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EXPERIMENTAL ARTICLES

Microbial Diversity in the Samples from Archeological Complexes of the Pazyryk Culture (IV–III Centuries BC) in Northwestern Mongolia

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Abstract—Prokaryotes were analyzed in the samples of ice and soils collected by the joint Russian–German–Mongolian expedition (2006) at the site of archeological excavations of two Pazyryk culture mounds in the upper Olon-Kurin-Gol River on the southern slope of the Saylyugem Mountains, Mongolian Altai. Phylogenetic analysis of the 16S rRNA gene fragments of the cultured bacteria revealed three major groups widespread in permafrost soils: *Firmicutes, Actinobacteria,* and *Gammaproteobacteria.* Analysis of the total DNA from the samples revealed nonculturable bacteria of the phyla *Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Actinobacteria, Chloroflexi, Gemmatoidetes, Firmicutes*, and *CFB* (*Cytophaga–Flavobacteria–Bacteroidetes*) isolated from the samples of permafrost soils (Arctic, tundra, and from Tibet highlands), groundwater, and Arctic and Antarctic ice cores.

Keywords: prokaryotes, isolates, Mongolian Altai, cloning, phylogenetic analysis

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Numerous publications deal with the species composition and diversity of microorganisms in the cryosphere objects [1–6]. Microorganisms preserved in ice or permafrost may not only stay in a frozen state for a long time, but are also able to reverse their metabolic activity. Archeological materials from the closed burials, where microorganisms of a specific period may be preserved in a frozen state for a long time, are especially interesting in this respect [7–9]. The typical inhabitants of soil, water, and normal microflora of animals and humans, as well as pathogenic microorganisms, were shown to retain viability under such extreme conditions [7–10].

During the archeological excavation of two burial mounds of the Pazyryk culture in the upper Olon-Kurin-Gol River on the southern slope of the Saylyugem Mountains, Mongolian Altai by the joint Russian–German–Mongolian expedition (2006), burial chambers with ice and permafrost soil particles were found. According to the data of radiocarbon analysis, the age of the burials was 2500 years [11]. Their preservation in a frozen state resulted from the peculiarities of the burial rite and from the cold arid climate dominating in the region during the last 3000–4000 years [12, 13]. Conservation in a frozen state preserved objects reflecting various aspects of the social, domestic, and economic lifestyle, which do not survive under other conditions.

During the excavation, biological material was collected from the burial chambers, as well as the samples of ice with frozen particles of soil and wood. The goal of the present work was to detect viable bacteria able to survive during prolonged freezing and to investigate their diversity using the classical microbiological and molecular genetic techniques.

MATERIALS AND METHODS

Sampling. Samples of ice containing small particles of soil and plant debris, as well as remains of the organic tissue of horses, were collected in the burial chambers. A total of the 11 samples taken are listed in Table 1. The samples were collected immediately after their uncovering, with precautions to avoid contamination. For sampling, sterile surgical gloves were used, as well as sterilized instruments stored in sealed plastic bags. The material was immediately placed in 50-mL sterile plastic test tubes with hermetic screw caps. Until the end of the expedition, the samples were stored in a dark place at 0–5 (night) and 5–15°C (day). During transit (72 h), the containers were stored at 20°C during day and 10°C at night. Afterwards the samples were stored at 4–6°C in the dark.

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Table 1. Sampling sites and phylogenetic position of the clones from the archeological complexes (northwestern Mongolia)

