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EXPERIMENTAL ARTICLES

Diversity of Bacterial Forms in Ice Wedge of the Mamontova Gora Glacial Complex (Central Yakutiya)

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Abstract—Electron microscopic investigation of four samples of ancient ice wedge from the Pleistocene glacial complex of Mamontova Gora (Yakutiya, Russia) revealed high diversity of bacteriomorphic particles. Their structural features included the presence of electron-transparent zones, presumably inclusions containing storage compounds, and microenvironments (capsules or external sheaths). These features may be a result of adaptive strategies providing for microbial survival under permafrost conditions. Predominance of rod-shaped forms morphologically resembling coryneform actinobacteria was found. X-ray microanalysis revealed organic origin of bacteriomorphic particles. Some particles were characterized by incomplete spectra of the major biogenic elements, resulting probably from low-temperature damage to the cellular structures. Total numbers of aerobic heterotrophic bacteria determined by plating on nutrient media were comparable to the values obtained for permafrost soils and Arctic ice. Predominance of coryneform actinobacteria was observed. Abundance of these evolutionarily early groups of actinobacteria may indicate the ancient origin of the microflora of the relic frozen rocks.

Keywords: permafrost rocks, ice wedge, bacteriomorphic particles, actinobacteria

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Numerous instances of recovery of viable microorganisms from perennial frozen rocks of the Earth's Arctic and Antarctic zones have been reported [1–4]. Due to its specific climatic conditions, permafrost may be considered among the most static environments, which are unique repositories ancient natural microbial communities.

One of the first microbiological studies of the permafrost samples was carried out in the early 20th century [5]. A question was then raised as to whether the microorganisms revealed were "real permafrost inhabitants or, they might be there at the stage of hidden life" due to the "conserving action of cold," [5]. The present-day notions of the history of permafrost formation and the data on the radiocarbon dating of frozen rocks make it possible to treat the organisms revealed in permafrost as the relict fossil forms [6]. However, the question about the possibility of their existence in an active state still remains open [6, 7].

Most of the microbiological permafrost studies were conducted using permafrost soil samples, whereas similar studies of ground ice of the cryolithozone are scarce and rather contradictory [6–10].

Ground ice occurring in permafrost rocks as independent formations is widespread in the cryolithozone. The types of ground ice are sufficiently diverse and are the result of complex cryogenic–geological processes. Among ground ices, the ice wedges formed in frost (temperature) cracks is the most widespread. A necessary condition for ice core formation is the penetration of frost cracks below the maximum depth of the seasonal thawing layer [11, 12]. Gigantic masses of the relict ice wedges incorporated into aleurite syngenetic permafrost are widely developed in Central and North Yakutiya and are referred to as the glacial complex [11, 12]. The ice wedge structure is determined by the process of wedge formation. Ice wedges may form both in the previously formed rocks under changes in the severity of permafrost conditions (epigenetic ice wedges) and simultaneously with the accumulation of sediments (syngenetic ice wedges) [11]. When ice wedge are formed, surface waters gain entrance into a crack during every cycle (usually annual) of crack formation, their filling with water, and its conversion to ice. Therefore, the ice composition may vary, depending on the conditions and the time of penetration of the surface waters into a crack. One of the well-known outcrop syngenetic ice wedges of the glacial complex of Central Yakutiya is the Mamontova Gora mountain. It is situated on the left bank of the river Aldan 500 km away from its estuary. Here, the river Aldan strips the Neogene–Pleistocene depth (aged from

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Table 1. Description of the samples

Sample no.	Age	Description
1	Upper Pleistocene	Ice core, mostly transparent with thin streaks of soil admixtures, whitish in color. Ice structure is granular and bubbly. Ice color is off-white
2	Upper Pleistocene	Cloudy gray, nontransparent ice core. The ice structure is granular and bubbly
3	Upper Pleistocene	The ice core material was sampled in the middle of a large thermokarst cirque (low terrace). Relatively transparent, contains an admixture of gray sand, the structure is granular and bubbly
4	Upper Pleistocene	The ice core material was sampled in a small thermokarst cirque (1 m from the core upper border). Nontransparent, with a large admixture of brownish gray sand. The ice structure is granular and bubbly

