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EXPERIMENTAL ARTICLES

Characterization of the Structure of the Prokaryotic Complex of Antarctic Permafrost by Molecular Genetic Techniques

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Abstract—A prokaryotic mesophilic organotrophic community responsible for 10% of the total microbial number determined by epifluorescence microscopy was reactivated in the samples of Antarctic permafrost retrieved from the environment favoring long-term preservation of microbial communities (7500 years). No culturable forms were obtained without resuscitation procedures (CFU = 0). *Proteobacteria, Actinobacteria,* and *Firmicutes* were the dominant microbial groups in the complex. Initiation of the reactivated microbial complex by addition of chitin (0.1% wt/vol) resulted in an increased share of metabolically active biomass (up to 50%) due to the functional domination of chitinolytics caused by the target resource. Thus, sequential application of resuscitation procedures and initiation of a specific physiological group (in this case, chitinolytics) to a permafrost-preserved microbial community made it possible to reveal a prokaryotic complex capable of reversion of metabolic activity (FISH data), to determine its phylogenetic structure by metagenomic analysis, and to isolate a pure culture of the dominant microorganism with high chitinolytic activity.

Keywords: permafrost soils, Antarctica, metagenomic analysis, fluorescent in situ hybridization (FISH), metabolically active prokaryotic complex

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In the recent years, microbiologists, as well as researchers working in neighboring fields of study, have been paying increasing attention to investigation of the mechanisms and forms of long-term microbial survival in permafrost subsoil sediments (Zvyagintsev et al., 1985; Gilichinskii and Duda, 2001; Tindall, 2004; Kryazhevskikh et al., 2012). The adaptive strategy employed by microorganisms to maintain viability over long periods of time is primarily based on the ability of spore-forming bacteria to switch to the dormant state characteristic of spores, i.e., specialized resting cells (Yergeau et al., 2012), or on the ability of non-spore-forming bacteria to form cystlike dormant cells (El'-Registan et al., 2005, 2006) or to switch to a nonculturable state (Kaprelyants et al., 1996). Formation of cystlike cells (CLC) for long-term survival and species conservation has been observed not only in non-spore-forming but also in spore-forming bacteria and occurs when spore formation is inhibited, e.g., under conditions of catabolite repression (El'-Registan et al., 2006). It should be noted that the images of bacterial fractions isolated from specimens of subsoil sediments of Arctic tundra obtained by direct electron microscopy revealed a close resemblance between the cells that had been conserved for thousands to millions of years (Votyakova et al., 1998; Suzina et al., 2001; Soina et al., 2004) and cystlike cells of gram-negative and gram-positive non-spore-forming bacteria obtained in laboratory models (Suzina et al., 2001; El'-Registan et al., 2005; Mulyukin et al., 2009, 2014).

Populations of dormant cells are always heterogeneous in their remaining ability to revert to growth with or without special reactivating procedures (Mulyukin et al., 2009, 2014). According to one of the current hypotheses, while microbial cells acquire and maintain the ametabolic state (anabiosis), these dormant forms accumulate autoinhibitors of spore germination; release of these substances from the cells removes the metabolic block, and the cells regain their ability to grow (El'-Registan et al., 2005, 2006). An important role in the restoration of colony-forming ability belongs to low-molecular-weight extracellular autoregulating agents that control certain stages of germination. For instance, it was shown that nonculturable *Micrococcus luteus* cells could be resuscitated by exposure to an extracellular regulatory metabolite, Rpf protein (resuscitation protein factor), which is not species-specific and is produced by a number of bac-

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Characteristics of the specimen studied. Chemical parameters

Specimen, age	Site of specimen collection	Depth of specimen collection, m	Water pH	C_{org}, %
Antarctic soil, 7500 years old	King George Island (Bellingshausen Station)	9	7.23–9.82 (maximum at the depth of 6.5 m)	0.1

terial species (Kaprelyants et al., 1996). From this point of view, investigation of nonculturable microorganisms of natural microbial communities, including permafrost ecosystems, essentially involves analysis of the factors of intercellular communication that control both the transition to the dormant state and its reversal (Kaprel'yants et al., 1985; Votyakova et al., 1994; El'-Registan et al., 2006).

It should also be pointed out that the biotechnological potential of microbial communities of Arctic and Antarctic permafrost rocks and subsoil sediments as natural microorganism collections has not been studied yet and is worth special consideration.