Sample no.	Sampling site	Phylum	Number of clones	Closest relative in GenBank database (% similarity)
1	Monument: Olon-Kurin-Gol-6, mound 2. Ice from the central part of the log construction with inclusions of small soil and wood residues. Depth ~200 cm	*Firmicutes* *Proteobacteria*, class *Gammaproteobacteria*	1 1	*Bacillus firmus* (100%) *Pseudomonas* sp. (100%)
2	Monument: Olon-Kurin-Gol-6, mound 2. Ice from the log construction with inclusions of wood, collected at the southern wall. Depth ~200 cm	*Firmicutes* *Proteobacteria*, class *Gammaproteobacteria*	2 2	*Bacillus* sp. (97%,98%) *Pseudomonas* sp. (98%)
3	Monument: Olon-Kurin-Gol-6, mound 2. Ice from the log construction with small inclusions of soil and wood particles, collected at the northern wall. Depth 210 cm. Sampling in three repeats	*Firmicutes*	2	*Bacillus cereus* (100%) *Bacillus* sp. (99%)
4	Monument: Olon-Kurin-Gol-6, mound 2. Residues of horse soft tissues. In the soil outside the northern wall. Depth 170–180 cm	*Firmicutes*	1	*Bacillus* sp. (100%)
5	Monument: Olon-Kurin-Gol-10, mound 1. Ice from the log construction. Ice crystals on the surface of the burial bed with inclusions of soil particles and small fibers. Depth ~150 cm	*Firmicutes*	1	*Bacillus* sp. (100%)
6	Monument: Olon-Kurin-Gol-10, mound 1. Ice from the log construction. Collected from below the burial bed. Sampling by fracturing the lens with frozen soil particles. Depth ~170 cm	*Actinobacteria* *Proteobacteria,* class *Deltaproteobacteria* *CFB*, supertype *Bacteroidetes/Chlorobi* *Proteobacteria*, class *Alphaproteobacteria*	1 3 2 7	*Rhodococcus* sp. (96%) Unc. clone (93%) Unc. clone (96%) Unc. clone (99%)
7	Monument: Olon-Kurin-Gol-10, mound 1. Ice from the northeastern corner of the log construction. Sampling from the ice layer with a corer. Depth ~180 cm	*Proteobacteria*, class *Gammaproteobacteria*	1	*Pseudomonas* sp. (100%)
8	Monument: Olon-Kurin-Gol-10, mound 1. Residues of organic tissues of horse no. 1. At the northern wall of the log construction at the outside in soil. Depth ~120–130 cm	*Firmicutes* *Actinobacteria* *Actinobacteria* *Proteobacteria*, class *Gammaproteobacteria* *Proteobacteria*, class *Alphaproteobacteria* *CFB,* supertype *Bacteroidetes/Chlorobi* *Chloroflexi* *Gemmatimonadetes* *Proteobacteria*	1 1 15 5 1 2 2 4 3	*Bacillus* sp. (99%) *Rothia* sp. (98%) Unc. clone (95%, 96%) *Afipia* sp. (98%) Unc. clone (96%, 97%) Unc. clone (94%) Unc. clone (91%, 94%) Unc. clone (93–99%) Unc. clone (93–99%)
9	Monument: Olon-Kurin-Gol-10, mound 1. Residues of organic tissues of horse no. 2. At the northern wall of the log construction at the outside in soil. Depth ~120–130 cm	*Firmicutes* *Firmicutes* *Actinobacteria* *Actinobacteria* *Proteobacteria* *Proteobacteria*, class *Gammaproteobacteria* *CFB*, group *Cytophagales/GSB*	2 15 2 3 6 2 2	*Bacillus* sp. (99%) *Paenibacillus* sp. (97%) *Rhodococcus* sp. (98%) Unc. clone (95%–100%) Unc. clone (93–99%) Unc. clone (98–99%) Unc. clone (97%)

Sample preparation. The test tubes were opened in a sterile box. Liquid samples were collected with sterile syringes. Solid residues of organic horse tissue (1 g) were resuspended in 100 mL of sterile physiological saline.

Detection and enumeration of bacteria. The melted ice samples were fixed according to the standard procedure [14], filtered through polycarbonate membranes (pore diameter 0.2 μm), and stained with CITIFLUOR with DAPI (4,6-diamidino-2-phenylindole) at the working concentration of 10 μg/mL for 5–8 min at room temperature. Total numbers of microbial cells (TNMC) were determined under an Olympus epifluorescence microscope using the Imagetest software package [15], which makes it possible to correct the range of investigated objects by discarding the false ones.

For isolation and enumeration of organotrophic bacteria, 1 mL of the suspension of organic horse tissue or of melted ice from the 10^{-3} dilution was plated on fish–peptone agar (FPA : 10) [16]. To isolate spore-forming *Bacillus* cultures, the samples were pasteurized (10 min at 80°C) prior to plating on FPA : 10 supplemented with $MnSO_4$ (http://www.dsmz.de). For selective isolation of *Bacillus cereus*, Bacillus-Cereus-Agar (Sifin code: TN 1245) supplemented with egg emulsion (code: TN 1316) and the Bacillus Cereus selective solution (code: TN 1315) was used. The plates were incubated at 22°C. Morphologically different well-isolated colonies were streak-inoculated on fresh media and incubated under the same conditions. The phenotypic properties (cell and colony morphology, motility, spore formation, and Gram reaction), as the physiological and biochemical characteristics, and the potential activity of extracellular enzymes (protease, amylase, lipase, lecithinase, alkaline phosphatase, and phospholipase) were studied using the standard techniques [17, 18]. The carbohydrate nutrient spectrum was determined using diagnostic media (BioKmopas-S, Russia, http://www.biokompas.ru). Identification of the isolates was carried out using the Bergey's manual [19]. Bacilli were identified according to the scheme proposed by Norris et al. [20] and using the Bergey's manual.

