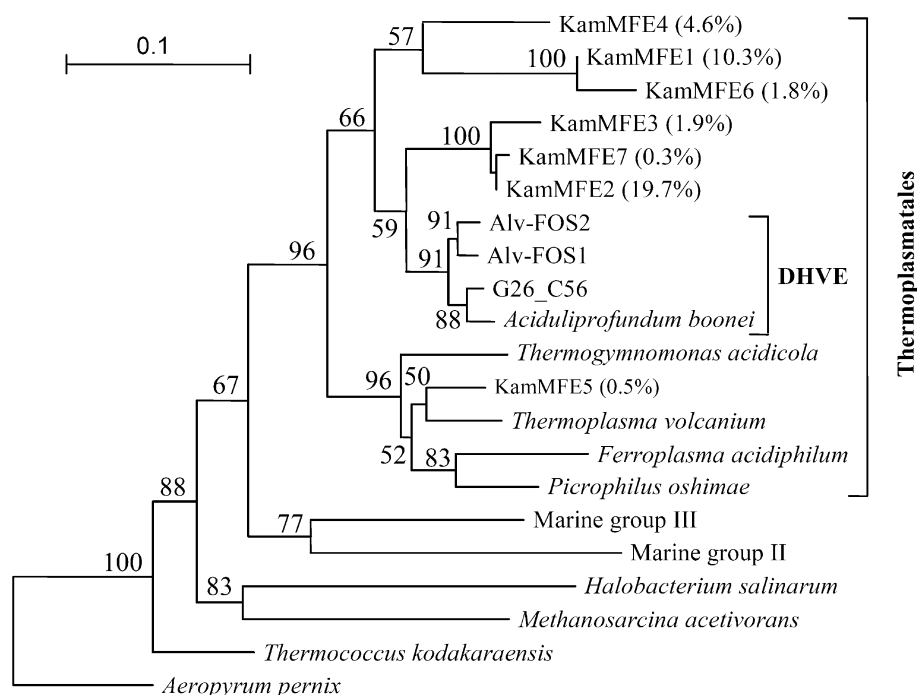


Fig. 3 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences from euryarchaeal clones found in this work (KamMFE1–KamMFE7) and representatives of Euryarchaeota. The shares of each clone/cluster in archaeal 16S rRNA sequences are shown in percents. Marine group II is represented by clone DeepAnt-JyKC7 (AY534910), marine group III by clone HF10_21C05 (DQ156479), and DHVE group

by clones G26_C56 (AF356635), Alv-FOS1 (DQ118403), and Alv-FOS2 (DQ082975). The 16S rRNA gene sequence of crenarchaeon *Aeropyrum pernix* was used as an outgroup. Numbers at nodes represent bootstrap values (100 replications of the original dataset), only values above 50% are shown. The scale bar represents 0.1 nucleotide changes per position