The goal of the present work was to evaluate the taxonomic structure, i.e., the abundance and phylogenetic diversity of a mesophilic organotrophic prokaryotic community isolated from Antarctic permafrost specimens, with the focus on the features of an initiated chitinolytic complex. The analysis was performed using fluorescent in situ hybridization (FISH) and pyrosequencing of the 16S rRNA gene fragments present in metagenomic DNA specimens.

MATERIALS AND METHODS

Subjects. The study concerned ancient microbial communities isolated from natural specimens of Antarctic permafrost rocks. The rocks represented the sediments of the marine terrace of King George Island (Bellingshausen Station) strongly washed with fluvioglacial waters. The temperature at the station varied from –8.2 to 0.5°C, constituting –3.85°C on the average. The annual average atmospheric pressure calculated from the empirical monthly distribution constitutes 991.24 Pa, varying insignificantly from 987.80 to 995.10 Pa. The wind velocity at the station ranged from 5.30 to 7.80 m/s, constituting 6.85 m/s on the average. The sediments studied were 7500 years old, with ice content ranging from 10 to 36% (*Spravochnik po klimatu Antarktidy*, 1981). Specimens were collected from the depth of 9 m, well B1-09. The organic carbon content was approximately 0.1%.

Characteristics of the specimens are listed in the table and on Fig. 1.

The composition of the prokaryotic community was determined for the following types of permafrost specimens: (1) not reactivated (2) reactivated, and (3) reactivated and subsequently initiated using chitin as a selective substrate. Resuscitation procedures involved rehydrating the cells, washing off the anabiosis autoinducers (with distilled water and 5% potassium pyrophosphate solution), and activating cell metabolism by adding methylresorcinol (50 μg/mL) and yeast autolysate (5% wt/vol) as described previously (Mulyukin et al., 2009; Kryazhevskikh et al., 2012).

Initiation of the chitinolytic microbial complex was performed by adding sterile medium (50 mL) that contained purified chitin (0.1% wt/vol; Sigma) as the sole source of carbon and nitrogen to a reactivated specimen (1 g) 2 h after reactivation. The resulting suspension was incubated with shaking (130 rpm) in a 250-mL flask at 23°C for 2 days (Manucharova, 2014). After incubation, the number of metabolically active prokaryotic cells was determined.

Total numbers of prokaryotic cells in soil samples were determined by fluorescence microscopy following specimen staining with different fluorochrome dyes (Manucharova, 2014; Manucharova et al., 2014) using an Axioskop 2 plus microscope (Zeiss, Germany) with the filter set 15 (excitation at $\lambda = 546$ nm) for Cy3-labeled probes and the filter set 09 (excitation at $\lambda = 450–490$ nm) for the cells stained with acridine orange.

Phylogenetic affiliation and the numbers of metabolically active cells in the microbial community of cryogenic sediments were determined using the molecular biological technique of fluorescent in situ hybridization (FISH) with RNA-specific labeled oligonucleotide probes (Amann and Ludwig, 2000; Manucharova et al., 2014). This method makes it possible to investigate the microbial diversity directly in situ, eliminating the need for plating on growth media, and to account for the live metabolically active cells. The set of probes included those specific to representatives of *Archaea* and *Eubacteria*, as well as to particular phylogenetic groups of *Eubacteria*. The numbers of bacteria of each phylogenetic group were determined using an Axioskop 2 plus fluorescence microscope with the filter set 15 (ZEISS).

Numbers of microbial cells contained in 1 g of soil sample were calculated as follows: $N = S_1an/vS_2c$, where N is the number of cells (the length of mycelium, μm) per 1 g of soil, S_1 is the area of the specimen (μm^2), a is the number of cells (the length of mycelium, μm) per field of vision (averaged by all specimens), n is the dilution factor (mL), v is the volume of the drop loaded on the slide (mL), S_2 is the area of the field of vision (μm^2), and c is the sample weight (g); specific mass of microorganisms was accepted as