DNA isolation from pure cultures and from each sample was carried out by the modified enzymatic lysis method with subsequent phenol–chloroform extraction [21] and by the (cetavlon) hexadecyltrimethylammonium bromide method as modified by Grachev et al. [22]. In the enzymatic lysis procedure, lysozyme and proteinase K were used, as well as the chemical detergent SDS and polyvinylpyrrolidone as an additional agent for binding humic acids during the mechanical grinding of the sample. For DNA isolation with cetavlon, the sample was homogenized at 60°C in a lysing buffer with high ionic strength (1.4 M NaCl) [22]. DNA obtained by both methods was used for subsequent analysis.

Polymerase chain reaction (PCR) was carried out as follows: 2 min at 94°C (1 cycle); 45 s at 92°C, 45 s at 52°C, 60 s at 72°C (30 cycles); 2 min at 72°C (1 cycle). The fragments of the 16S rRNA gene were amplified using the primers complementary to the most conservative sites: 27f (5'-AGAGTTTGATCMTGGCTC-3')–1350r (5'-GACGGGCGGTGTGTACAAG-3') and 500f (5'-CGTGCCAGCAGCCGCGGTAA-3')–1350r (5'-GACGGGCGGTGTGTACAAG-3') [23, 24]. Reaction mixture (25 μL) was supplemented with 10 pmol of the forward and reverse primer and 15 ng of the template DNA. The size and homogeneity of the amplification product were determined by electrophoresis in 1.5% agarose gel.

Cloning. PCR products were cloned using the InsTAclone™ PCR Cloning Kit (Fermentas) and GeneJET™ PCR Cloning Kit (Fermentas) according to the manufacturer's recommendations. The transformation was carried out using the chemically competent cells of *E. coli* XL1 according to the accepted procedure [21]. For screening, the cells were plated on LB medium with ampicillin (20 μg/mL). All the colonies were analyzed. Analysis of the cloned DNA was carried out using the universal plasmid primers: the forward M13/pUC (5'-GTAAAACGACGGCCAGT-3') and the reverse M13/pUC (5'-CAGGAAACAGC-TATGAC-3'); pJET1.2F (5'-CGACTCACTAYAGG-GAGAGCGGC-3') and pJET1.2R (5'-AAGAA-CATCGATTTTCCATGGCAG-3'), which were recommended by the manufacturer for cloning at 55°C annealing temperature.

Sequencing was carried out on a BigDye Terminator Kit v. 3.1 genome analyzer (Applied Biosystems) in the Interinstitute Sequencing Center (Institute of Chemical Biology and Fundamental Medicine, Siberian branch, Russian Academy of Sciences, Novosibirsk).

Phylogenetic analysis. The obtained sequences were compared to those of the NCBI database using BLASTN (http://www.ncbi.nlm.nih.gov/blast). The structures were analyzed using ClustalW V 1.4 (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was constructed using the sequences from similar environments, which were identified to the genus level. The search result also always included the related sequences of uncultured microorganisms characterized by molecular genetic techniques in natural communities. Comparison of the sequences and construction of phylogenetic trees were carried out by the neighbor-joining method using the MEGA 4.0 software package. Reliability of the branching order was assessed by bootstrap analysis using the relevant function of the package. Chimerical structures were determined by sequence analysis with PINTAIL (http://www.cardiff.ac.uk/biosci/research/biosoft).

The sequences were deposited to GenBank under accession nos. JN896864–JN896878, JQ012218–JQ012239.