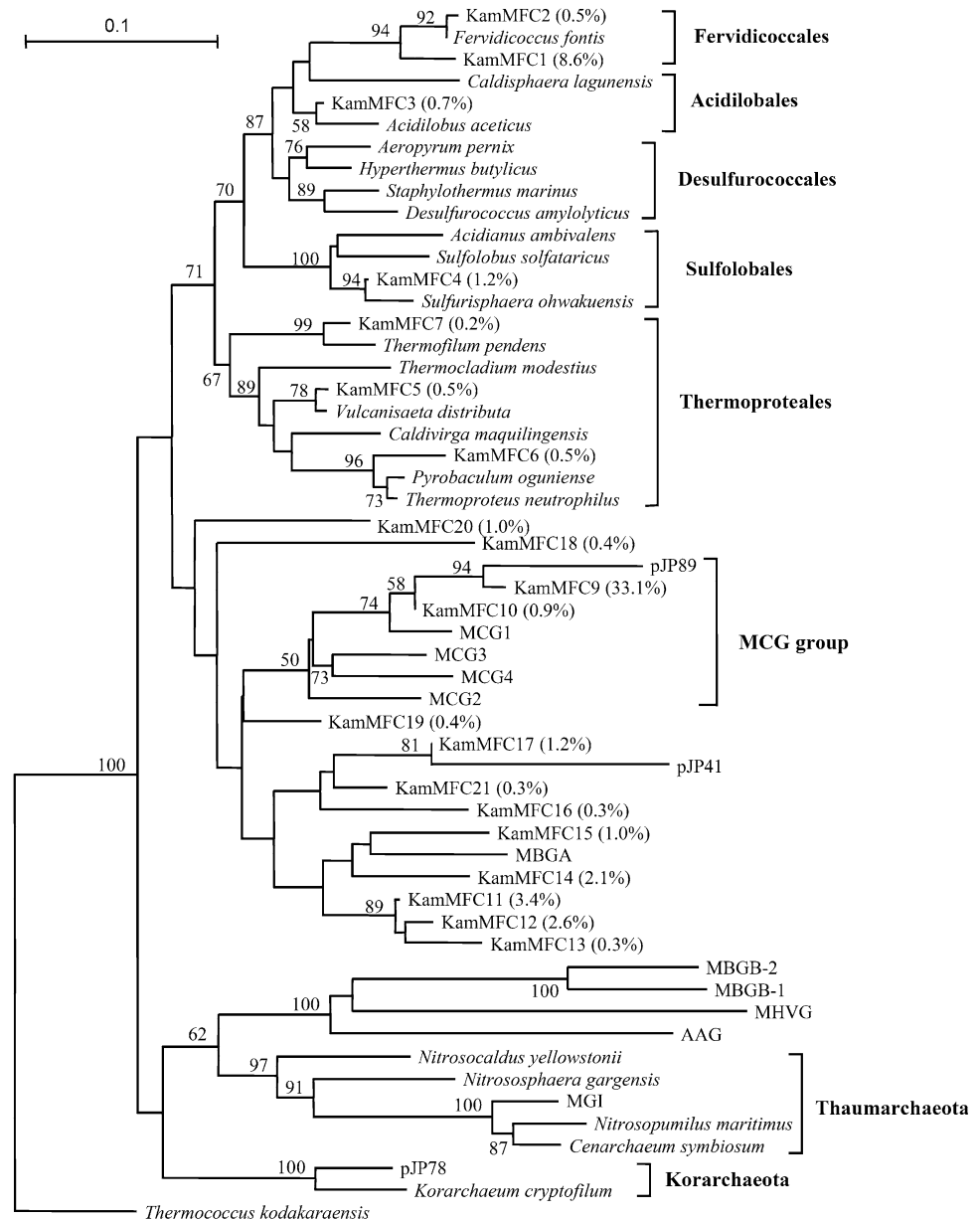
methane, we could assume the presence of these two reduced compounds also in the hot groundwater surrounding the pool.

Hot springs of the Uzon Caldera were found to be inhabited by various bacteria and archaea utilising the inorganic substrates of volcanic origin. Although phylogenetic relations do not necessarily correlate with physiological similarities, some distinct lineages with particular metabolic characteristics may be identified. Two groups of aerobic lithoautotrophs were dominating among bacteria in the thermal groundwater community: representatives of the genus *Acidithiobacillus* which oxidise inorganic sulfur compounds and metals, and *Verrucomicrobia* methanotrophs. Most of acidithiobacilli are mesophiles, but moderately thermophilic species are also known, e.g., sulfur-oxidising *Acidithiobacillus caldus* thriving at temperatures up to 50°C (Valdes et al. 2009). Microorganisms of genus *Acidithiobacillus* form sulfate as the end product of aerobic oxidation of reduced sulfur compounds and may be responsible for the moderately acidic pH (4.0) and high concentration of sulfate (Table S1) in the groundwater at the sampling site. Another group of lithoautotrophic bacteria is the thermoacidophilic methanotrophs belonging to the phylum *Verrucomicrobia*. Isolated almost simultaneously from three geographically remote volcanic areas,

these organisms grow by aerobic methane oxidation at pH range 0.8–6.0 and temperature from 40 to 65°C (Pol et al. 2007; Dunfield et al. 2007; Islam et al. 2008). One of these strains—*Methylophilum kamchatkensis*—was isolated from a hot spring in the Uzon Caldera (Eastern Thermal Field) with the temperature at the sampling site 52°C and pH 3.0 (Islam et al. 2008). Our work showed that methanotrophic *Verrucomicrobia* represented an environmentally relevant part of microbial community in thermal groundwater. They shared this habitat with *Acidithiobacillus* species, the latter maintaining moderately acidic pH at the level appropriate for methanotrophs.

