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EXPERIMENTAL ARTICLES

Diversity of Cultivable Bacteria Isolated from the Water Column and Bottom Sediments of the Kara Sea Shelf

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Abstract—In this work, the results of microbiological and molecular genetic investigation of the microorganisms inhabiting the Kara Sea and the adjacent Yenisei and Gydanskii Bays are presented. The microorganisms isolated from the samples collected in the studied area belonged to 4 phyla and 11 genera. Bacteria of two phyla, *Firmicutes* and *Actinobacteria*, prevailed; representatives of the *Gammaproteobacteria* and *Bacteroidetes* were isolated as well. According to their phenotypic properties, the obtained pure cultures were classified with the genera *Streptomyces, Rhodococcus, Micrococcus, Bacillus, Pseudomonas, Acinetobacter, Flavobacterium*, and *Marinococcus*. Analysis of the obtained nucleotide sequences of the 16S rRNA genes confirmed that the isolates belonged to the genus *Bacillus*. One strain was reidentified as *Brevibacillus laterosporus*, and two strains were identified *Aeromonas piscicola* and *Plantibacter* sp. The results of the study of the enzymatic activity of the obtained pure psychrotolerant cultures suggest that the microbial community is actively involved in the destruction processes occurring in the studied area.

Keywords: Kara Sea, microbial community, identification, 16S rRNA, enzymatic activity

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The microbiological studies of the Kara Sea were first conducted by Isachenko, who demonstrated that microorganisms were distributed throughout the entire water column and bottom sediments of the Arctic seas [1]. The first quantitative assessment of the microbial biomass of the Arctic seas, including the Kara Sea, was performed by Butkevich in the course of his 1935 expedition [2]. The author demonstrated that the number of bacterial cells in the water column of the Kara Sea was low (10^3–10^4 cells/mL). The numbers of saprophytic bacteria determined by Kriss [3, 4] were similar to the previously obtained values. The total number of microorganisms in the Kara Sea was one order of magnitude lower than in the other Arctic seas [5] and reached, in various years, thousands and tens of thousands cells in 1 mL. Similar results, $(20–40) \times 10^3$ cells/mL, were obtained in the southwestern part of the Kara Sea in 1981 [6].

In 1993, researchers from the Winogradsky Institute of Microbiology, Russian Academy of Sciences, studied the Kara Sea area and the flow area of two large rivers, Yenisei and Ob' [7]. The results obtained indicated that, in the marine zone of the area, the number of bacterial cells in the water ranged from 2–3 thousand to 250–280 thousand cells per 1 mL. The microbial processes of the carbon and sulfur cycles were also studied. The numbers and species composition of methanotrophs were determined by the immunofluorescence assay [8]. In August and September 2001, the total number of microorganisms (TNM) was determined. It did not exceed 0.5×10^6 cells/mL. The average bacterial production (BP) value in the Kara Sea was 2.4 μg C L^{-1}; in the Ob' estuary, the TNM and BP values reached 1.93×10^6 cells/mL (TNM) and 29.5 μg C L^{-1} (BP); in the Yenisei estuary, they reached 1.51×10^6 cells/mL (TNM) and 19.7 μg C L^{-1} (BP) [9].

In September 2007, a comprehensive study was carried out in order to determine the number of bacterioplankton (0.25×10^6 cells/mL), the rates of the key microbial processes of the carbon (BP, 0.15–0.2 μg C L^{-1} day) and sulfur cycles, methanogenesis (0.18–2.0 μL CH_4 L^{-1}), and methane oxidation (0.1–100 nL CH_4 dm^{-1}) in the surface water layer, to measure the rates of sulfate reduction (4–184 μg S dm^{-3} day^{-1}) and dark CO_2 assimilation, as well as to determine the carbon isotope composition of the suspended organic matter in the Kara Sea and the Ob' estuary [10].

Due to the exploration and development of natural resources in the Arctic shelf, the study of the structure and activity of the microbial community involved in the biogeochemical processes of the Kara Sea and adjacent bays is of urgent importance and interest.