16 million to several thousands of years) of alluvial sediments characterizing the whole period of Pleistocene glaciations. The left bank is an alluvial plane underlain by the ancient alluvium of the Paleo-Aldan river system with low, medium, and high terraces. In the course of field works, attention was mainly riveted to the 50-m level, where the Upper Quaternary sediments with ice wedges and the associated processes have attained the highest degree of development. In the profile of the 50-m Mamontova Gora terrace, horizontally and obliquely laminated alluvial sands with organogenic and pebble interlayers predominate. The Neogene–Middle Pleistocene sand layer is overlapped by Upper Pleistocene loam and silts containing repeated-wedge ice—the traces of syngenetic freezing. The sediments with ice wedges are 7 to 10 m thick. This gives evidence of continuous permafrost conditions during Pleistocene and suggests the possibility of detection of very old microorganisms [13].

The aim of the present work was to study the diversity of bacterial forms in the ice wedge samples of the Mamontova Gora glacial complex and to reveal in them the actinobacteria dominating in the permafrost ancient microbial community.

MATERIALS AND METHODS

The samples of ice wedge used in the study were approximately 25–40 thousand years old (Table 1) [14]. The samples were transported from the sampling site in heat-insulated containers with refrigerants in a frozen state. Prior to the experiments, the ice samples were stored at –20°C. The material for analyses was sampled from the central part of the samples under aseptic conditions.

The icy rock samples were studied by electron microscopy. The samples were contrasted with phosphotungstic acid [15] and examined under a JEM-100CXII electron microscope (JEOL, Japan).

Analysis of the elemental composition of the bacteriomorphic particles was carried out using a JEM-100CXII electron microscope equipped with an EM-ASID4D scanning console and an X-ray microanalyzer (Green Star) with an E 5423 detector (Link-System, United Kingdom) at 80 keV and ×20 000 magnification. The spectra obtained were processed using the bundled software package.

In order to assess the qualitative and quantitative composition of the cultivated aerobic bacteria with emphasis on actinobacteria, the standard nutrient media were used: synthetic medium containing glucose and starch–ammonium medium [16], synthetic medium with chitin [17], ISP1 liquid and with the addition of 2% agar-agar (Difco), and ISP3 (Difco) with 0.25% of yeast extract. The samples were inoculated in three replicates immediately after thawing. The incubation was carried out at 20, 28, and 37°C. The colony count on agar media and transfers of the isolates were carried out after two weeks of cultivation. The isolates were maintained on the ISP1 and on ISP3 agar media with yeast extract. The tolerance of the isolates to low temperature was studied by cultivation at 8°C. The morphological properties of the isolates, belonging to certain morphological–cultural groups, were studied using phase contrast microscopy (Axioplan, Carl Zeiss, Germany).

RESULTS

Visual observation of the samples revealed distinct differences in their appearance, probably resulting from the different conditions and the time of the surface waters ended up in a crack. For samples nos. 1 and 3, the presence of large interlayers of relatively pure ice, which alternated with thin ice interstratifications enriched with ground particles, was typical. On the contrary, samples nos. 2 and 4 were characterized by a high content of ground impurities, were nontransparent and of turbid, grayish-brown color. After thawing, the pH values of the icy material of the samples studied were 5.5–5.6, which is characteristic of the surface waters of the cryolithozone.

Electron microscopy revealed various bacteriomorphic particles in the samples studied (Figs. 1, 2). Particles resembling budding bacteria were observed with outgrowths in the form of buds at one of the