Table 2. Total number of microbial cells and cultured bacteria in the samples from the archeological complexes of the Pazyryk culture, northwestern Mongolia

Sample no.	TNMC, cells × 10^8	Organotrophs, CFU × 10^3	*Bacillus* spores, CFU	% of *Bacillus* spores of the number of organotrophs
		per 1 mL		
1	9.71	19	90	0.47
2	3.82	18	170	1
3	2.44	16	10	0.06
5	12.2	824	0	0
6	2.24	1032	300	0.03
7	2.09	7	0	0
		per 1 g		
4	12.8	350	200	0.06
8	12.4	30	0	0
9	12.6	6000	0	0

RESULTS AND DISCUSSION

Microbial numbers. Abundance of investigated microorganisms in the samples is shown in Table 2. TNMC values varied within an order of magnitude, with the highest numbers (1.24–1.28 × 10^9 cells/mL) in the residues of horseorganic tissue and the lowest, in the defrosted ice samples. Among the latter, elevated TNMC values were found in sample 5 (small ice crystals from the burial bed), where numerous cell aggregates were revealed by microscopy.

While all samples contained viable organotrophic bacteria, spores of bacilli were found only in some of them. The numbers of the former varied from 7 × 10^3 to 19 × 10^3 and from 3 × 10^4 to 6 × 10^6 cells/mL for melted ice samples and horse organic tissues, respectively. In all samples the number of viable organotrophs exceeded the number of spores. Their highest content (300 cells/mL) was found in the ice sample from the burial bed, while the lowest number (10 cells/mL) was detected in the ice sample from the northern wall of the burial chamber. No *Bacillus* cells or spores were found in the ice samples from the surface of the burial bed and from the northeastern angle of the log construction, nor in samples nos. 8 and 9 of the remains of the horse organic tissue. Previously, the presence of spore-forming bacteria has been reported in various Antarctic substrates, and the extreme capacity of bacterial spores for reactivation after prolonged anabiosis has been noted [2, 25]. In our case, prolonged storage of the samples and their defrosting prior to investigation could result in the germination of both spore-formers and other microorganisms, thus providing biased results on their abundance.

Morphological analysis and identification of the strains. From 9 samples (Table 1), 14 strains of organotrophic bacteria were isolated and classified into three groups according to their cell morphology and growth cycles. Almost all the strains (13 out of 14) grew at low temperature (4°C) and did not grow at elevated temperatures (37, 45, and 50°C), thus probably being psychrotolerant.

The first group consisted of two strains of gram-negative bacteria. These were single rod-shaped cells (0.4–0.65 μm wide and 0.8–1.3 μm long), motile, with polar flagella. Bacteria grew on the medium with 50 g/L NaCl and did not grow at 70 g/L NaCl. Both strains were aerobes, oxidase- and catalase-positive, and used sucrose, D(–)-mannitol, and D(+)-sorbitol as carbon sources. Strain z5_paz exhibited multiple enzymatic activity and produced protease, amylase, lipase, lecithinase, alkaline phosphatase, and phospholipase C, while strain z4_paz was inactive. Analysis of the 16S rRNA gene fragments revealed 100% similarity to the cultured pseudomonads: *Pseudomonas* sp. (FJ889630) form the soils of Svalbard (z4_paz) and *Pseudomonas* sp. Tibet-IB 13 (DQ177464) isolated from permafrost (z5_paz).

The second group consisted of three strains of gram-negative bacteria. Their cells were nonmotile rods (0.65 × 0.8 μm) or filaments with lateral projections, which on the fifth day of cultivation became cocci and curved rods (0.65 × 2.5 μm). Only one strain (z16_paz) grew at 70 g/L NaCl, produced phospholipase C, and did not possess oxidase activity. Comparative analysis of the sequences of the 16S rRNA gene fragments revealed that this group of strains z16_paz and z22_paz were most closely related (96–98%) to *Rhodococcus* (isolates EU768823 and FN377715, strains EU333891 and DQ858959) from permafrost, Antarctic soils, and snow water. Strain z23_paz exhibited high similarity (98%) to the soil bacterium *Rothia* (DQ822568).

The third group included nine strains of spore-forming gram-positive rods (0.8 ± 0.27 × 2.3 ± 0.3 μm). All strains of this group were catalase-positive and could produce extracellular enzymes (gelatinase