The goal of the present work was to assess the diversity of the cultivable microorganisms isolated from the Kara Sea shelf and the adjacent Yenisei and Gydanskii Bays by traditional microbiological methods and by using molecular biological techniques.

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MATERIALS AND METHODS

Samples of water and bottom sediments collected in the course of the expedition on the *Sovetskaya Arktika* research vessel (August–October 2009) from the Kara Sea shelf, Gydanskaya Guba Bay, and the Yenisei River basin (Fig. 1) were the subjects of this study.

Water samples were collected at 13 stations (W1–10 and W20–22) on the Kara Sea shelf; samples of the bottom sediments were collected at five stations (W7, 9, 10, 20, 22). In the Gydanskaya Guba Bay, samples of water and bottom sediments were collected from two sections, Cape Mongatalyang–Cape Mamonta (W11–15) and Cape Otvesniy–Cape Nyada-Salya (W16–19). In the Lower Yenisei area, samples were taken at 12 stations (W23–34); at three of them, samples of sandy bottom sediments were collected. Water samples were collected with bathometers into sterile bottles; samples of bottom sediments were taken using a bottom sampler and benthic corers. The samples of water and bottom sediments were processed within 3–6 h after sampling.

Isolation of microorganisms. Organotrophs were isolated using the Fish-pepton agar : water (1 : 10) (FPA : 10); psychrophiles were isolated within the temperature range of 4–6°C on R-2A : 10 Agar (Fluka Analytical, code: 17209); spore-forming bacteria of the genus *Bacillus* were isolated on milk agar [11] supplemented with 10 mg of $MnSO_4\ 5 \cdot H_2O$ per one liter of the medium (DSMZ, http://www.dsmz.de); *Bacillus* spores were obtained by pasteurization (at 65°C for 10 min) with the subsequent transfer to FPA : 10 supplemented with $MnSO_4 \cdot 5H_2O$.

Identification. The phenotypic properties of microorganisms (cell and colony morphology, motility, spore formation, physiological and biochemical properties, and Gram staining) were studied using the standard methods [12]; the enzymatic activity was assayed using the previously developed techniques [13]. The morphological analysis of bacterial cells and photographing were performed using an FEI Company Quanta 200 scanning electron microscope, as well as an Axiostar-plus light microscope (Carl Zeiss, Germany) equipped with a Power Shot A640 digital photographic camera (Canon, Japan) . The isolates were identified using the Bergey's Manual [14]. Spore-forming, gram-positive rods were identified using the scheme proposed by Norris et al. [12] and using the Bergey's Manual [15].

Molecular genetic methods. The DNA of new isolates was extracted using the modified technique based on the enzymatic lysis and subsequent phenol-chloroform extraction [16]. The polymerase chain reaction (PCR) parameters were as follows:

94°C for 2 min (1 cycle);

92°C for 45 s, 52°C for 45 s, 72°C for 60 s (30 cycles);

72°C for 2 min (1 cycle).

PCR amplification was carried out using the primers complementary to the most conservative 16S rDNA gene fragments [17, 18]:

27L, AGAGTTTGATCMTGGCTC;

500L, CGTGCCAGCAGCCGCGGTAA; and

1350R, GACGGGCGGTGTGTACAAG.

The size and purity of the product were assessed by electrophoresis in 1.5% agarose gel.

Sequencing was performed the Interinstitute Center for Sequencing (Novosibirsk). The obtained sequences were compared with the sequences from the GenBank database using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/blast). The structures were analyzed using the CLUSTAL W v. 1.4 software package (http://www.ebi.ac.uk/clustalw). Phylogenetic analysis was performed using the MEGA v. 4.0 software package by the neighbor-joining method and using the Kimura 2-parameters algorithm. The significance of the branching order was determined by bootstrap analysis of 100 alternative trees. The obtained nucleotide sequences of the 16S rRNA gene fragments were deposited in GenBank under the accession numbers JN203043–JN203049.

