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EXPERIMENTAL ARTICLES

Archaeal Diversity in Permafrost Deposits of Bunger Hills Oasis and King George Island (Antarctica) according to the 16S rRNA Gene Sequencing

E. S. Karaevskaya[a, 1], L. S. Demchenko[b], N. E. Demidov[a], E. M. Rivkina[a], S. A. Bulat[b], and D. A. Gilichinsky[a, †]

[a] *Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Russia*
[b] *Konstantinov Petersburg Nuclear Physics Institute, NRC "Kurchatov Institute", Gatchina, Russia*

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Abstract—Archaeal communities of permafrost deposits of King George Island and Bunger Hills Oasis (Antarctica) differing in the content of biogenic methane were analyzed using the clone libraries of two 16S rRNA gene regions. Phylotypes belonging to methanogenic archaea were identified in all horizons.

Keywords: archaea, biogenic methane, permafrost, Antarctica

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Antarctic oases are ice-free parts of the continent formed by permafrost sedimentary rocks. They occupy 0.3–2.0% of the Antarctic territory [1–3]. The organic carbon content in permafrost sediments varies from several basis points to 1.4% [1, 4]. The ice content of the rocks is 5–90% [1]; ice is present as intercalations or cement. The total microbial cell content determined by direct counting is 10^5–10^6 cells/g [5, 6]. For successful resuscitation of the potentially viable cells of these sediments in laboratory conditions, a large number of growth requirements should be taken into account, and nevertheless, only 0.001–0.01% of the total number of cells present can usually be cultured [7].

It was previously shown that DNA can be preserved in permafrost sediments for very long periods of time, from several thousands to several millions of years [6].

The purpose of this study was to investigate the diversity of archaea in the sediments in question by analyzing fragments of the 16S rRNA genes amplified by PCR with primers to the sequences specific for the domain *Archaea*.

MATERIALS AND METHODS

The study was performed with the samples containing biogenic methane [1]. The presence of biogenic methane in Arctic and Antarctic permafrost rocks of different ages [6, 8, 9] is important evidence of microbial biochemical activity that occurred in previous geological periods. Our study was aimed at describing the diversity of archaea in permafrost marine deposits of King George Island (Bellingshausen Station) and in sedimentary (presumably, lacustrine) deposits of Bunger Hills Oasis (Oazis II Station) by analysis of the clone libraries representing two 16S rRNA gene regions. This approach was previously applied to describe the bacterial communities in snow samples from the top of Mont Blanc [10] and in water samples from Radok Lake, the deepest Antarctic surface lake [11]. In this work, it was for the first time applied to characterize archaeal communities of Antarctic permafrost rock samples.

Well B1-09 on King George Island penetrated the marine terrace deposits previously washed with glacier meltwater. Deposits were composed of gravel with an underlying layer of sandy to loamy soils with debris inclusions that reached the depth of 7.5 m [1]. Ice content in the sediments varied from 10 to 36%. The well contained biogenic methane; the highest CH_4 content (0.41 mmol/mL) was observed in sediments at the depth of 6.6 m. The total dissolved solid content of the water fraction was 1–4 g/L, with Na^+ and SO_4^{2-} as predominating ions. The sediments were reduced, with Eh = –483 mV (Table 1).

Well A5-08 penetrated the lacustrine sedimentary rocks from the bed of a temporary stream that used to flow across a dried-up lake in the Bunger Hills Oasis (Oazis II Station). The sediments were composed of permafrost sands, sandy loams, and loamy soil varieties with inclusions of shingles, gravel, boulders, and fauna remnants. The predominant ions in the water fraction were Na^+, HCO_3^-, and SO_4^{2-}, the total dissolved solids level was 1–3 g/L. In the 1.5-m deep

[1] Corresponding author; e-mail: katya_k_s@mail.ru
[†] Deceased.