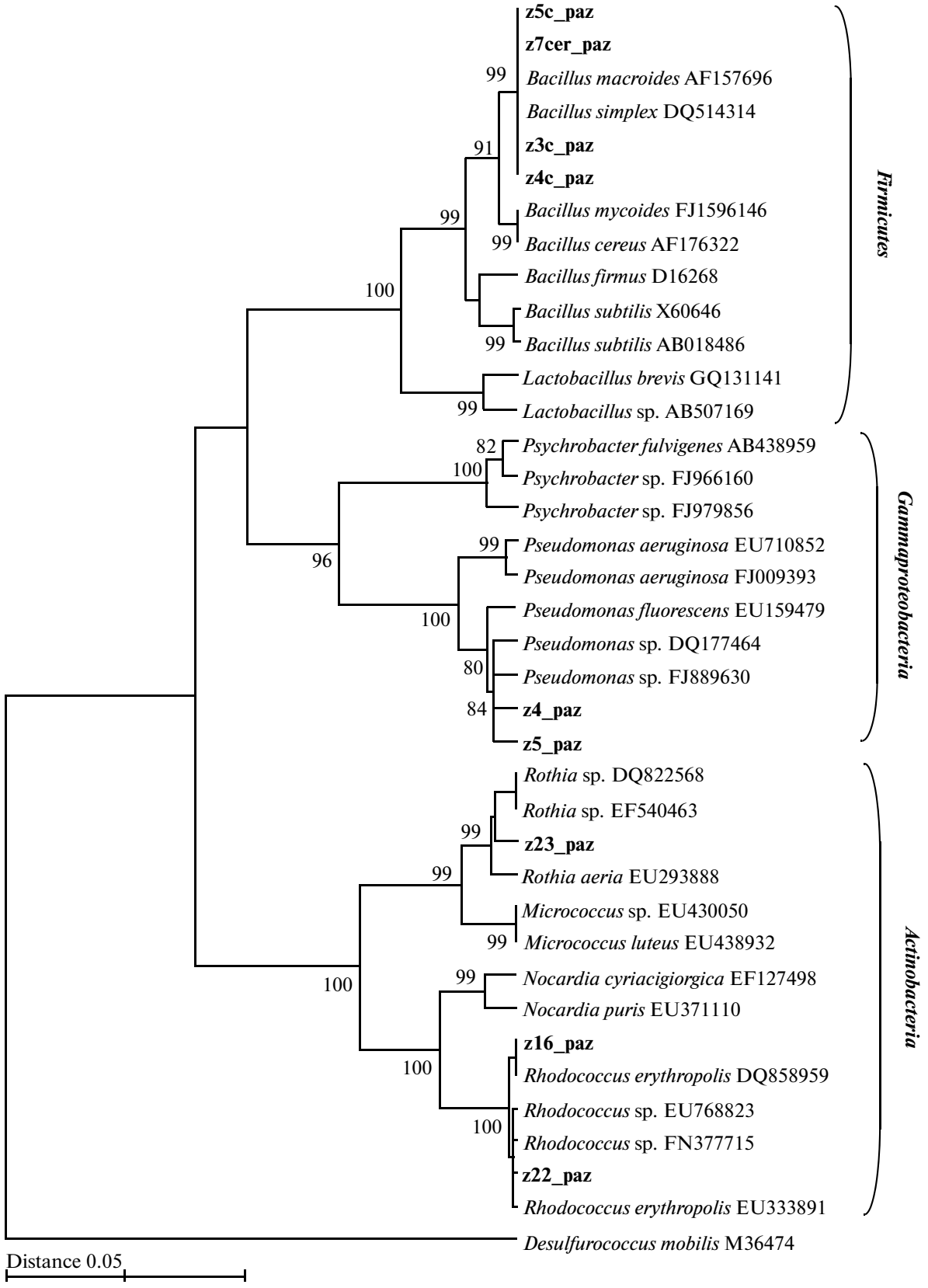

Fig. 1. Phylogenetic tree constructed for the 16S rRNA gene sequences of the cultured bacteria isolated from the samples from archeological complexes of the Pazyryk culture (IV–III centuries BC) in northwestern Mongolia. The tree was constructed using the neighbor-joining algorithm with a MEGA 4.0 software package.

