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EXPERIMENTAL ARTICLES

Molecular Analysis of Microbial Diversity in the Zavarzin Spring, Uzon Caldera, Kamchatka

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Abstract—The Zavarzin spring is situated in the caldera of the Uzon volcano, Kamchatka, and is characterized by a temperature of about 60°C, neutral pH, and high concentration of sulfur. The bottom of the spring is covered with a cyanobacterial mat. The structure of the microbial community of the water from the Zavarzin spring was qualitatively and quantitatively characterized by pyrosequencing of the V3 variable region of the 16S rRNA gene, which yielded 37 654 independent sequences. The microbial community includes about 900 bacterial and 90 archaeal genera. Bacteria comprised 95% of the microorganisms and archaea less than 5%. The largest part (32.3%) of the community was constituted by the chemolithoautotrophic bacteria *Aquificae* from the genera *Sulfurihydrogenibium* and *Thermosulfidibacter*. Among autotrophic microorganisms, members of *Thermodesulfobacteria* (7.3%), the gammaproteobacteria *Thiofaba* (7.6%), the deltaproteobacteria *Desulfurella* (2.6%), and the betaproteobacteria *Thiomonas* (0.6%) were also identified. Heterotrophic bacteria were represented by *Calditerrivibrio* (12.1%), *Thermotogae* (6.3%), the betaproteobacteria *Tepidimonas* (6.0%), *Deinococcus–Thermus* (4.4%), *Caldiserica* (1.7%), and *Dictyoglomi* (1.6%). About 1.9% of microorganisms belonged to the BRC1 phylum, which does not include cultured members, and 0.2% of bacteria formed a new phylogenetic branch of the phylum level, representatives of which have been found only in the Zavarzin spring. Members of all four archaeal phyla were identified: *Euryarchaeota* (42% of archaeal sequences), *Crenarchaeota* (50%), *Korarchaeota* (7.5%), and *Nanoarchaeota* (0.5%). Thus, in the Zavarzin spring, apart from photosynthesis carried out by the cyanobacterial mat, which covers the bottom, chemolithoautotrophic production of organic matter can occur. In aerobic conditions, it proceeds at the expense of the oxidation of sulfur and its reduced compounds, and in anaerobic conditions, at the expense of the oxidation of hydrogen with sulfur and sulfates as electron acceptors. The organic matter formed by autotrophic bacteria may be utilized by various organotrophic microorganisms, including both fermentative bacteria and organisms that carry out anaerobic respiration with sulfur and nitrate as electron acceptors.

Keywords: thermophiles, biodiversity, pyrosequencing, 16S rRNA, molecular methods, Zavarzin spring.

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Investigation of the microbial communities associated with thermal ecological niches is of interest both for fundamental microbiology, including evolutionary microbiology (due to the fact that many microorganisms living in these conditions belong to ancient evolutionary lineages of thermophilic bacteria and archaea), and for biotechnology.

Many new thermophilic bacteria and archaea belonging to various phylogenetic groups with diverse types of metabolism have been isolated from the hot springs of Kamchatka in the last three decades [1–3]. However, the biodiversity of the thermophilic microorganisms of Kamchatka is much less studied as compared with Yellowstone National Park in the United States and with Iceland. The Zavarzin spring, located in the Eastern thermal field of the Uzon caldera (Kronotsky National Biosphere Reserve), is a shallow basin of 4.5 × 2.3 m^2 fed by numerous thermal springs, due to which the water is intensely mixed. In the cooler zones at the edges of the spring, its bottom is covered with a cyanobacterial mat several millimeters thick. The water has a moderately high temperature (55–58°C) and neutral pH (6.3) and contains a high concentration of suspended sulfur [4]. The ion contents are as follows: SO_4^{2-} 0.335 mM; NO_3^-, 0.5 mM; and NH_4^+, 0.84 mM. The concentrations of the gases dissolved in the spring water are methane, 24 μM; hydrogen, 6.47 μM; and hydrogen sulfide, 0.12 μM (http://kamchatka.gly.uga.edu/index.php).