RESULTS AND DISCUSSION

As a result of our expedition (August–October 2009) to the Kara Sea shelf, Gydanskaya Guba Bay, and the Yenisei River basin, the quantitative characteristics of the numbers of microorganisms inhabiting the local ecosystems were obtained. The total number of microorganisms varied between 0.02 and 0.52 × 10^6 cells/mL; the number of organotrophs ranged from 33 to 2100 CFU/mL; the number of psychrophiles reached 2304 CFU/mL; the number of hydrocarbon-oxidizing did not exceed 1200 CFU/100 mL; the average number of spore-forming bacilli was 51% of the number of organotrophs [19, 20].

Microbiological analysis of the obtained samples of water and bottom sediments revealed that the growth of bacteria capable of developing at low temperatures was most active. We developed a collection consisting of 100 strains of organotrophic psychrotolerant microorganisms. The organotrophic community of the studied Arctic regions consisted of numerous representatives of various species. Out of 100 isolated cultures, 83 strains were identified to the genus level. The isolated actinobacteria were classified into three genera, *Streptomyces* (29 strains), *Rhodococcus* (4 strains), and *Micrococcus* (4 strains) (Figs. 2c, 2d); 29 strains were identified as members of the genus *Bacillus* (Fig. 3), one strain was identified as a representative of *Brevibacillus*, 8 strains belonged to the genus *Pseudomonas* (Fig. 2a), 2 strains were identified as members of *Acinetobacter* (Figs. 2b, 2e), 2 strains belonged to the genus *Flavobacterium*, 2 strains were identified as members of the genus *Marinococcus* (Fig. 2c), one strain was identified as a representative of *Aeromonas*,

Fig. 1. Area chart and location of the sampling stations (W1–W34) where water and bottom sediment samples were collected.

and one strain belonged to the genus *Plantibacter*. The microorganisms isolated from the bottom sediments were represented by the genera *Streptomyces* and *Bacillus*, which is typical of this ecological niche.

Special attention was given to spore-forming microorganisms (representatives of the genera *Streptomyces* and *Bacillus*) due to the fact that their spores were found to be most resistant to various extreme environmental conditions [21, 22], including low temperatures and high water salinity. The endospore-forming strains (30) were identified as members of the genus *Bacillus*. It should be noted that the studied area differs from other regions in the high diversity of bacteria belonging to this group. It was represented by six morphotypes identified using the following differentiating characteristics: (1) spore shape; (2) distinct inflation of sporangia; and (3) predominant position of spores in sporangia (table).

Six strains were identified to the species level, including Ap. 67-09 (*B. subtilis*), Ap. 11-09 (*B. fir-*