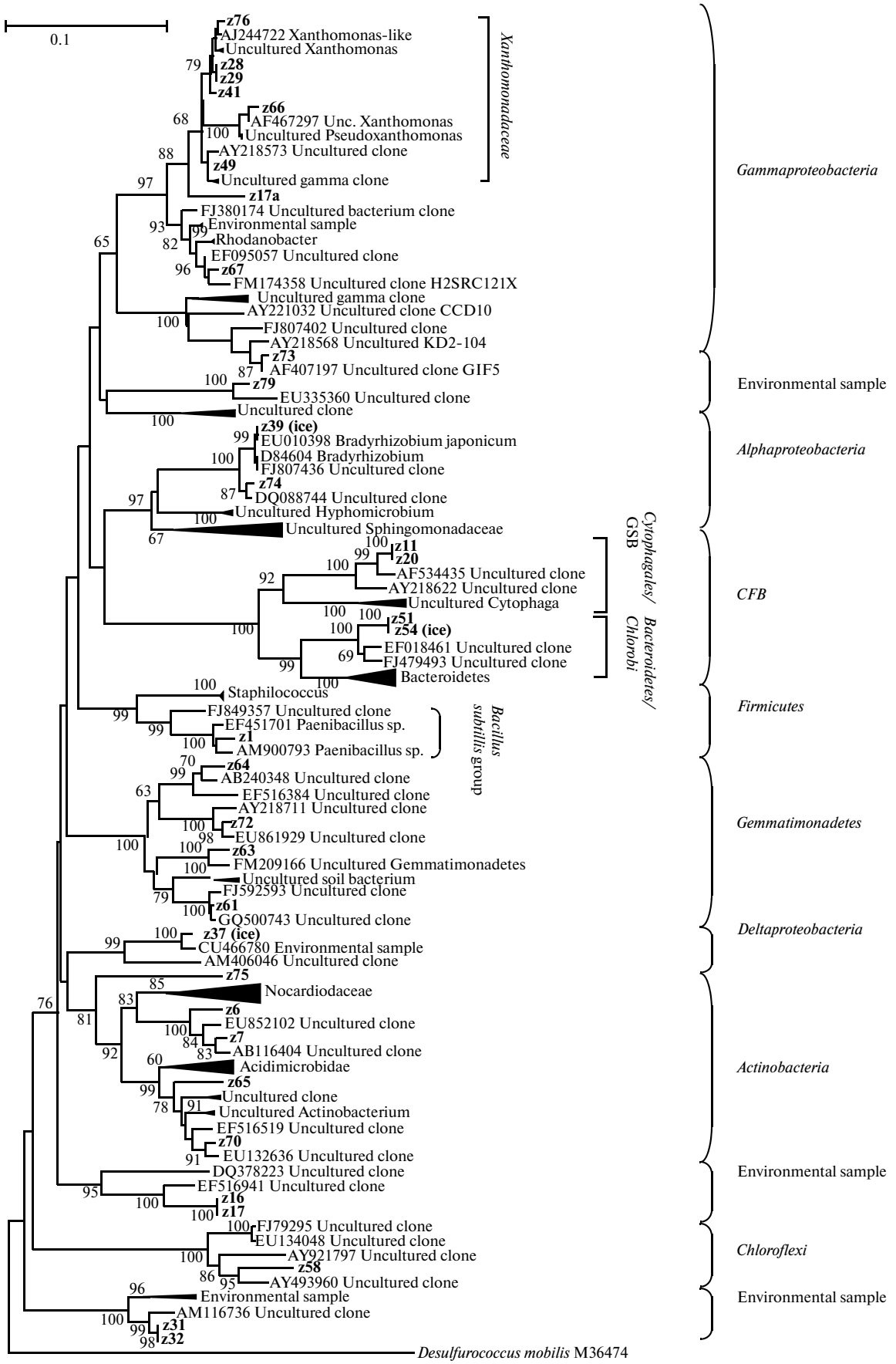
0.1
z76
AJ244722 Xanthomonas-like
Uncultured Xanthomonas
z28
z29
z41
z66
AF467297 Unc. Xanthomonas
Uncultured Pseudoxanthomonas
AY218573 Uncultured clone
z49
Uncultured gamma clone
z17a
FJ380174 Uncultured bacterium clone
Environmental sample
Rhodanobacter
EF095057 Uncultured clone
z67
FM174358 Uncultured clone H2SRC121X
Uncultured gamma clone
AY221032 Uncultured clone CCD10
FJ807402 Uncultured clone
AY218568 Uncultured KD2-104
z73
AF407197 Uncultured clone GIF5
z79
EU335360 Uncultured clone
Uncultured clone
z39 (ice)
EU010398 Bradyrhizobium japonicum
D84604 Bradyrhizobium
FJ807436 Uncultured clone
z74
DQ088744 Uncultured clone
Uncultured Hyphomicrobium
Uncultured Sphingomonadaceae
z11
z20
AF534435 Uncultured clone
AY218622 Uncultured clone
Uncultured Cytophaga
z51
z54 (ice)
EF018461 Uncultured clone
FJ479493 Uncultured clone
Bacteroidetes
Staphilococcus
FJ849357 Uncultured clone
EF451701 Paenibacillus sp.
z1
AM900793 Paenibacillus sp.
z64
AB240348 Uncultured clone
EF516384 Uncultured clone
AY218711 Uncultured clone
z72
EU861929 Uncultured clone
z63
FM209166 Uncultured Gemmatimonadetes
Uncultured soil bacterium
FJ592593 Uncultured clone
z61
GQ500743 Uncultured clone
z37 (ice)
CU466780 Environmental sample
AM406046 Uncultured clone
z75
Nocardiodaceae
z6
EU852102 Uncultured clone
z7
AB116404 Uncultured clone
Acidimicrobidae
z65
Uncultured clone
Uncultured Actinobacterium
EF516519 Uncultured clone
z70
EU132636 Uncultured clone
DQ378223 Uncultured clone
EF516941 Uncultured clone
z16
z17
FJ79295 Uncultured clone
EU134048 Uncultured clone
AY921797 Uncultured clone
z58
AY493960 Uncultured clone
Environmental sample
AM116736 Uncultured clone
z31
z32
Desulfurococcus mobilis M36474
Xanthomonadaceae
Cytophagales/ GSB
Bacteroidetes/ Chlorobi
Bacillus subtilis group
Gammaproteobacteria
Environmental sample
Alphaproteobacteria
CFB
Firmicutes
Gemmatimonadetes
Deltaproteobacteria
Actinobacteria
Environmental sample
Chloroflexi
Environmental sample