To characterize the composition of microbial communities, microbiological methods have been traditionally used that allow pure microbial cultures to be obtained and characterized microbiologically and biochemically. Later, molecular techniques based on

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PCR amplification of 16S rRNA genes and their sequencing came into practice; these methods do not require cultivation of microorganisms. Application of molecular techniques revealed that usually only 0.1–1% of the species occurring in the microbial communities are culturable under laboratory conditions; this is especially true for the communities of extreme habitats. Due to these investigations, the new phylogenetic groups of prokaryotes were identified, including taxa of high level, for many of which no culturable representatives have so far been isolated.

The molecular methods applied until recently in microbial ecology used cloning of 16S rRNA gene fragments with the following independent sequencing of the clones by capillary electrophoresis. This technique allowed several tens or hundreds of 16S rRNA sequences to be analyzed. However, many microbial communities have a more complex composition and include thousands of bacterial and archaeal species. An alternative to the conventional sequencing techniques is pyrosequencing, which allows one to carry out simultaneous determination of several hundreds of thousands of nucleotide sequences [5]. Pyrosequencing makes possible deep characterization of the microbial community, revealing both dominant microorganisms and minor components of the community, which may be ecologically important as well. The possibility arises of quantitatively determining the share of particular microbial groups, which is necessary for the reconstruction of the metabolic pathways of the community as a whole.

The goal of this work was to analyze the composition of the microbial community of the Zavarzin spring by pyrosequencing of 16S rRNA gene fragments.

MATERIALS AND METHODS

Sampling. Water samples from the Zavarzin spring (GPS coordinates, 54 29.883 N, 160 00.874 E) were taken in August 2008. The samples were collected from the surface water layer to preclude contamination with the bottom sediments covered by a cyanobacterial mat. These samples (50 ml) were fixed by adding formaldehyde to a concentration of 5%.

PCR amplification and sequencing. The metagenomic microbial DNA was isolated by the technique developed in the Centre "Bioengineering", Russian Academy of Sciences, for soil samples [6]. For PCR amplification of 16S rRNA gene fragments, including the variable V3 region, the "universal" primers U341F (5'-CCTACGGGRSGCAGCAG) and U515R (5'-TTACCGCGGCKGCTGVCAC) were used. The PCR reaction mixture (50 μl) contained 2.5 U of *Taq* DNA polymerase, 0.2 mM $MgCl_2$, dNTPs (0.1 mM each), and primers (1mM each). Amplification was performed on an Eppendorf Mastercycler (Eppendorf, Germany) in the following regime: initial denaturation at 96°C for 1 min, 30 cycles of at 96°C for 40 s, 50°C for 40 s, and 72°C for 1 min, and the final elongation at 72°C for 5 min.

The PCR products were separated by electrophoresis in an agarose gel.

Pyrosequencing of the resulting amplification products was carried out according to "shotgun library" (Roche) protocol using a GS LR70 Sequencing Kit. A total of 37 654 sequences were determined.

Analysis of the diversity and taxonomic composition of the community. The obtained data were analyzed using the RDP Classifier software package [7]. At the first stage, the identified sequences were divided into bacterial and archaeal ones using the RDP Naive Bayesian rRNA Classifier Version 2.0 available online (http://rdp.cme.msu.edu/classifier/classifier.jsp). After that, bacterial and archaeal sequences were analyzed separately.

To determine the diversity of the community and its taxonomic composition, the Pyrosequencing pipeline (http://pyro.cme.msu.edu/), a component of the RDP Classifier program package, was used [7]. For this purpose, the obtained sequences were aligned and cluster analysis of them was undertaken with the use of the Complete Linkage Clustering program (a component of RDP Classifier). Clustering was carried out at various levels: the distance between the clusters was varied from 0 to 0.3 with a step of 0.01; therefore, the clusters corresponded to taxa of different ranks.