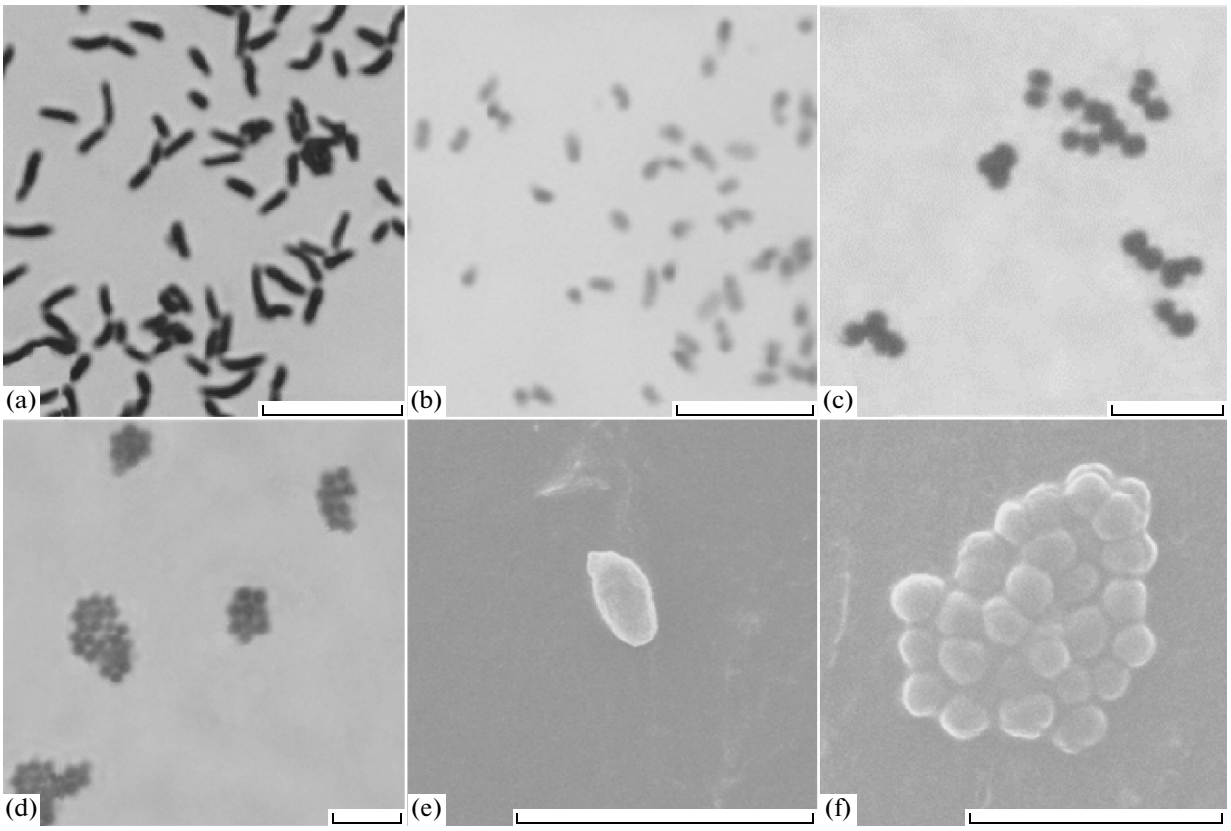

Fig. 2. Cell morphology of the strains isolated from the water and bottom sediment samples collected in the Kara Sea shelf. Light microscopy (a)–(d), scanning electron microscopy (e)–(f): (a) strain Ap. 43-09, *Pseudomonas*; (b), (e) strain Ap. 7-09, *Acinetobacter*; (c) strain Ap. 30-09 *Marinococcus*; (d), (f) strain Ap. 54-09, *Micrococcus*. Scale bar, 5 μm.

mus), Ap. 68-09 (*B. megaterium*), Ap. 38-09 (*B. psychrodurans*), Ap. 45-09 (*B. marisflavi*), and Ap. 58-09 (*B. circulans*).

The isolated *Streptomyces* strains formed aerial mycelium of different colors: white (7 strains), whitish-brown (3 strains), brown (8 strains), pink (5 strains), cream-colored (4 strains), and gray (2 strains). The substrate mycelium of 22 *Streptomyces* cultures was colorless. Only three strains forming pink, brown, and cream-colored aerial mycelium formed pink substrate mycelium; the substrate mycelium of 4 strains forming white, pink, and brown aerial mycelium was dark brown. None of the studied strains produced soluble and melanoid pigments. The sporangia of these strains were of different shapes and were monopodially located on the aerial mycelium. Three main types of sporangia (short straight or slightly curved; long straight and branched; and in the form of hooks, loops, and irregular helices) were detected. Spores of all the studied strains were surrounded by smooth capsules. On the basis of their morphological and cultural properties, the isolated strains were classified into the genus *Streptomyces*.

Analysis of the nucleotide sequences of the 16S rRNA gene fragments of seven pure cultures isolated from the samples of bottom sediments collected at 5 stations on the Kara Sea shelf and in the Yenisei Bay was carried out. These strains belonged to the phyla *Firmicutes* and *Actinobacteria*, class *Gammaproteobacteria*, genera *Brevibacillus, Bacillus, Aeromonas*, and *Plantibacter*.

The nucleotide sequences of the 16S rRNA gene fragments (1300 bp) of all strains were analyzed. The sequences showed high homology (99–100%) with the DNA of bacteria isolated from various natural ecosystems. The phylogenetic tree was constructed on the basis of the sequences with the highest levels of similarity, taking into account the similarity of habitats and taxonomy.

Out of the seven studied strains, five strains were identified as bacteria of the order *Bacillales*. Strain Ap. 17-09 showed 100% similarity to the bacterium isolated from polluted soil (*Bacillus* sp., AM293003). Ap. 42-09 showed high similarity (100%) to *Bacillus* sp. (AB576891) isolated from high mountain snow. Strain Ap. 47-09 showed 99% similarity to *Bacillus* sp. (EU308307) isolated from a saltery in Greece.