Fig. 2. Phylogenetic tree constructed for fragments of the 16S rRNA gene sequences (800 bp) of uncultured bacterial clones from the samples from archeological complexes of the Pazyryk culture (IV–III centuries BC) in northwestern Mongolia. The tree was constructed using the neighbor-joining algorithm with a MEGA 4.0 software package.

and phospholipase C); three strains exhibited nitrate reductase activity. The strains of this group differed in morphology of their vegetative cells, sporangia, and spore position. Strains z1c_paz, z3c_paz, and z5c_paz were different from the other *Bacillus* isolates. The first strain had ellipsoidal spores, which resulted in a pronounced terminal swelling of the sporangium cell. Strain z3c_paz formed chains of 5–6 cells, 0.7 × 2.2 μm each. Its spores were ellipsoidal, 0.9 × 0.7 μm, and were usually located in the center of the cell. Strain z5c_paz formed long filaments of up to 30 cells, 1.0 × 2.6 μm each. Ellipsoidal spores were located centrally. According to its morphological, physiological, and biochemical characteristics, strain z1c_paz fit to the description of *Bacillus firmus*, while strains z3c_paz and z5c_paz resembled *B. cereus*. Phylogenetic analysis revealed that all strains formed a separate subgroup on the phylogenetic tree within the *Firmicutes* phylum and were close to morphogroup I of group I *B. subtilis* (Fig. 1). According to their 16S rRNA nucleotide sequences, four strains (z3c_paz, z4c_paz, z5c_paz, and z7c_paz) were most similar (97–99%) to the cultured *B. macroides* (AF157696) and *B. simplex* (DQ514314) isolated from soils (Table 1).

Analysis of the fragment of the 16S rRNA gene from the total DNA. From the samples of total DNA (samples 6, 8, and 9), 73 clones were obtained which contained the inserts of expected length. Phylogenetic analysis of the total DNA revealed the presence of bacteria of the phyla *CFB* (*Cytophaga–Flavobacteria–Bacteroidetes*), *Chloroflexi, Actinobacteria, Firmicutes, Gemmatimonadetes*, as well as of the *Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria*, and the sequences of uncultured bacteria (Table 1). These sequences exhibited similarity to those of the cultured bacteria isolated from frozen soils (Arctic, tundra soils, and Tibet highlands), groundwater, and Arctic and Antarctic ice cores (97–100%).

Analysis of DNA from molten ice revealed 12 sequences characteristic of the *Alphaproteobacteria, Deltaproteobacteria,* and the *CFB* (*Cytophaga–Flavobacteria–Bacteroidetes*) phylum. Three of these (designated z37) had 93% similarity to the clone CU466772 isolated from a natural community (further on, the number of identical sequences is given in parentheses). On the phylogenetic tree, this group of clones forms a *Delta/Epsilonproteobacteria* subgroup within the class *Deltaproteobacteria* (Fig. 2). Two clones (z54) belonged to the *CFB* supertype *Bacteroidetes/Chlorobi* and exhibited 96% similarity to the uncultured soil bacterium (DQ450753) retrieved from the frozen soils of the Alpine tundra. A group of clones z39 (7) had 99% similarity to the clone isolated from a water meadow soil (EF515952) and forms a cluster with members of the *Alphaproteobacteria*. On the phylogenetic tree, this group of sequences was grouped together with the soil *Bradyrhizobium* involved in the nitrogen cycle.