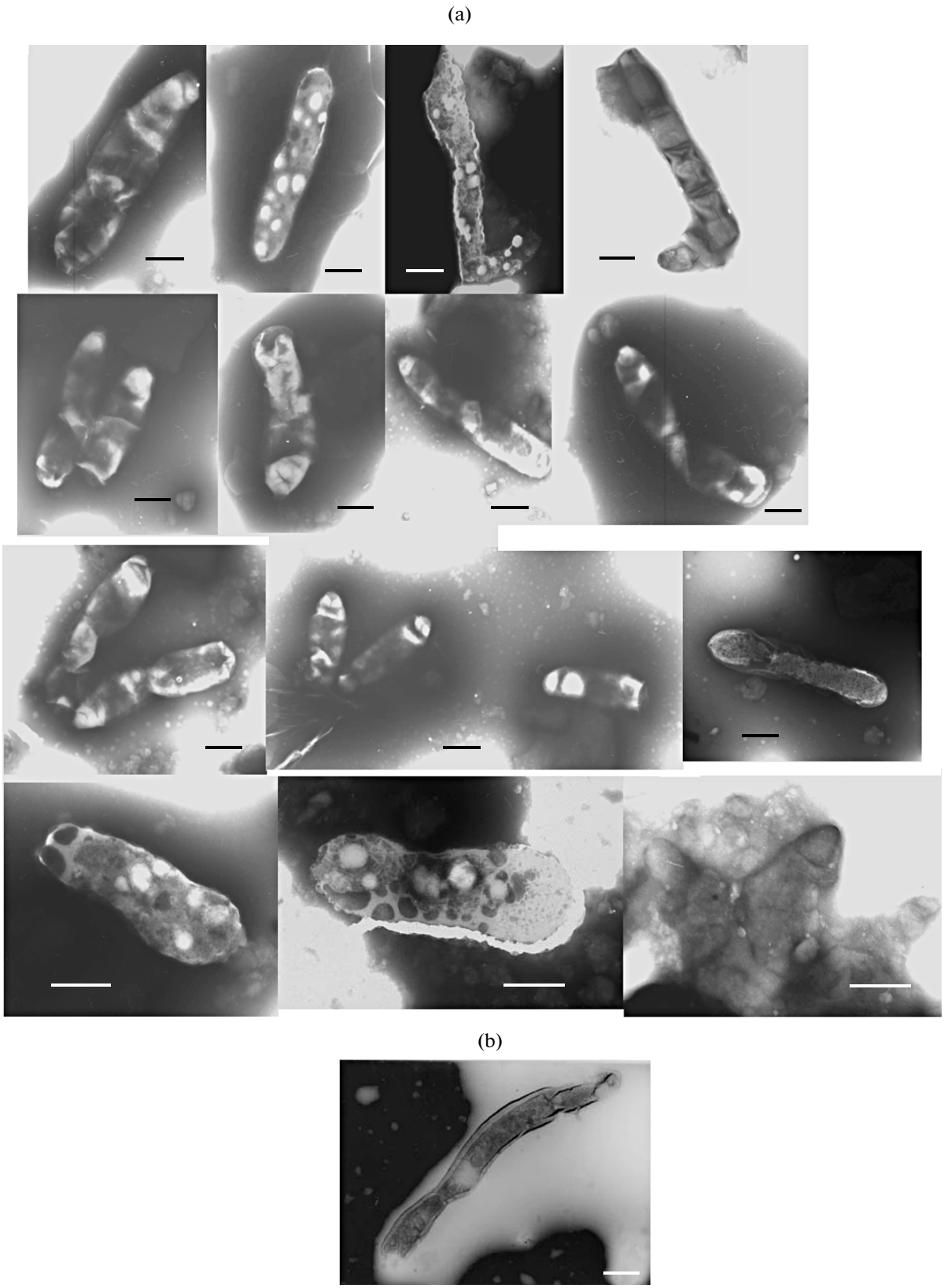

Fig. 1. Bacteriomorphic particles, morphologically similar to the cells of actinobacteria: coryneform rods (a) and forms similar to the hyphal fragments of mycelial actinobacteria (b). The length of the scale bar is 300 nm.