Strain Ap. 20-09 showed 99% similarity to *Bacillus* sp. (AB533800) isolated from a saline lake in California. According to the obtained phylogenetic tree (Fig. 4), the strains Ap. 20-09, Ap. 42-09, and Ap. 47-09 belonged to the morphological group I of

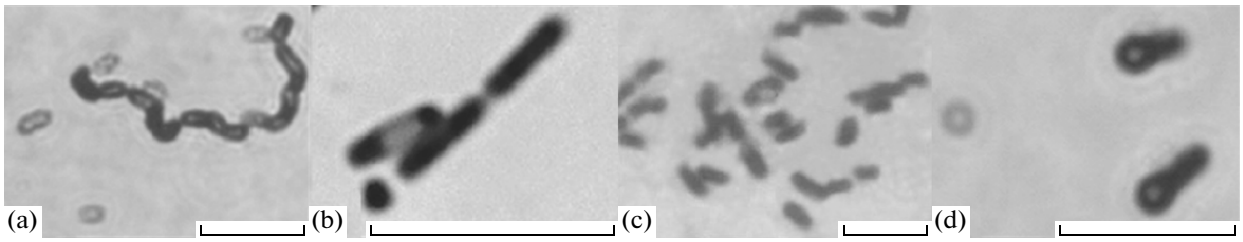

Fig. 3. Morphological diversity of *Bacillus* cells with spores isolated from the samples of water and bottom sediments collected in the Kara Sea shelf. Light microscopy: (a) morphotype I (Ap. 5-09); (b) morphotype II (Ap. 48-09); (c) morphotype III (Ap. 58-09); (d) morphotype VI (Ap.47-09). Scale bar, 5 μm.

B. subtilis and were divided into two separate subgroups: one group clustered with the *B. pumilis* subgroup (Ap. 42-09 and Ap. 47-09), another one clustered with the *B. firmus* (Ap. 20-09) subgroup. Strain Ap. 16-09 was found to be closely related to representatives of another phylogenetic subdivision of *Bacillus* species, "group 4", which is presently classified as the genus *Brevibacillus*. The nucleotide sequence of this strain showed 100% similarity to that of *Brevibacillus laterosporus* (DQ371289, China). In the phylogenetic tree, the sequences of the newly isolated strains fell into a cluster with the bacterial strains isolated from marine habitats (*B. firmus*, FJ88301 and *B. oceanisediminis*, HQ234336), soil ecosystems (*B. pumilis*, FN870069, *B. arenosi*, EF690414, and *Brevibacillus laterosporus*, FR823413), and bottom sediments of natural springs (*Brevibacillus* sp., GQ284340).

Members of the family *Bacillaceae* are able to degrade the substrates from plant and animal sources and to hydrolyze a wide spectrum of biopolymers. It is well known that, as difficult-to-degrade compounds accumulate, bacteria of the genus *Bacillus* begin to predominate in various ecological niches.

Analysis of the 16S rRNA gene sequences of strain Ap. 46-09 confirmed that this strain belonged to the class *Gammaproteobacteria*, family *Aeromonadaceae*, and showed 99% similarity to *Aeromonas piscicola* from the type strain collection (HQ832417, Spain). In the obtained phylogenetic tree, this strain fell into a cluster with the strains isolated from marine bottom sediments, sediments of a freshwater lake, and sewage (*Oceanisphaera litoralis*, AJ550470, *Tolumonas auensis*, X92889, and *Zobellella taiwanensis*, FJ999669, respectively). Members of the family *Aeromonadaceae* produce hydrolytic enzymes, including amylase, DNase, chitinase, elastase, esterase, peptidase, etc.

Strain Ap. 55-09 fell into a cluster with the representatives of the phylum *Actinobacteria*, family *Microbacteriaceae*; its closest homologue is *Plantibacter* sp. (AM396918; 99% similarity), discovered in Antarctica.