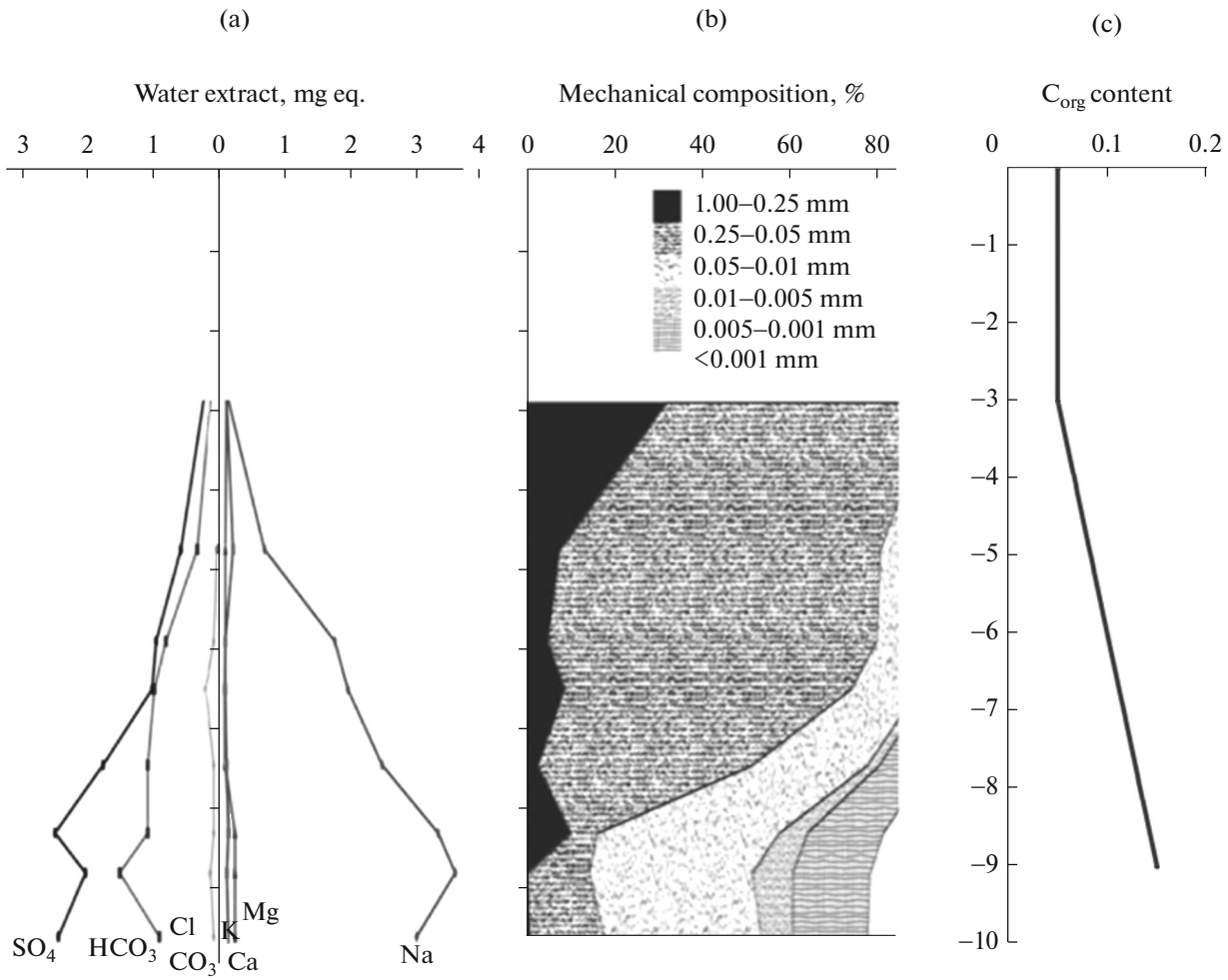

Fig. 1. Chemical and physical characteristics of soil at specimen collection sites (depth, 9 m): content of salts in water extract, mg eq (a); granulometric composition, % particles of given size (b); and organic carbon content, % (c).

1 g/cm^3 and the accepted water content in the cells was 80%. The biomass of microbial cells was calculated based on the following parameters for dry biomass: 2×10^{-14} g per one bacterial cell of 0.1 μm^3 in volume, and 3.9×10^{-8} g per 1 m of actinomycete mycelium 0.5 μm in diameter (Polyanskaya et al., 1995).