Table 1. Characteristics or the permafrost sediments

Well/depth, m	Location	Sediment age, ka	Temperature at the site, °C	pH	C_{org}, %	Eh, mV	CH_4, mol/mL	$\delta^{13}C$, %
B1-09/6.6	Bellingshausen Station, 62.2° S, 59.0° W	7.5	–0.6	10.1	0.104	–356	0.411	–92.1
B1-09/9.4				9.7	0.284	–414	0.095	–94.1
A5-08/1.5	Oasis II Station, 66.3° S 100.8° E	23.7	–7.9	8.1	0	+267	0.001	ND
A5-08/5.8				9.1	0	–489	0	ND

layer, the environment was oxidized (Eh = +267 mV), while at the depth of 5.8 m, sediments were reduced (Eh = –489 mV) (Table 1). No methane was detected in this well. Altogether, four samples obtained from the depths of 6.6 and 9.4 m for well B1-09 and from 1.5 and 5.8 m for well A5-08 were analyzed.

The age of the sediments was determined by AMS radiocarbon dating in the Radiocarbon Laboratory of Zurich University (Switzerland). Gas specimens were collected using headspace degassing in 150 mL syringes [12]. Methane content was determined on a KhPM-4 gas chromatograph (Russia) equipped with a flame ionization detector [9]. The carbon isotope composition ($\delta^{13}C$) of methane and carbon dioxide was determined on a Delta^{plus} XL,GC Combustion III system for gas chromatography–mass spectrometry (ThermoFinnigan, Germany) in the Center of Isotopic Research of Karpinsky Russian Geological Research Institute. The absolute $^{13}C/^{12}C$ ratio determined for the international VPDB standard [13] was used as the reference value (0.0112372). The total carbon abundance was determined by dry incineration using an AN-7529 express analyzer (Russia). The total cell numbers in the samples were determined by direct counts in preparations stained with 4,6-diamidino-2-phenylindole (DAPI) and acridine orange (1 : 10000 dilution) fluorescent dyes. DAPI-stained cell suspensions were examined in a thin film between a slide and a coverslip; for acridine orange staining, cell suspensions were fixed on a slide plate by passing through a Bunsen burner flame and then stained. Each specimen was analyzed in three replicates; the cells were counted using a Carl Zeiss Axiostar plus microscope in at least 30 fields of vision.

The number of bacteria was calculated using the following formula:

$$N = (4 \cdot a \cdot n \cdot 10^{10})/S \cdot b,$$

where N is the number of bacteria in 1 g soil, a is the average number of cells in a field of vision, n is the dilution, 4 stands for the total specimen area of 4 cm^2 (4×10^8 μm^2), S is the area of the field of vision (17662 μm^2), and b is the specimen mass (g).

Samples obtained from well B1-09 were cultured in the standard PBBM growth medium for methanogens, which contained the following (g/L): NaCl, 0.9; $MgCl_2$, 0.2; $CaCl_2$, 0.1; NH_4Cl, 1.0; trace element solution (medium 318; DSMZ), 10 mL; vitamin solution (medium 141; DSMZ), 5 mL; and resazurin solution, 1 mL. Phosphate buffer solutions were sterilized for 5–10 min at 0.5 atm and added to the medium after sterilization (30 min at 1 atm). Phosphate buffers were prepared with (1) 15.0 g KH_2PO_4 in 100 mL distilled water, and (2) 29 g K_2HPO_4 in 100 mL distilled water. The cells were cultured in an H_2/CO_2 atmosphere (4 : 1, 150 kPa) at 4°C and 20°C [14].

DNA isolation from the sediment samples was performed in a certified laboratory with class 10 000 clean rooms and class 100 laminar flow hoods, (Laboratoire de Glaciologie et Geophysique de l'Environnement, Grenoble, France) [15]. Cells were disintegrated mechanically using a FastPrep homogenizer (MP Biomedicals, United States). Genomic DNA was isolated using the PowerSoil DNA isolation kit (MoBio Labs, United States). Fragments of archaeal 16S rRNA genes were amplified with two pairs of degenerate primers: SABAr4Fm (1) + 958Ra (2) to the v1–v5 region and Gmar347Fnew (3) + 985Ra to the v3–v5 region (630 and 975 bp, respectively). The primer sequences were as follows: (1) 5'- TTCYSGTTGATYCYGCSRGA, (2) 5'-YCCGGCGTTGAMTCCAATT and (3) 5'-GGGYGCASCAGGCGCGAA.