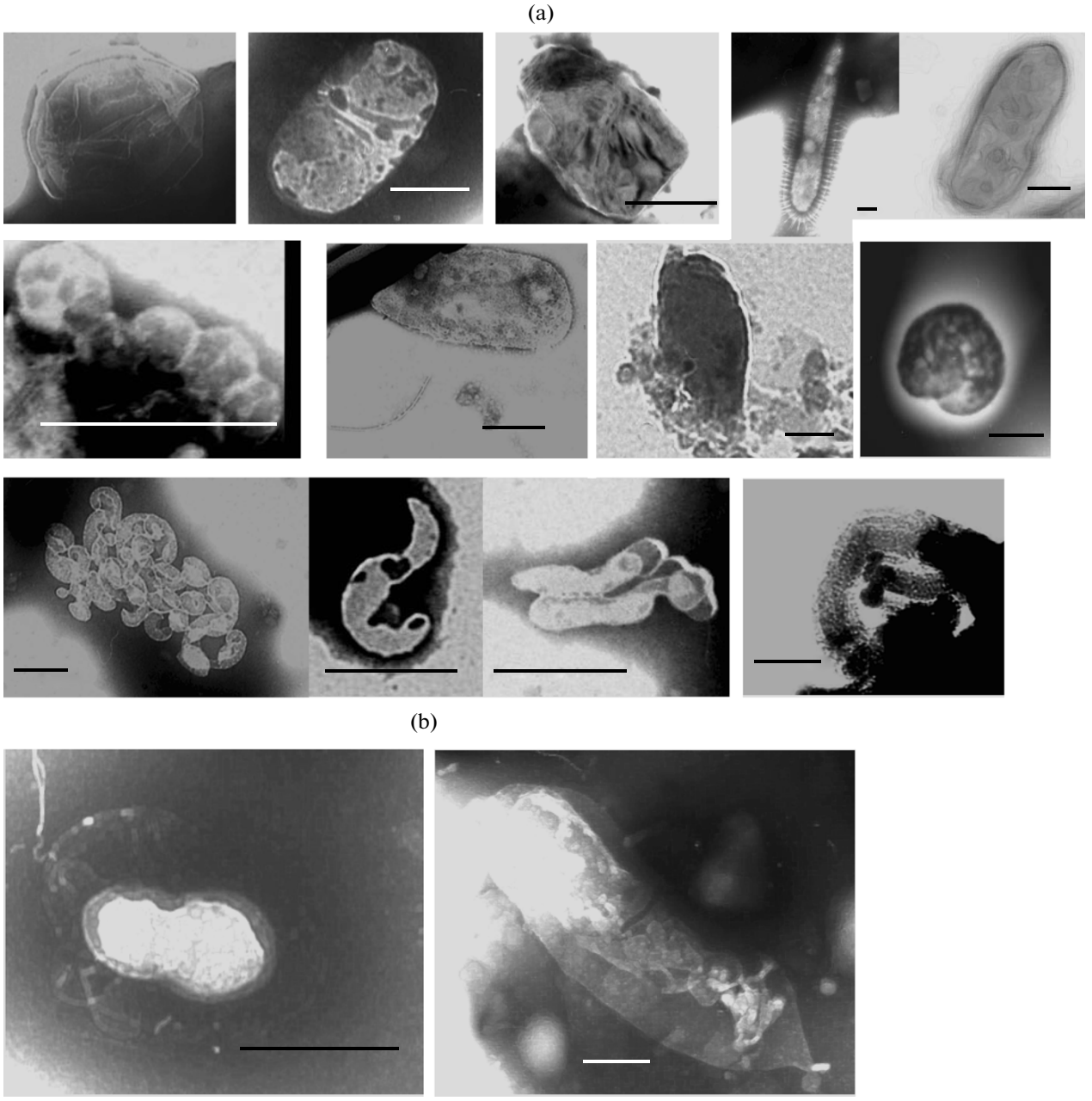

Fig. 2. Morphological diversity of bacterial forms in the ice wedge samples (a) and the presence of the microsurroundings in the form of capsules and the external covering (b). The length of the scale bar is 300 nm.

poles, as were oval particles, sometimes with thread-like appendages, supposedly, pili. Pear-shaped bacteriomorphs and chains of coccoid forms were also present in the samples. Sample no. 2 exhibited the greatest diversity of bacteriomorphic subjects. Rod-shaped particles, similar in shape and characteristic grouping to the cells of coryneform actinobacteria, were dominant (Fig. 1a). The particles resembling the hyphal fragments of mycelial actinobacteria were revealed in the same sample (Fig. 1b). Coiled bacteriomorphs, both as single structures and in aggregates, were also found in great numbers (Fig. 2a). Moreover, we noted the presence of structures in the form of an electron-transparent external covering and capsules around bacteriomorphic particles (Fig. 2b).

The elemental composition of the bacteriomorphic particles indicated their organic origin, since the main biogenic elements (P, S, K, and Ca) were revealed in their X-ray spectra (Fig. 3). Decreased content or the incomplete spectrum of biogenic elements noted for certain bacteriomorphic particles resulted probably from the cryoinjuries of cell structures. Experiments with actinobacterial isolates in the stationary and