Phylogenetic composition of the prokaryotic community was determined using high-throughput sequencing of variable 16S rRNA gene fragments in metagenomic DNA specimens. Total DNA was isolated by conventional techniques using a PowerSoil DNA Isolation Kit (MO BIO, United States) as recommended by the manufacturer. Fragments of the 16S rRNA genes were amplified using degenerate primers complementary to both bacterial and archaeal sequences: PRK341F (CCTACGGGRBGCASCAG) and PRK806R (GGACTACYVGGGTATCTAAT). The fragments obtained by PCR were purified on QIAquick columns according to the manufacturer's instructions. Each fragment was dissolved in 50 μL TE buffer; the obtained quantity was sufficient for subsequent analysis. Pyrosequencing of the amplicons was performed on a GS FLX (Roche, United States) using the GS FLX Titanium Sequencing Kit XL+ and the GS Titanium PicoTiterPlate Kit 70–75 according to the Titanium protocol. The procedure was conducted as proposed in the Sequencing Method Manual (version from June, 2013). Taxonomic classification of the obtained sequences was performed using the RDP Classifier program (Glenn, 2011).

Culturable forms of bacteria were identified by plating the specimens on solid media, and CFU numbers per 1 g specimen were registered. Cells were grown on trypticase soy agar (TSA), tenfold diluted TSA (TSA/10), LB agar (2.5%), or a selective medium containing chitin as a sole carbon and nitrogen source.

RESULTS AND DISCUSSION

Restoration of metabolic activity in microbial communities of Antarctica after long-term dormancy. The analyzed sample of permafrost sediments did not contain microbial cells capable of colony formation on solid media in the absence of resuscitation procedures

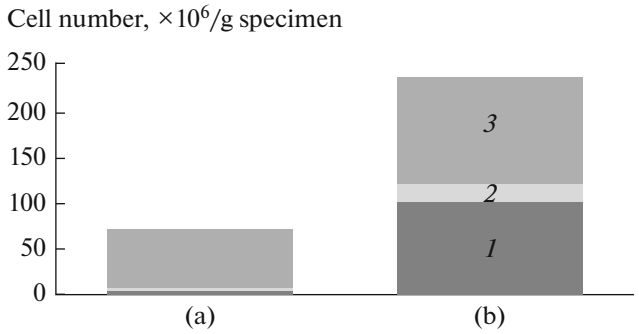

Fig. 2. Numbers of metabolically active prokaryotes (cells/g specimen) identified with probes specific to *Eubacteria* (*1*), *Archaea* (*2*), and total number of cells (including inactive ones) assessed by acridine orange staining (*3*) of a permafrost specimen following resuscitation (a) and chitin initiation (b).

(CFU = 0). For this specimen, we compared the total number of cells registered by microscopy after acridine orange staining and the number of metabolically active cells determined by FISH with the probes specific to representatives of *Bacteria* and *Archaea*. It was found that the total number of prokaryotic microorganisms in the non-reactivated specimen, regardless of their physiological condition (including spores and cysts) was as high as 6×10^7 cells/g, which corresponded to 14 μg of total prokaryotic biomass per 1 g specimen. The number of metabolically active prokaryotes in a defrosted specimen that had not been subjected to any preliminary treatment or cell resuscitation procedures was 1×10^6 cells/g, that is, 5% of the total prokaryotic population as assessed by fluorescence microscopy. Similar results were obtained previously in our study of permafrost subsoil sediments of the Kolyma Lowlands (Kryazhevskikh et al., 2012).

In the reactivated specimen, the total cell number was the same, whereas the portion of metabolically active cells increased nearly tenfold. According to the results of FISH performed in reactivated specimens, the number of cells containing ribosomal RNA that hybridized to oligonucleotide probes was as high as 8×10^6 cells/g soil (Fig. 2a), which corresponds to 1 μg biomass per 1 g specimen. Thus, the biomass of the metabolically active prokaryotic population in permafrost sediment potentially capable of reproduction after resuscitation procedures reached 10% of the total biomass (Fig. 3a). These results confirmed our observations on the reactivation of durably dormant microbial communities of permafrost subsoil sediments of the Kolyma Lowlands (Kryazhevskikh et al., 2012). A novel aspect of the present work was investigation of the structure of the prokaryotic complex initiated by introducing chitin as a selective substrate.