This strategy was chosen after preliminary experiments on studying bacterial diversity by PCR amplification of different 16S rRNA gene fragments [11], which showed that at least two different gene regions should be analyzed to obtain a more comprehensive description of diversity at the phylotype level.

Amplicons were cloned into vectors of the TOPO TA Cloning® Kit for Sequencing (Invitrogen, United States). The coverage of clone libraries was previously determined by ribotyping with three restriction endonucleases *Alu*I, *Hpa*II, and *Hae*III (Fermentas, Lithuania) and corrected to greater values; and the ultimate level of coverage was determined by analyzing the obtained nucleotide sequences combined into phylotypes. Sequencing was performed by LGC Genomics GmbH (Germany) and Evrogen (Russia). Sequence alignment and comparison, as well as phylotype identification, were performed with CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2) and

BLAST algorithm available at the GenBank database (http://blast.ncbi.nlm.nih.gov). Nucleotide sequences with more than 98% identity were combined into a single phylotype.

RESULTS AND DISCUSSION

According to radiocarbon analysis data, the maximum age of the deposits penetrated by well B1-09 was 7485 ± 40 years, and those in well A5-08, as 23705 ± 110 years [1].

Permafrost sediment samples from well A5-08 contained 10^5–10^7 microbial cells/g and the number of cells varied at different horizons, while samples from well B1-09 contained 10^8 cells/g, with uniform microbial abundance throughout the profile. The higher abundance of microbial cells observed in well B1-09 probably resulted from the younger age of these deposits and from the higher temperature of permafrost (–0.6°C) at this site (Table 2).

A total of seven clone libraries were constructed for four soil samples, representing two amplicon types for each horizon, except for the sample from 5.8-m depth of well A5-08, which did not give rise to any PCR products representing the v1–v5 region of the 16S rRNA gene, apparently due to the lack of DNA with the relevant binding sites. Each library contained 16 to 30 clones with the coverage of 93% and higher (Table 2); i.e., the archaeal diversity was not large. The sequences that showed more than 98% identity to the phylotypes from the archaeal contaminant library [15] were classified as putative contaminants.

In this work, we considered the so-called true phylotypes isolated from the specimens studied. We also introduced the notions of dominant and minor phylotypes for those phylotypes that were represented by at least three clones and by less than three clones, respectively. Presumably, the biogeochemical functioning of ancient microbial communities was primarily determined by the preserved dominant phylotypes.

The obtained sequences were uploaded into the NCBI database under accession numbers KF201629–KF201632 and KF201634–KF201644. It should be noted that our analysis of clone libraries representing different regions of the same gene (v1–v5 and v3–v5) identified mainly non-overlapping phylotypes of archaea, as was previously shown by molecular genetic studies of bacterial diversity in other habitats [10]. Therefore, for further description of each horizon, data from the two types of libraries were combined.

As a result, the methane-rich upper horizon (6.6 m) of well B1-09 (Bellingshausen) was found to contain archaea of ten phylotypes (five dominant and five minor), and eleven phylotypes (six dominant and five minor) were detected in the lower horizon (9.4 m) (Table 2). The dominant phylotypes of the two horizons were all different, except for two closely related phylotypes of the genus *Methanobrevibacter*, and for *Nitrososphaera* sp. phylotypes, which were classified as contaminants. In addition, two phylotypes of the class *Methanobacteria* were dominant in the upper horizon but minor in the lower one (Table 2).

Altogether, from the total number of phylotypes detected, only a few could be identified at the genus or species level. For well B1-09, these were two dominant phylotypes (both putative contaminants) and two minor ones (one putative contaminant) for the 6.6 m deep horizon and five dominant (two putative contaminants) and one minor phylotype from the 9.4 m deep horizon. For well A5-08, two phylotypes from the 1.5 m horizon were identified, one of them putatively a contaminant.