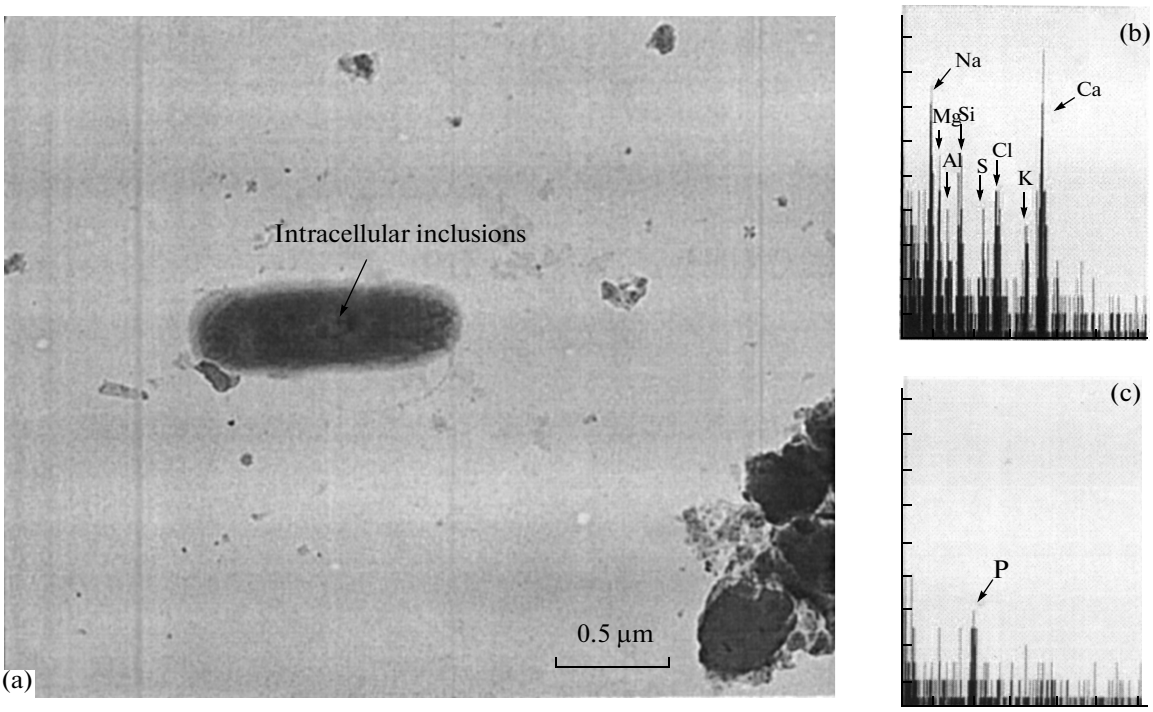

Fig. 3. A bacteriomorphic particle (a). X-ray spectra of the bacteriomorphic particle: integral spectrum of the elemental composition of the whole particle (b); spectrum of the elemental composition of the intracellular inclusions (c).

exponential growth phase of their cultures showed that freezing–thawing resulted in considerable changes in the elemental composition of bacterial cells towards a decrease in their intercellular content. Importantly, the cells of the isolates studied were different in the degree and character of injuries. These injuries were not lethal, since the isolate retained their viability when plated onto the nutrient media. The changes in the ratios of biogenic elements in the cells of these organisms are shown on Fig. 4. These findings reflected the ratio of the peak areas calculated using the relevant software. For example, in the mycelial cells of the isolate no. 1 sampled in the exponential growth phase, the K/Ca ratio decreased insignificantly after the freezing–thawing procedure, with the P/S ratio decreasing more than twofold. A different character of changes in the elemental composition was noted for the stationary-phase cells of isolate no. 2, which had a characteristic coccus-rod-coccus life cycle. After the freezing–thawing procedure, a significant decrease in the K/Ca ratio (more than 4.5-fold) was observed; P/S ratio decreased more than 1.5-fold. These changes indicated the differences in the sensitivity/resistance of bacteria of various cell morphotypes to the stressful effect of freezing–thawing, depending on their physiological state.

Microbiological investigation of the aerobic heterotrophic microflora of the thawed material from the permanently frozen ice samples demonstrated that the number of the colony-forming units of viable organisms was an average of 10^2–10^3 CFU/mL (Table 2). The maximal values (3.4×10^3 CFU/mL) of the number of viable organisms and their diversity were noted in sample no. 2.