Lithoautotrophic bacteria made more than a half of bacterial population; however, their actual share was only about 20%, when recalculated for the whole microbial community where archaea made more than 70% of total. Other microorganisms present in the community were either organotrophs (*Fervidococcales*, *Geobacillus*, *Actinobacteria*), or their function could not be predicted based on taxonomic affiliation (organisms related to uncultured bacteria and archaea). Thus, it could be assumed that either unknown groups of thermophilic lithoautotrophs were present in the community, or the community was only partly supported by the energy of inorganic electron donors, and additional organic energy

Fig. 4 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences from crenarchaeal clones found in this work (KamMFC) and representatives of *Crenarchaeota*. The shares of each clone/cluster in archaeal 16S rRNA sequences are shown in percents. MCG1 group is represented by clone ODP1227A19.02 (AB177016); MCG2 by clone OHKA15.43 (AB094561); MCG3 by clone ODP1227A18.19 (AB177012); MCG4 by clone ODP1227A1.01 (AB176996); MBGA by clone ACA10-0cm (AF119143); MBGB-1 by clone:OHKA4.94 (AB094544); MBGB-2 by clone ODP1227A2.32 (AB177025); MHVG by clone OHKA1.28 (AB094525); AAG by clone pMCA256 (AB019717); MGI by clone SAGMA-9 (AB050239). Phylogenetic positions of environmental clones pJP89 (L25305), pJP41 (L25301) and pJP78 (L25303) are also shown. The 16S rRNA gene sequence of euryarchaeon *Thermococcus kodakaraensis* was used as an outgroup. Numbers at nodes represent bootstrap values (100 replications of the original dataset), only values above 50% are shown. The scale bar represents 0.1 nucleotide changes per position



sources were provided with meteoric water coming from surrounding cold zones.

A specific feature of the community studied was the dominance of archaea. Typically, archaea are much less abundant than bacteria in neutral high temperature hot springs (e.g., Hugenholtz et al. 1998). In moderately thermoacidophilic community of the Uzon Caldera groundwater archaea were dominating. Their most numerous groups were related to “uncultivated” archaeal lineages known only as environmental 16S rRNA clones. One euryarchaeal group formed separate lineages distantly affiliated with *Thermoplasmatales*. Closely related clones (97–99% sequence identity) were found in different thermal environments worldwide, including acidic hot springs and hydrothermally modified soil. So far, all *Thermoplasmatales*

known in laboratory cultures were organotrophs, utilising organic compounds of different complexity. Thus, it is possible that uncultured relatives of *Thermoplasmatales* detected in the groundwater of the East Thermal Field are also organotrophs; this assumption supports the hypothesis on the allochthonous organic matter feeding the thermal ground community.

Of special interest is the presence of pJP89-related clones (KamMFC9 in Fig. 4) as one of the most abundant groups in the community. These organisms, representing the crenarchaeal MCG1 lineage, are widely spread in thermal environments all over the world. The closely related organisms were found in hot springs in the Yellowstone National Park (USA), Iceland, Bulgaria (Barns et al. 1994; Meyer-Dombard et al. 2005; Spear et al.

2005; Kvist et al. 2007; Perevalova et al. 2008), anaerobic digester in France (Godon et al. 1997). The diagram illustrating the distribution of pJP89-related organisms in pH–temperature coordinates (Fig. S3) shows that most of them were revealed in neutral high-temperature environments. However, our work was so far the only one showing that pJP89-related organisms made an environmentally relevant group. It could be assumed that moderately thermoacidophilic environment provided optimal conditions for growth of these organisms, while in a high-temperature neutral hot springs they were present as minor components (proved also by Hugenholtz et al. 1998 work on Obsidian Pool). Clones with sequences identical to KamMFC9 were identified in Zavarzin Pool at Uzon (T 58°C, pH 6.0), but there they also formed a minor fraction of archaea (our unpublished data).

Thus, a wide distribution of pJP89-related organisms and uncultured groups in the order *Thermoplasmatales* indicated the environmental significance of these lineages. This assumption was further supported by our data showing the quantitative domination of these organisms in the moderately thermoacidic groundwater of Uzon Caldera. These data should stimulate further attempts of metabolic characterisation of pJP89 and uncultured *Euryarchaeota* groups—either by cultivation or by metagenomic approaches.

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