Evaluation of the taxonomic complexity of the community was carried out using the Rarefaction program (a component of RDP Classifier) by means of analysis of graphs in which the number of phylotypes (i.e., the number of clusters) detected at various cluster distances was plotted as a function of the number of analyzed sequences. Evaluation of the biodiversity also was carried out by calculating the Chao1 index using the RDP Classifier [8].

To characterize the taxonomic composition of the community, the nucleotide sequence corresponding to the cluster center (i.e., the sequence having a minimal sum of square distances to other sequences included in the cluster) was found for each cluster using the Dereplicate Request program (RDP Classifier). This sampling of representative sequences was performed for clusters obtained by cluster analysis at a distance parameter of 0.2.

Taxonomic assignment of the sequences representing clusters was carried out by comparing them with the use of BLASTN with the 16S rRNA sequences available in GenBank. If the analyzed sequence shared more than 97% homology with 16S rRNA of a validly described microorganism, the cluster was attributed to the corresponding genus. In the absence of such a homolog, the taxonomic position of the cluster was determined by constructing a phylogenetic tree that included the representative sequence of the cluster and a set of selected 16S rRNA sequences of bacteria or archaea. The alignment of sequences was made using the Clustal X software [9]. Phylogenetic trees were

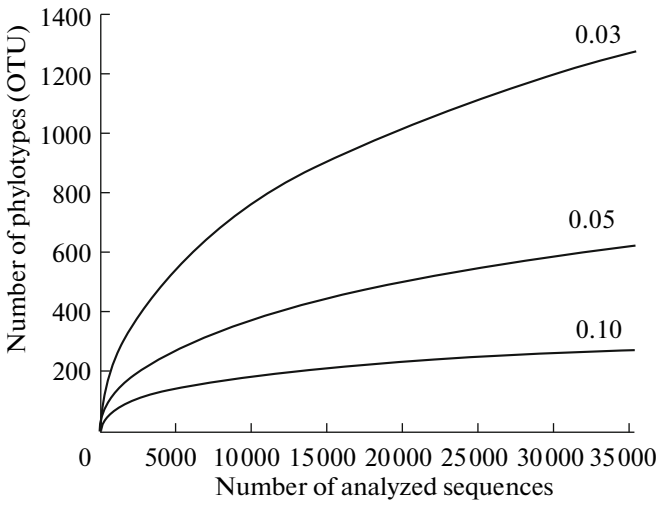

Fig. 1. Evaluation of the diversity of bacteria of the microbial community of the Zavarzin spring. The numbers of phylotypes of different levels (cluster distances are 0.03, 0.05, and 0.1) are shown as functions of the number of analyzed sequences of the 16S rRNA V3 region.

constructed by the neighbor-joining method using the Treecon program [10]. Bootstrap values were used in assessment of phylogenetic trees authenticity.

RESULTS AND DISCUSSION

Evaluation of the bacterial and archaeal diversity in the microbial community of the Zavarzin spring. A total of 37 654 sequences were determined by pyrosequencing of the V3 region of the 16S rRNA gene. Using the RDP Naive Bayesian rRNA Classifier Version 2.0, 35 702 sequences were attributed to bacteria and 1666 sequences to archaea; 286 sequences did not exhibit similarity to 16S rRNA and were excluded from further analysis. Thus, archaea constituted about 4.5% of the Zavarzin spring microbial community. The prevalence of bacteria also proved typical for the neutral and weakly alkaline moderate-temperature (50–80°C) springs of Yellowstone National Park [11]; archaea can dominate most extreme habitats with temperature higher than 80°C or high acidity (see, e.g., [12]).

The taxonomic complexity of the community was assessed by calculating the Chao1 index and by obtaining the Rarefaction curves showing the number of phylotypes (clusters) detected at various cluster distances as a function of the number of analyzed sequences. The Chao1 index of the bacterial community diversity was 1816 at the species level (cluster distance of 0.03) and 877 at the genus level (cluster distance of 0.05). As can be concluded from the curves in Fig. 1, the scale of sequencing achieved in our work was still insufficient for complete characterization of the community diversity: with the increase in the number of analyzed sequences, the rarefaction curves did not reach a plateau, at least in cases of species- and genus-level clustering. The bacterial diversity in the Zavarzin spring is comparable to the complexity of bacterial communities of submarine hydrothermal vents [5] and soil [13] and is several times higher than that found in alkaline hot springs of Yellowstone National Park [14]. Archaea comprised a minor part of the community and were less diverse: the Chao1 index was 167 for the species level and 89 for the genus level.