In the methane-poor horizon at the depth of 6.6 m, there was only one dominant phylotype, which showed 90% identity to *Methanosarcina* sp. (*Euryarchaeota*), a methanogen capable of utilizing all three methanogenesis pathways: hydrogenotrophic, acetoclastic, and methylotrophic [16]. Two other phylotypes from different horizons were classified as *Methanobrevibacter* (*Euryarchaeota*, hydrogenotrophic methanogenesis) [17]. Three phylotypes from the 6.6 m horizon belonged to *Nitrososphaera* (*Thaumarchaeota*), aerobic archaea that oxidize ammonium to nitrite (AMMOX) [18]; all three coincided with the phylotypes included in the contaminant database [15]. Two more dominant phylotypes from the 9.4 m horizon remained unidentified; i.e., it was not possible to classify them into any known taxonomic group.

For one of these phylotypes, the 16S rRNA gene sequence showed a remote similarity (83%) to the genus *Methanobrevibacter* of the class *Methanobacteriales*, while the other was similar to *Thermoplasma* (86%) of the class *Thermoplasmata* (*Euryarchaeota*), which includes acidophilic and sulfur-oxidizing species, mainly thermophilic ones [19].

Thus, assuming that the dominant phylotypes reflect the functioning of the corresponding ecosystem (in the case considered, at the soil temperature of –0.6°C), the methane-rich horizon at the depth of 9.4 m was probably characterized by hydrogenotrophic methane production by archaea, while in the methane-poor horizon at 6.6 m, methanogenesis probably occurred via different pathways and was probably accompanied by anaerobic ammonium oxidation to nitrite. The fact that methane found in the sediments was enriched with ^{12}C indicated that it has largely been produced by the hydrogenotrophic pathway [20]. However, to obtain a complete picture and to explain the higher levels of methane in the upper horizon, the data on the dominant bacterial phylotypes are also required.

In well A5-08 (Bunger Hills Oasis), two dominant archaean phylotypes were identified in the 1.5-m deep horizon, and one dominant phylotype was identified in the 5.8-m deep horizon; they did not coincide

Table 2. Phylotypes of archaea present in soil cores, identified by two 16S pRNA gene fragments

16S rRNA gene fragment	Number of clones—GenBank acc. no. of the 16S rRNA fragment	v3–v5/v1–v5 library coverage, %	Most closely related culturable organism—GenBank acc. no. of the 16S rRNA gene sequence—% identity to the phylotype identified/phylotype no. of the most closely related unculturable microorganism	Properties of species/genus	Source of species/genus isolation	Reference
Sample taken at the depth of 6.6 m from well B1-09 (Bellingshausen Station, King George Island)						
v3–v5	7	100/94	*Nitrososphaera* sp.*—JF748724—96%/JX099324—99%	Ammonium-oxidizing archaea	Agricultural soil, Korea/mineral soil, Atakama	[23]/[24]
v3–v5	3		*Nitrososphaera gargensis** – GU797786—96%/JN562356—100%		Natural habitats/wetland subsurface flow	[25]/[26]
v1–v5, v3–v5	1, 10—KF201638		*Methanothermobacter tenebrarum* – AB523785—80%; *Methanobrevibacter* sp.—AB009821—83%/FJ264536—99%	Hydrogenotrophic thermophilic methanogen; symbiotic methanogen	Gas-associated formation water, Japan; termite nests	[27]; [28]/[29]
v1–v5	9—KF201639		*Methanobrevibacter ruminantium*—DQ150253—3%; *Methanobrevibacter arboriphilus*—AY196663—80%/GQ356907—99%	Heterotrophic and hydrogenotrophic methanogens	Biogas fermentation reactor; methane-containing wetwood of living trees; anaerobic digested sludge	[30]; [31]/[29]
v3–v5	3—KF201631		*Thermoplasma* sp.—FJ823135—86%/FJ873201—100%; *Candidatus* Methanogranum caenicola—AB749767—83%	Thermophilic acidophile, facultative aerobe capable of sulfur reduction/hydrogenotrophic methanol hydrolysis	Human intestine/cold seep sediments of the Okhotsk Sea; anaerobic intestinal mucus	[32]/[33]; [34]
v3–v5	2—KF201630		*Staphylothermus marinus*—NR075046—87%/GU270138—100%	Hyperthermophilic sulfur-dependent anaerobic heterotroph	Hydrothermal vent of the East Pacific Rise/deep methane-rich seep sediments of the Okhotsk Sea	[35]/[36]
v1–v5	2—KF201641		*Methanosarcina semesiae*—NR028182—98%/GQ356887—99%	Dimethyl sulfide-consuming methanogen	Mangrove sediments	[37]/[29]