According to their morphological, physiological, and biochemical properties, strains Ap. 16-09, Ap. 17-09, Ap. 20-09, Ap. 42-09, and Ap. 47-09 were classified into the genus *Bacillus*; the results of phylogenetic analysis of these strains confirmed their classification as members of this genus, except for Ap. 16-09, which was reidentified as *Brevibacillus laterosporus*. According to the results of 16S rRNA sequencing, strains Ap. 46-09 and Ap. 55-09 were classified into the species *Aeromonas piscicola* and *Plantibacter* sp., respectively. We failed to identify these strains to the species level using physiological and biochemical methods.

The results of our study of the enzymatic activity of the new isolates provide indirect information on the

Description of the morphotypes of bacteria of the genus *Bacillus*

Morphotype	Spore shape	Distinct inflation of sporangia	Predominant position of spores in sporangia	Number of strains
Morphotype I Fig. 3a	Oval	Not detected, spores are surrounded by thick capsules	Whole internal space of the sporangium	12
Morphotype II Fig. 3b	Oval	Not detected	Central, terminal	3
Morphotype III Fig. 3c	Oval	Inflation in the lateral part of the sporangium, lateral	Central, terminal, lateral	6
Morphotype IV	Oval	Insignificant	Central, terminal	4
Morphotype V	Oval	Detected, spores are surrounded by thick capsules	Terminal	2
Morphotype VI Fig. 3d	Sphere	Detected, spores with thick envelopes	Terminal, central	3