The most favorable incubation temperatures for reactivation of heterotrophs were 20 and 28°C. The maximal number of colonies was found on agarized media ISP1 and ISP3 with yeast extract. Bacteria of different groups were revealed in the samples: gram-negative, gram-positive, sporulating and nonsporulating, rod-shaped and coccoid, as well as polymorphic, sometimes with rudimentary branching, rod-shaped microorganisms with the coccus-rod-coccus cycle of development characteristic of coryneform actinobacteria [18]. Single colonies of spore-forming mycelial actinobacteria were also found (Table 3). Nonpigmented or slightly pigmented colonies of coryneform actinobacteria, which accounted for about 70% of the number of all the colonies revealed (on ISP1 and ISP3 agar media), numerically predominated in the samples. Samples nos. 2, 3, and 4, yielded uniform single colonies of microscopic fungi. The isolates of the gram-positive spore-forming bacteria, the microscopic fungi, and, particularly, the coryneform bacteria showed the capacity for active surface growth at a lowered temperature of 8°C. The growth of the latter group was observed as early as the third day of cultivation.

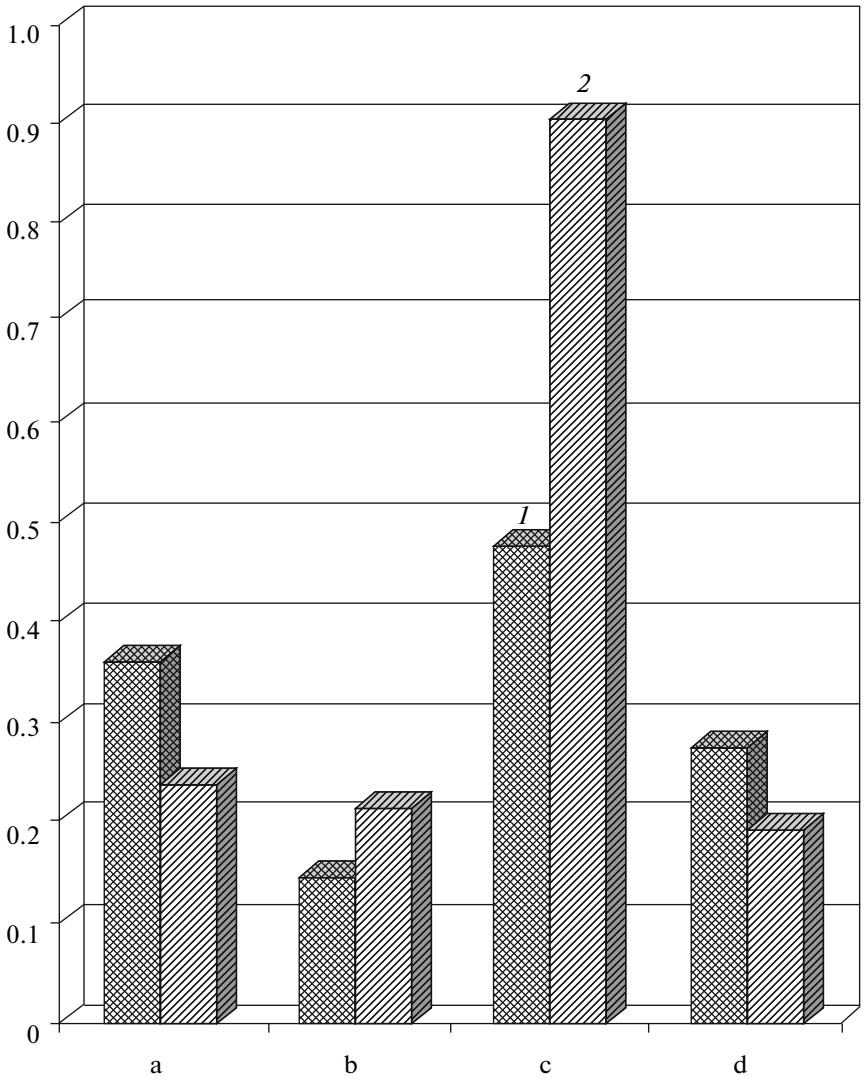

Fig. 4. Effect of the freezing–thawing procedure on the biogenic element ratio in the cells of the model cultures of actinobacteria. The P/S (*1*) and K/Ca ratios (*2*) in the cells of isolate no. 1 before freezing (a) and after thawing (b); in the cells of isolate no. 2 before freezing (c) and after thawing (d). *1*, P/S; *2*, K/Ca.

DISCUSSION

The samples of ice from the ice wedge of the syngenetic type in the depth of the Upper Pleistocene sediments containing loams with silt interlayers were used for microbiological study [13]. Differences in color, transparency, porosity, and the content of organomineral particles were noted in the course of the experiments. The heterogeneity of ice in the samples studied resulted from the process of accumulation of ice at the expense of surface waters as well as from the conditions of its formation: the temperature gradients, deformation of permafrost rocks, and a gamut of other physical and geological processes [11].