Table 2. (Contd.)

16S rRNA gene fragment	Number of clones—GenBank acc. no. of the 16S rRNA fragment	v3–v5/v1–v5 library coverage, %	Most closely related culturable organism—GenBank acc. no. of the 16S rRNA gene sequence—% identity to the phylotype identified/phylotype no. of the most closely related unculturable microorganism	Properties of species/genus	Source of species/genus isolation	Reference
v3–v5	2-KF201638		*Methanothermobacter tenebrarum* AB523785 – 83%; *Methanothermobacter thermautotrophicus* – AY196661 – 82%/ FJ264536 – 99%	Hydrogenotrophic thermophilic methanogen; hydrogenotrophic moderately thermophilic methanogen	Water associated with natural gas fields; human intestine/marine habitats	[27]; [38]/ [29]
v3–v5	2-KF201632		*Methanoculleus thermophilus* – EF118904 – 79%; *Methanogenium thermophilum* – M59129 – 79%/ AB825643 – 97%	Thermophilic anaerobic methanogen; marine thermophilic methanogen	Soil, freshwater sediments; marine sediments/deep marine sediments	[39]; [40/[41]
v1–v5	1, 1		*Nitrososphaera* sp.* – FR773158 – 96%/ GQ871360 – 99%, HM584831 – 99%	Ammonium-oxidizing archaea	Soil/soil treated with inorganic fertilizer and manure	[42]/[43]
Sample taken at the depth of 9.4 m from well B1-09 (Bellingshausen Station, King George Island)						
v3–v5	8	98/93	*Nitrososphaera* sp.* – JF748724 – 96%	Ammonium-oxidizing archaea	Agricultural soil	[23]
v3–v5, v1–v5	21, 16		*Nitrososphaera gargensis** – GU797786 – 96%/ JN562356 – 100%, HM584831 – 99%		Soil/ wetland subsurface flow	[42]/[26]
v3–v5	4		*Nitrososphaera* sp.* – FR773158 – 96%/ GQ871360 – 99%		Soil/soil treated with inorganic fertilizer and manure	[42]/[43]
v3–v5, v1–v5	16, 5-KF201636		*Nitrososphaera viennensis* – FR773157 – 95%/ EU309862 – 100%		Soil/rhizosphere of the freshwater macrophyte *Littorella uniflora*	[42]/[44]
v3–v5	5-KF201635		*Methanosarcina* sp. – AB598272 – 99%	Anaerobic methanogen using all three types of methanogenesis	Methane-rich marine sediment cores collected near the Shimokita peninsula, Japan	[45]
v1–v5	3-KF201640		*Methanobrevibacter ruminantium* – DQ150253 – 75%/ GQ356823 – 99%	Heterotrophic and hydrogenotrophic methanogen	Ruminant intestine	[30]/[29]

Table 2. (Contd.)