In the experiment with chitin initiation of reactivated specimens, both the total number of prokaryotes (2.3×10^8 cells/g) and the share of metabolically active cells increased. The number of cells whose RNA hybridized to the oligonucleotide probes used was 1×10^8 (Fig. 2b), corresponding to 50% of the total biomass of the prokaryotic community revealed by acridine orange staining (Fig. 3b).

Thus, while resuscitation procedures restore the metabolic activity of "conserved" microbial communities, subsequent initiation of prokaryotic complexes with a particular type of activity with a selective substrate provides the community with a functional load by introducing a target resource into the system. Depending on the particular biotechnological purpose, it is possible to obtain communities with different functional properties in order to isolate the active producers and to create controlled bacterial systems.

Structure of the prokaryotic complex of the permafrost soil of Antarctica. The introduction of molecular techniques into the microbiological practice resulted

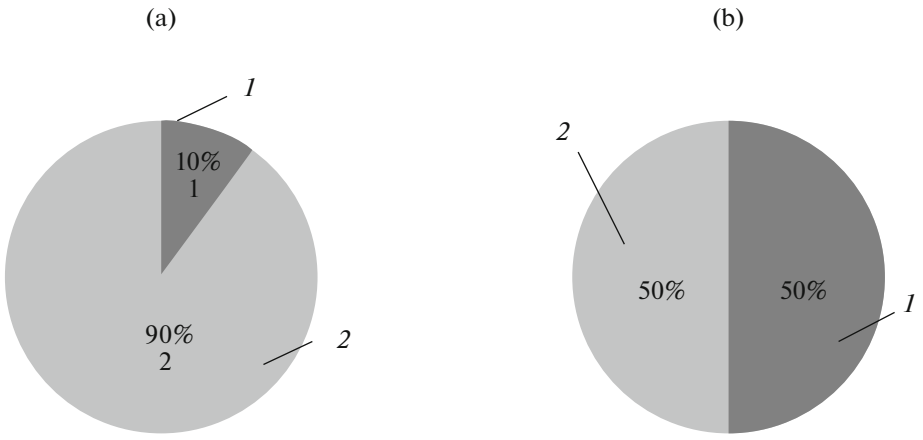

Fig. 3. The portion of biomass attributed to metabolically active unicellular prokaryotes (*1*) and unidentified cells (including inactive ones) (*2*) in a specimen following resuscitation (a) and initiation (b) as assessed by FISH.

in enhanced understanding of the structural diversity of microbial communities, including the organisms in a nonculturable state, a percentage of which is normally considerable in natural systems, especially in prokaryotic complexes (Manucharova, 2014). It should be noted that the methods used in this work, FISH and metagenomic sequencing, complement each other and provide the possibility to considerably improve the estimates of the abundance and biomass of metabolically active cells of these prokaryotic complexes and to assess their phylogenetic affiliation.

Analysis of the taxonomic structure of the prokaryotic metabiome determined by metagenomic sequencing of DNA specimens obtained from all variants of permafrost sediment samples showed that, at the domain level, bacteria constituted an overwhelming majority of the microbial community. Altogether, we obtained 50 000 partial 16S rRNA gene sequences with an average length over 400 bp. Among them, 1000 sequences represented the domain *Archaea* and 49 000 belonged to bacteria. A preliminary search of an archaeal clone library based on DNA amplified with the primers specific to the v3-v5 fragment of the bacterial 16S rRNA gene showed that the specimen contained fragments resembling those of the phylum *Euryarchaeota*, family *Halobacteriaceae*. Previous studies also reported the presence of archaea of the phylum *Euryarchaeota*, methanogens and thermophiles, in permafrost soil samples from littoral oases of Antarctica (Karaevskaya et al., 2014).

Metagenomic DNA isolated from the reactivated and the initiated specimens was found to contain the 16S rRNA gene sequence fragments characteristic of the following phylogenetic groups of *Bacteria*: *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, and *Acidobacteria*. It was shown that, at the phylum level, the predominant groups of the microbiome were *Proteobacteria* (Σα, β, γ, and δ), *Firmicutes,* and *Actinobacteria*, constituting 69, 18.05, and 7.96% of the total number of prokaryotes detected, respectively, according to the data from total DNA sequencing.

