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Relict Microorganisms of Cryolithozone as Possible Objects of Gerontology

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Abstract—Permafrost is widely distributed in the Northern Hemisphere and arrives after hundreds of thousands and millions of years. Permafrost contains live microorganisms, which are not frozen due to the relatively high ambient temperatures (from –2 to –8°C) but are immobilized; thus, their age seems to be close to the age of the permafrost. The longevity of relict microbial cells is obviously related to their mechanism of protection against heat, radiation, free radicals, and other damaging agents. A strain of *Bacillus* sp. was isolated from the permafrost aged about 3 million years and its 16S rDNA sequence was identified, followed by preliminary testing of the bacterial culture in *Drosophila melanogaster* and mice. The experiments showed immune stimulation and improvement of the physical condition. This fact, together with the age of microbial cells, suggests the consideration of relict microorganisms as objects of gerontology.

Keywords: relict microorganisms, permafrost, stimulation of immune system, vitality
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INTRODUCTION

Investigation of the mechanisms of aging is still a relevant fundamental problem; hence, the study of cells that can survive through many thousands of years may be of interest for gerontology. The discovery of live relict cells is an exceptional event *per se*. Bacteria, being in essence the simplified semblances of eukaryotic cells, were considered to live or remain viable for a long time. However, this assumption has been confirmed only recently. As is shown, anthrax spores are preserved for about 105 years [7, 16]. Bacterial colonies isolated from amber were aged 40 million years and more [12]. However, the above single findings do not leave us so firmly convinced of the phenomenal longevity of bacteria as permafrost studies. Permafrost occupies a vast territory; it takes about 70% of area of the Russian Federation. Its temperatures are mainly about –2...–8°C and its age in some places is millions of years [3, 11].

The evidence of vitality of microorganisms in permafrost appeared long ago [1, 6, 7, 11]. Bacteria, fungi, diatoms, and other microorganisms were found at the *Vostok* Antarctic station in 1979 [1]. Bacterial metabolism in permafrost was observed at about –20°C [11]. Other references also demonstrated the vitality of bacteria at temperatures below 0°C [6–8, 10, 14]. Microorganisms are resistant to freezing; many of them endure it easily [6, 8]. It is known that part of water in the materials (more than 10%) remains unfrozen at temperatures below –20°C [5]. Without denying the probability of development of microorganisms in permafrost rocks, we should note that their growth is hampered. As we know, the growth of aging cultures stops even in laboratory conditions. Crystallization of the water and cessation of metabolism diminish the ability for growth [6]. The width of nonfreezing water interlayers at –2 and –4°C is about 0.01–0.10 μm [5] (i.e., much less than the sizes of microorganisms). These conduction paths are actually unavailable for life support, and any noticeable transfer of cells in such material is out of the question. Therefore, we can be sure that the bacteria in permafrost rocks are fossil, relict organisms [11]. Their age is confirmed by geological conditions, history of formation of frozen strata, carbon-14 dating, results of the study of optical isomers of amino acids and, indirectly, biodiversity [14].

The nature of longevity of microorganisms in ancient permafrost cannot be exhaustively explained. It is believed that no chemical and biological reactions

take place in an organism in a state of anabiosis [6, 8]. However, we do know that most proteins in a living cell are unstable [2, 13], being alive for minutes and, more rarely, days. Genetic structures are liable to mutations, and reparations are not as effective to prevent accumulation of damages [9]. Cell structures are affected by free radicals and radiation. Thermal motion of atoms and molecules in water solution is an independent destructive factor: permafrost temperatures are far from absolute zero [2]. The cytoplasm of cells does not freeze at such temperatures [6, 8, 10]. It is obvious that an organism in anabiosis is also liable to degradation and decay. The data on the heat stability of nucleotides [15] show that due to the instability of cytosine, the period of time when the DNA chain remains functional is hardly longer than several hundreds of years. Taking a certain average mutation rate into account [9], nearly all genes in a bacterial chromosome will be modified in 1000 years and no traces of the genetic apparatus will remain in a million years, even subject to the functioning of repair systems. In the absence of nutrients, cells can rely only on the electrical charge of the cytoplasmic membrane, but it will suffice to synthesize only 100–150 ATP molecules. Evidently, this energy is insufficient for long-term preservation of integrity of cell structures. Ancient DNA of mummies, mammoths, insects in amber, and other organisms were found to be destroyed [12, 18]. Calculations show that even minor DNA fragments (100–500 nucleotides) can be preserved for no more than 10000 years in the usual climate and up to 100000 years at the most in cold regions [18]. Thus, it is unclear how bacteria survive in millennial permafrost. One should suppose the existence of mechanisms preventing the accumulation of damages. We believed it would be worthwhile to study the influence of these microorganisms on higher organisms, the more so that the immune responses of the latter could be relevant.

This work describes bacteria found in the ancient permafrost strata of Yakutia, Republic of Sakha, Russia, and presents preliminary results of testing the biological activity of bacterial culture in Drosophilas and laboratory mice.