The composition of the bacterial community was characterized by taxonomic classification of the representative cluster sequences (Table 1). About 50% of the sequences exhibited more than 97% identity with 16S rRNAs of cultured microorganisms; in this case, the cluster was attributed to the corresponding genus. In the absence of such a homolog, taxonomic classification was carried out by constructing a phylogenetic tree that included a set of selected 16S rRNA sequences of bacteria.

The largest part of the community (32.3% of all microbial 16S rRNA sequences) was comprised of two genera from the phylum *Aquificae: Sulfurihydrogenibium* (21.6%) and *Thermosulfidibacter* (10.7%). *Aquificae* usually dominate in hot springs with neutral pH (as demonstrated, e.g., for Yellowstone National Park); their relative share increases with increase in temperature and exceeds 90% in some springs [15]. In particular, *Sulfurihydrogenibium* representatives mainly occur in neutral springs with a temperature of up to 75°C [16]. These are microaerophilic microorganisms, either obligate chemolithoautotrophs that oxidize sulfur and its compounds using oxygen as electron acceptor or facultative heterotrophs [17]. In contrast, *Thermosulfidibacter* representatives are anaerobic and oxidize hydrogen by reduction of sulfur [18].

Representatives of *Thermodesulfobacteria* (genus *Caldimicrobium*) comprise about 7.3% of the microbial community. *Caldimicrobium rimae*, isolated from the Treshchinnyi spring of the Uzon caldera, is a lithoautotroph that fixes CO_2 and oxidizes hydrogen with sulfur or thiosulfate as electron acceptors [19]. The same type of metabolism is typical of the betaproteobacteria of the genus *Thiomonas* [20], the fraction of which in the community is 0.6%.

Gammaproteobacteria of the genus *Thiofaba*, which comprise 7.6% of the total microbial community, are chemolithoautotrophic and aerobic. They fix CO_2 at the expense of oxidation of sulfur and its reduced compounds [21]. Betaproteobacteria of the genus *Tepidimonas* comprise about 6.0% of the community. They are moderately thermophilic aerobic chemolithoheterotrophs that obtain energy from oxidation of thiosulfate and other sulfur compounds to sulfate [22].

Deltaproteobacteria of the genus *Desulfurella* represent about 2.6% of the community. *Desulfurella acetivorans*, the first described bacterium of this genus, was isolated from the cyanobacterial mat of the Zavar-

Table 1. Composition of the bacterial community of the Zavarzin spring

Phylum	Number of sequences	Share (%)*	Closest 16S rRNA homolog**	% Identity
Aquificae	8076	21.61	*Sulfurihydrogenibium* sp. UZ3-5	98
	3894	10.42	*Thermosulfidibacter takaii*	96
	90	0.24	*Thermosulfidibacter takaii*	96
Deferribacteres	3003	8.04	*Calditerrivibrio nitroreducens*	98
	1535	4.11	*Calditerrivibrio nitroreducens*	96
Gammaproteobacteria	1954	5.23	*Thiofaba tepidiphila*	99
	876	2.34	*Thiofaba tepidiphila*	98
	998	2.67	*Pseudomonas fluorescens*	99
	200	0.54	*Yersinia kristensenii*	98
	77	0.21	*Acidithiobacillus ferrooxidans*	96
Thermodesulfobacteria	1802	4.82	*Caldimicrobium rimae*	99
	936	2.50	*Caldimicrobium rimae*	96
Betaproteobacteria	2243	6.00	*Tepidimonas* sp. AA2	98
	212	0.57	*Thiomonas* sp. ML2-96	96
Thermotogae	2343	6.27	*Fervidobacterium* sp. CBS-4	99
Deinococcus-Thermus	1627	4.35	*Thermus* sp. BXW	98
Deltaproteobacteria	982	2.63	*Desulfurella multipotens*	98
	142	0.38	*Desulfobulbus mediterraneus*	90
BRC1	719	1.92	Uncultured bacterium clone ZB_P13_D08	97
Caldiserica	643	1.72	*Caldisericum exile*	97
Dictyoglomi	616	1.65	*Dictyoglomus* sp. 1512	99
New lineage	389	1.04	*Hydrogenivirga caldilitoris* strain IBSK3	84
Verrucomicrobia	379	1.01	Bacterium Ellin5102	84