These results were confirmed by the data obtained by FISH (Figs. 4a, 4b): the most numerous groups of metabolically active cells were *Proteobacteria* (Σα, β, γ, and δ), *Firmicutes,* and *Actinobacteria*. Importantly, the number of metabolically active cells of the above groups of *Eubacteria* detected in the initiated specimen of permafrost soil (Fig. 4b) was over an order of magnitude higher than in the reactivated specimen (Fig. 4a). Apart from bacteria with known systematic positions, the specimens also contained the sequences that could not be identified at the phylum level;;the share of the latter was lower in the substrate-initiated specimens.

A more detailed analysis of the taxonomic structure of the prokaryotic community of the initiated specimen according to the metagenomic sequencing

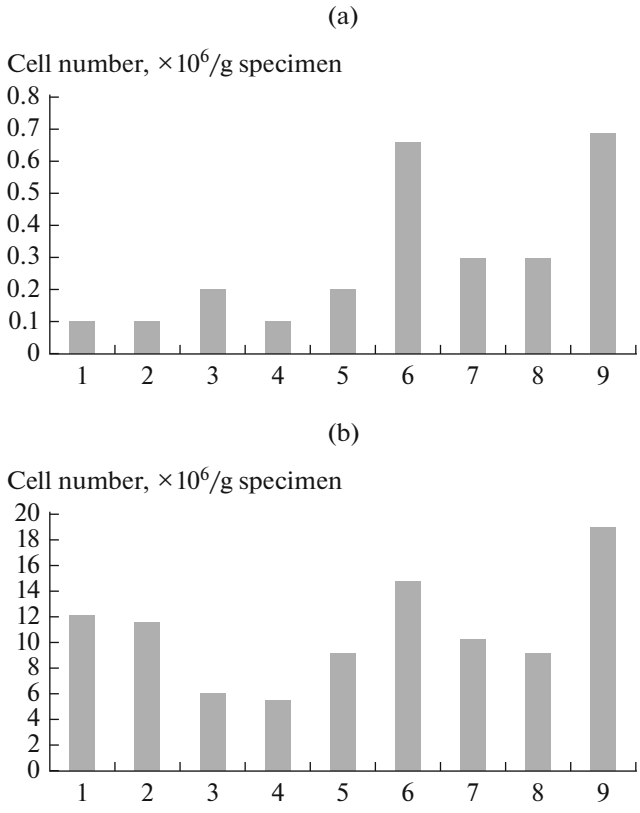

Fig. 4. Numbers of cells representing different phylogenetic groups of the *Bacteria* domain in a specimen following resuscitation (a) and chitin initiation (b): *Alphaproteobacteria* (*1*), *Betaproteobacteria* (2), *Gammaproteobacteria* (3), *Deltaproteobacteria* (4), *Bacteroidetes* (5), *Firmicutes* (6), *Acidobacteria* (*7*), *Verrucomicrobia* (8), and *Actinobacteria* (9).

data showed that, among proteobacteria, the dominant groups were alpha and beta represented by species of the genera *Burkholderia*, *Cupriavidus,* and *Variovorax*, while *Firmicutes* were mainly represented by the genus *Brevibacillus* (family *Paenibacillaceae*).

After plating the initiated specimen on the selective solid medium containing chitin as the only source of carbon and nitrogen, predominant, morphologically uniform colonies were isolated. We determined the taxonomic position of these microorganisms capable of reverting to growth on solid chitin-containing media and found that they belonged to *Firmicutes*, in agreement with the data obtained by molecular biological techniques: FISH and metagenomic analysis. Based on the 16S rRNA gene sequence, the representatives of *Firmicutes* that exhibited the dominant colony morphology traits and were isolated as a pure culture could be classified into the genus *Brevibacillus,* showing the closest homology to *B. brevis* (Fig. 5).

Thus, the complex of procedures employed in this study, including resuscitation of microbial communities of permafrost soils of Antarctica after long-term