Electron microscopic study showed that sample no. 2 containing a considerable number of mechanical inclusions was characterized by a great variety of the bacterial forms. In terms of morphology, the bacteriomorphic particles revealed could be divided into four main groups: rod-shaped, rounded, coiled, and thread-like. Coryneform structures dominated among the rod-shaped ones.

The particles had a number of features, which could be regarded as manifestations of the adaptive strategy of survival at low temperatures. For example, coryneform bacteriomorphs contained a large number of electron-transparent zones. It is known from the practice of electron microscopic studies of the cells of coryneform actinobacteria that these zones are caused by the substances of lipid nature [18]. Accumulation of storage nutrient compounds in the vegetative cells of microorganisms is known to increase their resistance to freezing and other damaging factors [19]. We also noted the presence of capsule formations or formations resembling external sheaths around the bacterio-

Table 2. Number of aerobic heterotrophic bacteria (CFU/mL) in the Mamontova Gora ice wedge samples

Sample no.	1			2			3			4		
Nutrient medium	Cultivation temperature, °C											
	20	28	37	20	28	37	20	28	37	20	28	37
Medium with chitin	0.84×10^2	2.8×10^2	0	0.8×10^3	0.5×10^3	0	0.3×10^3	0.1×10^3	0	0.8×10^3	0.5×10^3	0
Synthetic medium 1 with glucose	0.8×10^2	7.4×10	1.4	1.7×10^3	0.7×10^3	0	0.5×10^3	0.4×10^3	0	1.1×10^3	0.5×10^3	9×10
Starch-ammonium	0.4×10^2	1.2×10^2	0	0.8×10^3	0.3×10^3	0	0.3×10^3	0.3×10^3	0	1.2×10^3	0.3×10^3	9×10
ISP1 (agar)	1.8×10^3	1.2×10^2	4	3.8×10^3	3.4×10^3	6×10	0.8×10^3	0.9×10^3	9×10	2.6×10^3	2.0×10^3	2×10^2
ISP3 with yeast extract	1.5×10^3	1.8×10^2	4	2.4×10^3	3.0×10^3	8×10	0.6×10^3	0.9×10^3	6×10	2.4×10^3	2.7×10^3	1×10^2

Table 3. The main groups of aerobic heterotrophic microorganisms revealed in the ice wedge samples of the Mamontova Gora ice complex

Group of microorganisms	Cell morphology	Number of colonies, % of the total number of colonies revealed on nutrient media ISP1 (ISP3 with yeast extract)
Gram-positive non-spore-forming bacteria	Single, arranged in pairs, in chains, cocci in conglomerates	4 (6)
Actinobacteria	Rod-shaped ($0.3–0.6 \times 1.0^{-7}$ μm), straight or slightly curved, club- or baton-shaped. The daughter cells are formed by cleaving or bending; located in the V-like or palisade fashion, sometimes branching with a characteristic coccus-rod-coccus cycle of development; sometimes motile	65 (68)
Mycelial actinobacteria	Nonfragmenting mycelium; long chains of spores on aerial mycelium	<1
Gram-positive spore-forming bacteria	Bacillary-type rod-shaped cells grouped in chains	27 (23)
Gram-negative non-spore-forming bacteria	Small rod-shaped, non-spore-forming, nonmotile cells	3 (2)
Fungi	Whitish multinucleated mycelium with sporangia	<1

morphic particles. The formation of a specific microenvironment may also be one of the mechanisms of preservation of viability by microorganisms under the extreme permafrost conditions [19]. The decisive role of extracellular polysaccharides as cryoprotective compounds in the survival strategy under the conditions of low temperatures and elevated pressure may be seen in the psychrophilic organism *Colwellia psychrerythraea* [20]. The survival strategy may vary in different species of microorganisms and is primarily determined by their subcellular structural organization and genetic characteristics.