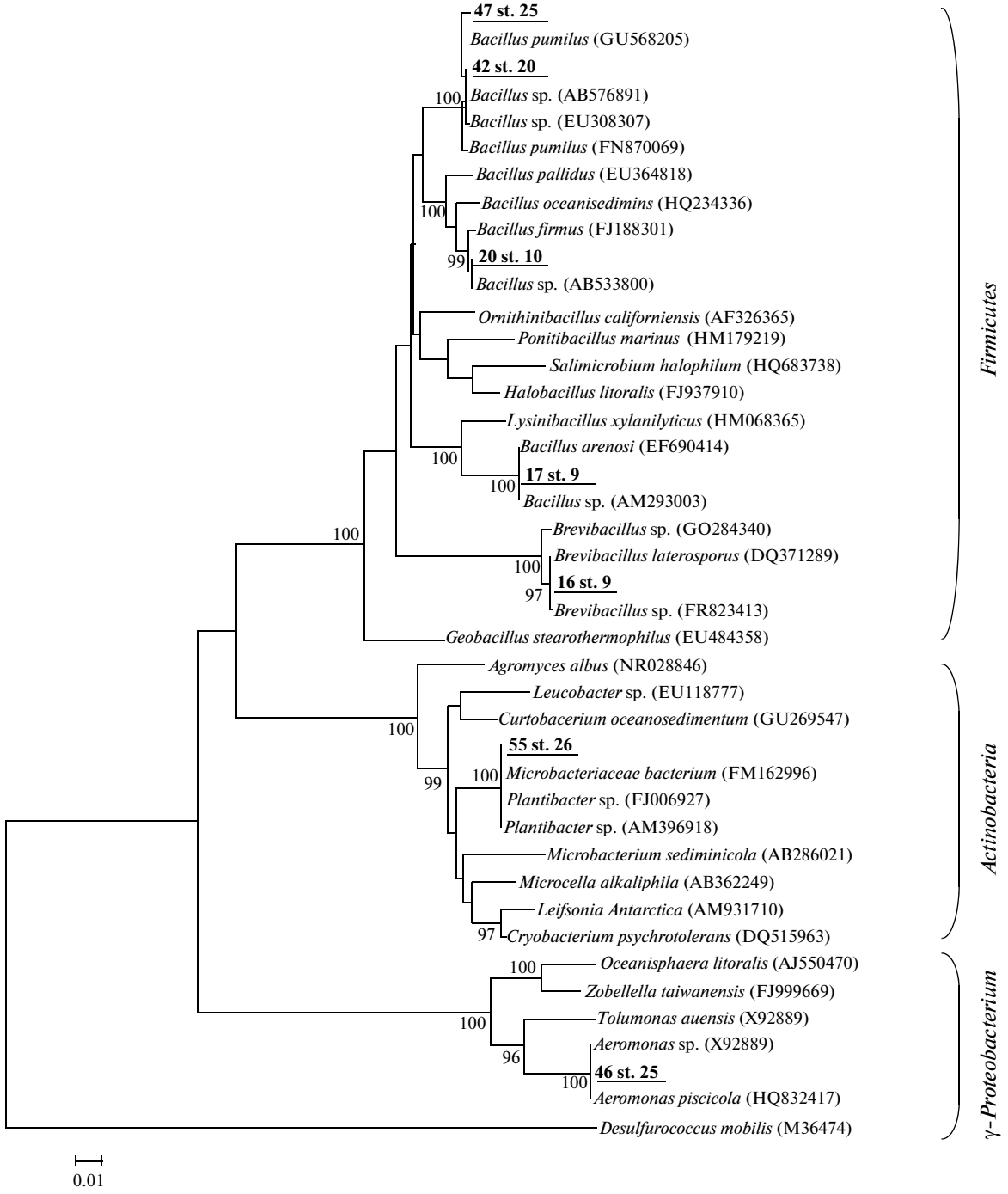

Fig. 4. Phylogenetic tree of the 16S rRNA genes of bacteria isolated from the bottom sediments of the Kara Sea. The dendrogram was constructed with the neighbor-joining method using the MEGA 4.0 software package. Scale bar, one substitution per 100 nucleotides. Only bootstrap values above 95% were considered significant.

destruction processes occurring on the Kara Sea shelf, as well as on the involvement of members of the cultivable organotrophic community in these processes.

The results of our experiments showed that the pure cultures of 100 strains isolated from water and bottom sediments exhibited multiple enzymatic activity: 60 and 58% of the strains exhibited protease and phosphatase activities, respectively, 55% of the strains exhibited lecithinase activity, 41% of the strains exhibited amylolytic activity, and 25% of the strains exhibited lipase activity. We would like to emphasize that although each of these strains exhibited several activities, none of them was able to produce all the studied enzymes. It was demonstrated that the microorgan-

isms isolated from both water and bottom sediments were active producers of proteases, phosphatases, amylases, lecithinases, and, to a lesser extent, lipases.

Hence, the results of our experiments indicate that the cultivable organotrophic community actively participates in the destruction processes occurring in the regions under study. This is not surprising, since the predominant groups of actinobacteria and bacteria of the genus *Bacillus* are able to utilize difficult-to-degrade organic compounds, to actively function and survive under extreme environmental conditions, and to produce enzymes involved in the degradation of various organic compounds.

The results of analysis of the strain collection obtained from the Kara Sea shelf, Yenisei Bay, and Yenisei River basin support the conclusion that the microbial community inhabiting the water column of the shelf and the bay is mixed and consists of microorganisms capable of growth on nutrient media with or without NaCl. From the samples collected above the Sopochnaya Karga settlement–Cape Natzoy section (Fig. 1), microorganisms capable of growth only on NaCl-free media were isolated. Such microorganisms constituted a half of the cultivable community inhabiting the Yenisei River basin (49%) and 29% of the community inhabiting the Kara Sea shelf. The distribution of organotrophic bacteria in the shelf and the Lower Yenisei area probably depends on rapid mixing of fresh and marine waters where the rates of accumulation and decomposition of organic matter are quite high.

Microorganisms isolated from the Kara Sea shelf and the adjacent Yenisey and Gydanskiy Bays using traditional microbiological methods are represented principally by the phyla *Firmicutes* and *Actinobacteria*, as well as by the *Gammaproteobacteria* and *Bacteroidetes*, belonging to the genera *Streptomyces*, *Rhodococcus, Micrococcus, Bacillus, Pseudomonas, Acinetobacter, Flavobacterium*, and *Marinococcus*. Analysis of the nucleotide sequences of the 16S rRNA gene fragments confirmed affiliation of the studied strains with the genus *Bacillus*. One strain was reidentified as *Brevibacillus laterosporus*. The results of the molecular biological studies enabled us to identify the strains *Aeromonas piscicola* and *Plantibacter* sp.

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