Notes: * of the total number of microorganisms.

** the closest cultured organism is indicated (except for phylum BRC1).

zin spring 20 years ago [4]. *Desulfurella* spp. can grow lithoautotrophically with molecular hydrogen and sulfur and can also oxidize nonfermentable organic compounds, including acetate and other organic acids, using sulfur as an electron acceptor.

Several groups of anaerobic and aerobic organotrophs known to occur in thermal habitats all over the world, make a moderate contribution to the bacterial community of the Zavarzin spring. About 6.3% of the sequences were attributed to the genus *Fervidobacterium* (phylum *Thermotogae*); the bacteria of this genus are known to carry out anaerobic fermentation of organic compounds, including proteins and polysaccharides [23]. Many representatives of the genus *Fervidobacterium* synthesize extracellular proteolytic enzymes that are able to hydrolyze such stable proteins as keratins [24]. Bacteria of the genus *Thermus* (phylum *Deinococcus–Thermus*) and of the genus *Dictyoglomus* (phylum *Dictyoglomi*), the amounts of which in the community are 4.4 and 1.6%, respectively, are also organotrophic.

The members of the second major group of bacteria of the Zavarzin spring (12.1%) belong to the genus *Calditerrivibrio* (phylum *Deferribacteres*); they are also likely to be organotrophs (Table 2). The earlier-described bacteria of this genus are organotrophic anaerobes that use nitrate as an electron acceptor and reduce it to ammonium [25]. The abundance of *Calditerrivibrio* in the Zavarzin spring may be caused by the high concentration of nitrate ions in the water (0.5 mM), one of the highest nitrate concentrations in Uzon springs.

Approximately 1.7% of the microorganisms represent the genus *Caldisericum*, (phylum *Caldiserica*, formerly OP5). This phylum is mainly represented by uncultured organisms from various thermal habitats [11]. The only cultured representative is *Caldisericum exile* AZM16c01 isolated from a hot spring in Japan; this microorganism is chemoheterotrophic and oxidizes organic substrates using sulfur and sulfite as an electron acceptors [26].

Table 2. Main types of metabolism of the bacteria from the Zavarzin spring

Reaction to oxygen	Carbon source	Electron donors	Electron acceptors	Microorganisms	Share of the group (%)
Aerobes	CO_2, organic compounds	sulfur	oxygen	*Sulfurihydrogenibium Thiofaba*	29.2
	organic compounds	thiosulfate	oxygen	*Tepidimonas*	6.0
Anaerobes	CO_2	hydrogen	sulfur	*Thermosulfidibacter Caldimicrobium Thiomonas*	18.6
	organic compounds	organic compounds	protons, sulfur (fermentation)	*Fervidobacterium Thermus Dictyoglomus*	12.3
	organic compounds	organic compounds, hydrogen	sulfur (with CO_2 and H_2S as products)	*Desulfurella Caldisericum*	4.4
	organic compounds	organic compounds	nitrate	*Calditerrivibrio*	12.2

About 1.9% of the microorganisms were attributed to the phylum BRC1, which currently does not include cultured representatives. Bacteria of this phylum have been detected in various thermal habitats, including the Zavarzin spring, e.g., GenBank accession number GQ328636. However, the metabolism of these bacteria remains unknown. About 1% of the sequences formed a separate phylum-level branch in the 16S rRNA phylogenetic tree, the homology with the closest cultured organism, *Hydrogenivirga* sp. 128-5-R1-6 of the phylum *Aquificae*, being as low as 84%. Phylogenetically close uncultured bacteria (93-96% 16S rRNA identity) have been detected in the springs of Yellowstone National Park.