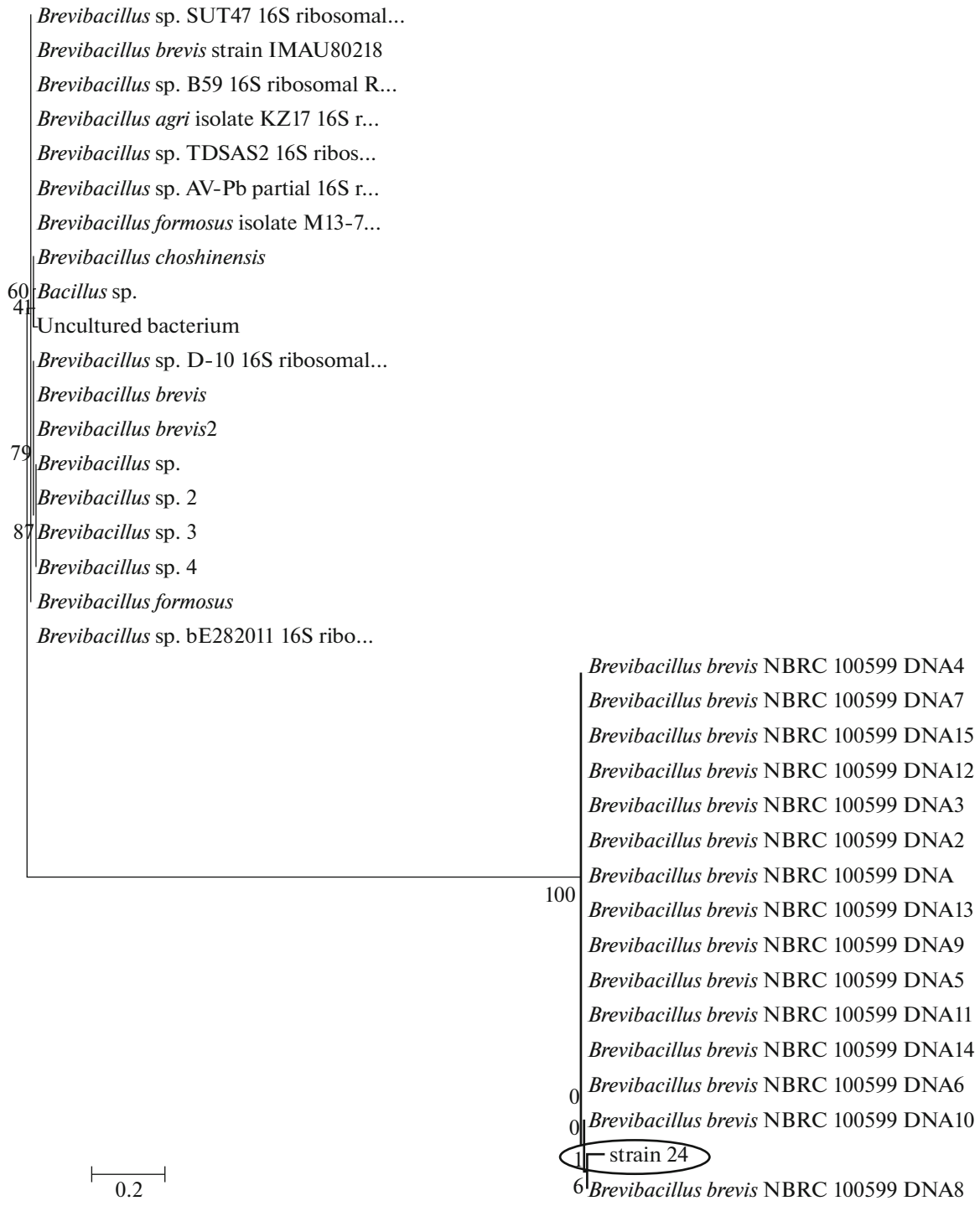

Fig. 5. Phylogenetic position of the pure culture isolate that predominated on the selective medium (chitin).

dormancy and subsequent initiation of a microbial group with certain physiological properties, resulted in quantitative predominance of the latter group in the growing community. Using this approach to specimen preparation for molecular biological analysis, we were able to achieve the following goals: (1) to identify the prokaryotic groups that quantitatively predominate in the community; (2) to detect the prokaryotic complex capable of reverting to a certain type of metabolic activity, in this case, to chitinolysis; and (3) to isolate the most active microbial species predominant in the chitinolytic complex as a pure culture and to determine its taxonomic position. In this context, resuscitation can be considered as the stage where the com-

munity reverts to its potential biological activity, while initiation with a selective substrate serves as the stage triggering a particular functional step in the succession of the community. On the whole, our results demonstrate the possibility to make use of the structural and functional potential of ancient microbial communities, which have hardly been studied at the present time but may prove very promising tools for a variety of biotechnological tasks.

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