From the DNA from organic horse residues (samples nos. 8 and 9), members of the classes *Alphaproteobacteria* and *Gammaproteobacteria*, of the phyla *Actinobacteria*, *CFB, Chloroflexi, Gemmatimonadetes*, and *Firmicutes* were retrieved, as well as the sequences similar to unidentified bacterial clones from environmental communities. Eight sequences (z28, z29, z41, z49, z66, z67, z73, and z76) belonged to the class *Gammaproteobacteria*. An uncultured soil bacterium of the genus *Thermomonas* (EU665166) was among the closest relatives of clones z28 and z29 (98% similarity). The sequence of z66 had 98% similarity to the AB245360 sequence (*Pseudoxanthomonas*). Clone z76 exhibited 98% similarity to the uncultured bacterium AM935417 of the genus *Luteimonas*. On the phylogenetic tree, these sequences formed a branch comprising the members of the family *Xanthomonadaceae*. The sequence z67 was 97% similar to the *Rhodanobacter* sequence from lake sediments (AY921834). The sequence z79 formed a branch with unidentified bacteria (97% similarity) within the class *Gammaproteobacteria*.

The sequence z51 had 96% similarity to the sequence DQ450753 from tundra soils, which belongs to the *CFB* phylum, supertype *Bacteroidetes/Chlorobi*. The sequences z11 and z20 were most closely related to bacterial clones with 98% similarity to uncultured forms of the *Cytophaga–GSB* (green sulfur bacteria) subgroup.

The sequence of the clone z74 exhibited 98% similarity to the uncultured bacterium GQ388829 of the genus *Afipia*, family *Bradyrhizobiaceae*. Members of this genus are clustered together with the second subgroup of the class *Alphaproteobacteria*.

The group of z70 clone sequences was among the most numerous ones (15). It exhibited similarity to the soil clones EU132636 (96%) and AY921962 (95%) of the phylum *Actinobacteria*. Clone sequences z6 (3), z7 (3), and z65 (2) also had high similarity (100% EF688389, 98% AY922100, and 95% CU194268, respectively) to members of the *Actinobacteria*.

Four sequences (z58) had low similarity (94%) to the uncultured clone AM934908 of the phylum *Chloroflexi*. Clones z31f, z32f, z16, and z17 formed a group together with unidentified sequences of the natural community with uncertain taxonomic position.

Clones z61, z64, and z72 were most closely related to the sequence of uncultured soil microorganisms of the recently described monophyletic group *Gemmati-*

monadetes within the *Bacteria* domain. Members of this group are widespread in nature, occurring in lake and marine sediments, gas hydrates, arctic bacterioplankton, as symbionts of marine sponges, etc. [26].

Members of the *Firmicutes* were also identified in the total DNA of organic horse residues. The sequences of a group of clones z1 (15) had 97% similarity to the isolate *Paenibacillus* sp. KAR72 (EF451701), which is phylogenetically related to morphogroup II within the *Bacillus subtilis* group I.

Thus, our analysis revealed the presence of viable bacteria, some of which were cultured and identified to the genus level, in the samples which have been preserved in a frozen state for ~2500 years. Phylogenetic analysis of the fragments of the 16S rRNA gene sequences of the cultured bacteria and of the total DNA revealed the presence of the typical organisms from the microflora of permafrost soils, which were described earlier [1–4, 27, 28]. They had high similarity to bacteria detected in Siberian permafrost, tundra, soils of Tibet highlands, and the Arctic and Antarctic samples of groundwater and ice cores [27–32].

No pathogenic bacteria were found in the samples, although some isolated strains had such enzymes as gelatinase and phospholipase, which are indirect indicators of pathogenicity [33]. Identified bacterial species are widespread in soils, water, and within the normal microflora of animals and humans; their viable homologues were found in Antarctic glaciers [2]. Higher values of TNMC and the number of organotrophic bacteria in molten ice, compared to those found in permafrost soils, may result from prolonged storage of defrosted samples prior to investigation, which caused cell growth.

This, our data demonstrate that members of the detected taxa have a high adaptive capacity for survival in a frozen state for over 2500 years.

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