16S rRNA gene fragment	Number of clones—GenBank acc. no. of the 16S rRNA fragment	v3–v5/v1–v5 library coverage, %	Most closely related culturable organism—GenBank acc. no. of the 16S rRNA gene sequence—% identity to the phylotype identified/phylotype no. of the most closely related unculturable microorganism	Properties of species/genus	Source of species/genus isolation	Reference
v1–v5	2-KF201637		*Methanogenium cariaci* – NR104730 – 99%		Methane-rich marine sediments	[46]
v3–v5	2-KF201638		*Methanothermobacter tenebrarum* – AB523785 – 80%/ FJ264536 – 99%	Hydrogenotrophic thermophilic methanogen	Water associated with natural gas fields	[27]/[29]
v1–v5	1-KF201642		*Methanosaeta* sp. – AJ133791 – 91%/ GQ356823 – 99%	Acetoclastic methanogen	Subglacial sediments	[47]/[29]
v3–v5	1-KF201634		*Methanobrevibacter arboriphilus* – AY196663 – 84%/ GU270190 – 100%	Hydrogenotrophic and heterotrophic methanogen	Soils, anaerobic water ecosystems	[31]/[36]
v1–v5	1-KF201639		*Methanobrevibacter ruminantium* – DQ150253 – 83%; *Methanobrevibacter* sp. – AB009827 – 83%/ GQ356907 – 99%	Heterotrophic and hydrogenotrophic methanogen	Ruminant intestine	[30]/[29]
Sample taken at the depth of 1.5 m from well A5-08 (Oasis II Station, Bunger Hills)						
v3–v5	15	100/100	*Nitrososphaera* sp.* – JF748724 – 96%/ AY923076 – 99%	Ammonium-oxidizing archaea	Agricultural soil/rock varnish in the Whipple Mountains, California	[23]/[48]
v1–v5	8-KF201643		*Methanosarcina lacustris* – AY260431 – 98%/ GU122859 – 99%	Psychrotolerant methanogen	Anaerobic lake sediments	[21]/[49]
Sample taken at the depth of 5.8 m from well A5-08 (Oazis II Station, Bunger Hills Oasis)						
v3–v5	19-KF201644	100/–	*Methanomethylovorans hollandica* – AY260433 – 90%/ KC604451 – 99%	Methylotrophic strictly anaerobic methanogen	Freshwater sediments/groundwater	[22]/[50]

* Phylotypes conspecific (over 98% identity) with sequences of the contaminant database [15].

(Table 2). The dominant phylotypes of the upper horizon were identified as belonging to *Nitrososphaera* [18] (identical to the phylotypes present in the contaminant database [15]) and as *Methanosarcina lacustris*, a "multifaceted" psychrotolerant methanogen of lacustrine habitats [21] (98.3% identity). The unidentified dominant phylotype of the lower horizon belonged to *Methanomicrobia*, probably, to *Methanosarcinales*: the closest known species, *Methanomethylovorans hollandica* (89.4% identity) is another multifaceted methanogen [22]. Assuming that the dominant phylotypes determine the mode of functioning of an ecosystem, we suppose that, although neither horizon currently contained methane, methanogenesis might have been occurring in these sediments before their freezing.

Thus, we identified the dominant phylotypes most closely related to methanogenic archaea in permafrost rocks of marine and presumably lacustrine origin. Within marine sediments of King George Island, there were present a wide variety of dominant and minor phylotypes related to the genera *Methanosarcina, Nitrososphaera, Methanobrevibacter, Methanogenium, Methanolobus*, and *Methanoculleus*, two dominant phylotypes of the class *Methanomicrobia*, as well as a phylotypes close to ammonium-oxidizing, thermophilic and acidophilic archaea. Such level of diversity is apparently determined by several factors, including the meltwater washing of sea sediments before freezing, environment temperatures close to zero, and a relatively young age of the frozen sediments.

In the Bunger Hills Oasis sediment sample with a high redox potential (Table 1), the diversity of archaea was very low: we detected only two representatives of *Methanomicrobia* and, moreover, no DNA fragments were amplified in PCR with primers to the v1–v5 region of the 16S rRNA gene. Apparently, the corresponding fragments of archaeal DNA were poorly preserved in these sediments.

We did not observe methanogenesis in enrichment cultures from well B1-09 samples that have been growing in the standard medium for methanogens for over two years. It is possible that we failed to find the optimal growth conditions for culturing these methanogens, or, alternatively, they did not retain viability in the relatively warm conditions (–0.6°C) of the sediments in question. The obtained list of dominant archaeal phylotypes should help to optimize selective conditions for their cultivation and could possibly enable us to cultivate at least some of these microorganisms.

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