The X-ray microanalysis of bacteriomorphic particles in the ice core samples confirmed their organic origin. However, in a number of cases, these forms were characterized by an incomplete spectrum of the main biogenic elements. An abnormally low value or the absence of the phosphorus and potassium peaks in the X-ray spectra of some particles were noted, which could probably result from the damage of vitally essential cell structures, primarily, of the cell membrane

affected by low temperatures. It was noted that damaged and dead cells occurred not infrequently in the permafrost samples [21]. Our model experiments revealed that freezing–thawing caused a significant decrease in the phosphorus and potassium content in the cells of bacteria isolated from permafrost. The degree of this decrease was determined, to a significant degree, by the physiological state of the cultures at the time of low-temperature treatment. Apparently, the significant P/S decrease in the cells of the exponential-phase culture was indicative of an affection of the lipid component of the cell membranes, an impairment of the energy status of the cells, and the loss of the phosphorus-containing cell components [22]. At the same time, the K/Ca ratio remained virtually unchanged, indicating the preservation of the barrier function of the cell membrane. A different picture of a change in the elemental composition influenced by the freezing–thawing procedure could be observed in the stationary-phase bacterial cells. A significant K/Ca decrease (more than 4-fold) indicated the loss of K^+ as a result of damage to the cytoplasmic membrane and formation of defective zones impairing its barrier functions [22]. However, the damage to the cellular structures may be nonlethal and eliminated by the cell reparation systems under growth-favorable conditions.

Detection of viable microorganisms preserving the intact cell membrane, cell integrity, and the capacity for reverting to metabolic activity and proliferation is of special importance for investigation of the permafrost microflora. It is established that the viable microflora of syncryogenic permafrost is characterized by resistance to the stressful effect of the freezing–thawing procedure and may indicate the origin of permanently frozen rocks [23]. The results of this work showed that the number of the colony-forming units (CFU) of the heterotrophic aerobic bacteria was commensurable with the number of similar microorganisms revealed earlier in frozen rocks and in ice [8–10, 21]. Our data agree with the results of the previous studies of frozen rocks in which bacterial heterotrophic cenoses were found to consist mainly of the coryneform forms of actinobacteria with a significant content of non-spore-forming rods and an absolute absence of spores [8]. Psychrotolerance of the isolates of coryneform bacteria was also noted in this study. Earlier, the high resistance of coryneform bacteria to freezing and their capacity for long-term preservation of viability under the dietary deficiency conditions was reported [18].

According to comparative genomics of actinobacteria [24], coryneform bacteria represent the evolutionarily earlier forms of actinobacteria compared to the mycelial representatives of this group. Coryneform actinobacteria numerically predominate in the microflora of permafrost rocks [8]. On the contrary, the study of the present-day microbial population of the permafrost soils of Central Yakutiya showed predominance of the mycelial spore-forming forms of actinobacteria in the lower mineral horizon of the soil profile, with an increase in their number to a depth of roof permafrost [25]. This difference in the composition of the actinomycete component of the bacterial microflora may serve as a confirmation of the absence of pronounced microbial migration from the upper horizons into the depth of perennially frozen rocks. Predominance of the evolutionarily earlier forms of actinobacteria in the composition of bacterial cenoses of the frozen rocks suggests that this index may be probably used to confirm their ancient origin. In this connection, the literature data on the identification of the fragments of the ancient bacterial DNA isolated from permafrost samples are worth noting. It was shown that among the DNA fragments preserved, the fragments with a high level of similarity to the DNA of coryneform bacteria constitute the greater part, in particular, the representatives of the genus *Arthrobacter* [26].

The permafrost rock conditions rule out the possibility of active growth and development of microorganisms. The amount of unfrozen water, a component necessary for the activity of biological structures, is very scarce. It is mainly present as thin interlayers, which are approximately 0.01–0.1 μm thick [12]. The preservation of cell structures under these conditions is possible at the expense of internal cryoprotectors contained in the cytoplasm, the specific microenvironment, as well as the presence of films of unfreezing water. The possibility of carrying out certain biochemical processes by microorganisms, primarily, the reparation of injuries of life-supporting cell structures, should not be ruled out.

The presence of viable microorganisms in frozen rocks gives evidence of a new, as yet unknown form of preservation of life without division during tremendously long periods of time. Thus, we may conclude that the microorganisms living in permanently frozen rocks represent ancient organisms, rather than new generations [12].

Apparently, the cells of microorganisms, which could be localized at the boundaries of ice crystals or in the air cavities between them, represented the ancient forms whose period of preservation coincided with the period of ice core formation. The syncryogenic origin makes it possible to consider the Mamontova Gora ice wedges as one of the unique natural banks of ancient microorganisms.

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