Another phylum-level phylogenetic branch was formed by 0.2% of bacteria. A search in GenBank revealed seven close (98% identity) 16S rRNA sequences of uncultured microorganisms from the Zavarzin spring (GenBank accession numbers GQ328713, CQ328694, GQ328549, GQ328495, GQ328450, GQ328488, GQ328421), while the homology with 16S rRNA sequences of other microorganisms from various habitats did not exceed 86% (data of October 24, 2010). Therefore, this phylogenetic branch of bacteria, for which we propose the designation Z01, is endemic to the Zavarzin spring.

Less than 1% of the sequences were attributed to other taxonomic groups: to the phyla *Verrucomicrobia, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes*, and *Bacterioidetes* and to the proteobacteria of the alpha and epsilon classes. Cyanobacteria, which form

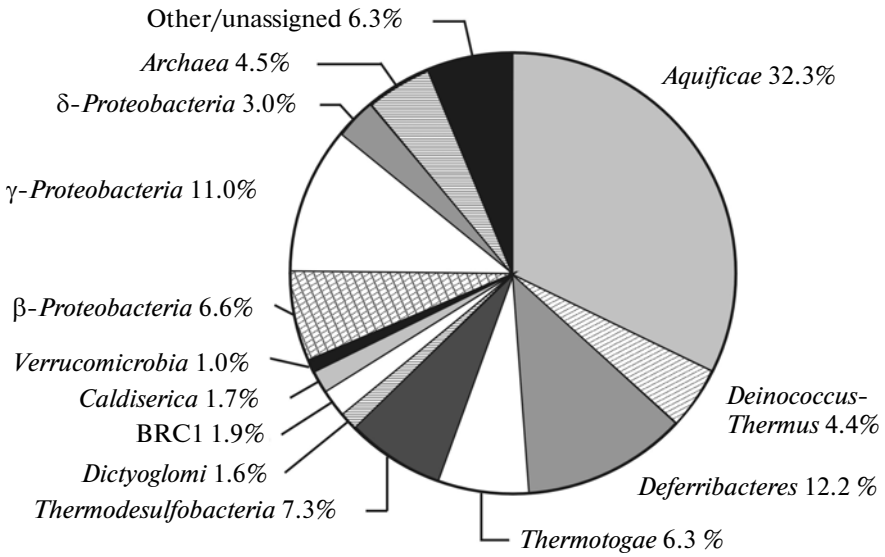

Fig. 2. Composition of the microbial community of the Zavarzin spring.

a mat at the bottom of the spring, comprised less than 0.2% of the microorganisms of the water phase.

Composition of the archaeal community. Archaea comprised 4.5% of the microbial community and included members of all four phyla: *Euryarchaeota* (42% of archaeal sequences), *Crenarchaeota* (50%), *Korarchaeota* (7.5%), and *Nanoarchaeota* (0.5%). Almost all of the euryarchaea belonged to the phylogenetic branch affiliated with the order *Thermoplasmatales* but remote from cultured organisms from this group. The order *Thermoplasmatales* includes moderately thermophilic, acidophilic, aerobic, and facultatively anaerobic archaea that oxidize organic substrates aerobically or, under anaerobic conditions, at the expense of sulfur reduction [27]. The members of this order have also been found in the ground water of the third region of the Uzon caldera Eastern thermal field [28]. Crenarchaea were represented mainly by the genus *Pyrobaculum* (25.2% of the archaea). The members of this genus are in most cases facultative anaerobes and lithoautotrophs oxidizing hydrogen and organic substances with sulfur or oxidized compounds thereof [27]. Microorganisms belonging to uncultured lineages of crenarchaea were also identified (about 18% of archaea). Representatives of the deep phylogenetic branch of archaea, *Korarchaeota*, detected in the hot springs of Yellowstone National Park [29], Iceland, and Kamchatka [30] also constituted a significant part of the archaeal community.

Metabolism of the microbial community of the Zavarzin spring. Phylogenetic analysis performed in this work allowed us to classify most of the community members at the genus or family level. Although phylogenetic proximity in many cases does not correlate with similarity of microbial metabolic pathways, the obtained data allow us to suggest hypotheses on the ecological interrelations between the major microbial groups found.

The temperature of the spring is lower than the boundary limiting photosynthesis (around 70°C). Therefore, primary production of organic matter can proceed photosynthetically and chemolithoautotrophically at the expense of oxidizing the reduced compounds of volcanic origin supplied by the geothermal fluid. Photosynthesis in the Zavarzin spring is performed by the cyanobacterial mat that covers the bottom; in the spring water, the concentration of phototrophs (cyanobacteria and *Chloroflexi*) was very low. Chemolithoautotrophic production occurs under aerobic and microaerobic conditions at the expense of oxidation of sulfur and reduced sulfur compounds (*Sulfurihydrogenibium, Thiofaba*) and, in the anaerobic zone, at the expense of hydrogen oxidation with sulfur as the electron acceptor (*Thermosulfidibacter*, *Caldimicrobium, Thiomonas*). Methane, another potential energy source, is unlikely to be utilized in the Zavarzin spring (55–58°C, pH 6.3), since we have found none of the known methanotrophic bacteria, including the thermoacidophilic methanotrophs of the phylum *Verrucomicrobia* identified in Uzon springs that have a similar temperature but a lower pH value (2–4) [31]. On the whole, the share of chemolithoautotrophic bacteria in the Zavarzin spring was about one-half of the total number of microorganisms (Table 2).

The organic matter synthesized by photosynthetic and chemolithoautotrophic microorganisms, as well as that arriving with the surface waters from the adjacent low-temperature zones, can be utilized by various organotrophic microorganisms, represented mainly by anaerobes. Organotrophs include both bacteria that ferment organic substrates (*Fervidobacterium*, *Dictyoglomus, Caldisericum*) and bacteria that perform complete oxidation of organic substrates by oxygen (*Thermus*), sulfur (*Desulfurella*), or nitrate (*Thermus, Calditerrivibrio*) as electron acceptors. Elemental sulfur may enter the spring from the surrounding sulfur hillocks or may be formed via chemical or biological oxidation of hydrogen sulfide present in the spring water. The virtually complete absence of known sulfate-reducing microorganisms may be accounted for by the low concentration of sulfate. In contrast, bacteria phylogenetically close to known nitrate reducers comprise a significant part of the community, which correlates with the high concentration of nitrate in the spring water. However, the origin of the nitrate is unclear, since the community lacks known thermophilic microorganisms that could drive the first (archaea of the phylum *Thaumarchaeota*) and second (bacteria of the phylum *Nitrospira*) phases of nitrification and thus oxidize ammonium present in the spring water.

On the whole, the Zavarzin spring is characterized by very high diversity of thermophilic prokaryotes. This habitat harbors microorganisms of various high-level taxa with diverse types of metabolism. No absolute prevalence of a single microbial group was observed, in contrast to high-temperature springs of the Yellowstone National Park, where *Aquificales* were shown to comprise more then 95% of the communities [15]. This distinction may be explained by the comparatively moderate temperature and pH values in the Zavarzin spring and by the diversity of primary production processes occurring there: chemosynthesis performed by chemolithoautotrophs in the spring water layers and photosynthesis carried out by phototrophic microorganisms of the cyanobacterial mats. The large number of phylogenetically diverse groups, none of which predominate, indicates that microorganisms of the Zavarzin spring form a well-balanced complex community in which each group occupies its own ecological niche. Such a community may be considered a model of the first ecosystems of the ancient Earth and should be an object of further detailed studies.

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