MATERIALS AND METHODS FOR ISOLATING MICROORGANISMS

Samples for the study of microorganisms in frozen rocks were taken from outcrops and subsurface structures in several regions. One of the outcrops is located on the left bank of the Aldan River, 325 km upstream to its confluence with the Lena River on the Mamontova Gora. Samples were taken 0.9–1.0 m deeper than the layer of seasonal thawing out. The outcrop was destroyed by the river (more than 1 m per year); therefore, the sampled sediments were obviously in a state of permafrost. At the same time, there is the annual spring failure washing-off that prevents obstructions and the mixture of rocks. The latter are fine-grained sands, and their age corresponds to the middle Miocene: 10–12 million years [4]. The fall of temperature and frost penetration into sediments began here in the end of Pliocene, about 3.0–3.5 million years ago [3]. Later on, the sediments did not thaw out because of the cold climate of Yakutia. According to data from paleoclimatic reconstructions of the region, the average annual temperatures in Pleistocene were −12 to −32°C in winter and +12 to +16°C in summer [3]. Thus, the age of permafrost on the Mamontova Gora may be up to 3.5 million years. Moreover, samples were taken from the younger ice wedge of Yakutia from the underground of the Melnikov Institute of Geocryology, Siberian Branch of RAS (Yakutsk), and from underground ice in the Fox Permafrost Tunnel (Alaska).

Frozen rock samples were taken with maximum possible precautions for the field conditions, using metal instruments (bores, forceps, and scalpels) sterilized with alcohol and burned-in flame. For surface sterilization, a sample of about 50 g was placed into a glass with 96% ethanol solution, then put into a burner flame, and packed into a sterile test tube. Moreover, the 4–5-kg monoliths of frozen rocks were taken. Selected rocks were stored at −5°C (i.e., close to the natural conditions). Samples were transported in a frozen state in thermally controlled containers with cooling agents.

Samples of different dilutions in sterile conditions were added to Petri dishes containing *YPD*, *MRS*, and *NA* media. Samples were also added into a liquid meat–peptone broth under anaerobic and aerobic conditions.

DNA was extracted using the Fast DNA kit for soil (BIO 101 Inc., Vista, CA, USA). The 16S rRNA gene fragments were amplified by polymerase chain reaction with bacterial primers. The reaction was performed in a 20-jil volume by the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA); the amplicons were exposed to electrophoresis and purified by the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified amplicons were cloned using the pCR2.1 vector, *E. coli* culture, and TA cloning kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Plasmid DNA-carrying 165 rDNA was obtained from 24-h culture using the Spin Mini prep Kit (Quiagen, Crawley, UK). Purified plasmid DNAs were sequenced in an ABI PRISM 3100 Genetic Analyzer using the Big Dye Terminator Cycle-Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The sequence length was 1488 bp. The sequence obtained was compared to other sequences using BLAST (Basic

Local Alignment Search Tool). The phylogenetic tree was constructed using the CLUSTAL W software package [17].

RESULTS OF ISOLATION, GROWTH STUDY, AND IDENTIFICATION OF MICROORGANISMS

A cultivated bacterium capable of aerobic and anaerobic growth was found in frozen Miocene sediments on the Mamontova Gora; its optimal growth temperature was defined as +37°C. The bacteria are comparatively large rods (1.0–1.5 × 3–6 μm), which are joined into chains in the culture (Fig. 1) and can form spores. The microorganism is immobile and Gram positive. It is referred to the genus *Bacillus* but seems to be a novel species. The 165 rRNA nucleotide sequence of the bacillus was deposited in the DDBJ/EMBL/GeneBank and assigned number AB178889, identification number 20040510203204.24251. The isolated bacillus shows the maximum species similarity to *Bacillus simplex* and *B. macroides*: the homology between their 165 rRNA is 96–97%.

The growth of bacilli at low temperatures was observed previously [8]. It is known that *Bacillus anthracis* easily endures freezing [7]. The optimal growth temperature of the bacillus found is rather high. Bacillary spores are known to be most resistant [16]; thus, *B. thuringiensis* and *B. macroides* were found in amber with an absolute age of 120 million years [12]. Therefore, the finding of a living bacillus in the ancient permafrost of the Mamontova Gora is not altogether surprising. Spores are typical of Gram-positive genera *Bacillus*, *Clostridium*, and *Streptomyces* [16]; recently it has been shown that these genera are formed by Gram-negative microorganisms as well. Thus, spore formation is a widespread mechanism of survival, probably involving the horizontal transfer of genetic information.

Tens of microbial species were isolated by the foregoing method from the much younger ice wedge of Yakutia and Alaska aged about 25–40 thousand years [14]. Most of the isolated bacteria are Gram positive and close to *Arthrobacter* and *Micrococcus* sp., and the fungi are close to *Geomyces* sp.

THE NATURE OF THE LONGEVITY OF MICROORGANISMS

The molecular bases of thermal stability of biological materials are an unsolved problem [2, 13, 15]. Protein stability is determined by free energy G, which is characterized by value $G = 5–15$ kcal/mol [2]. The portion of destroyed regions of the structure related to the portion of ordered regions (k) seems to be the following:

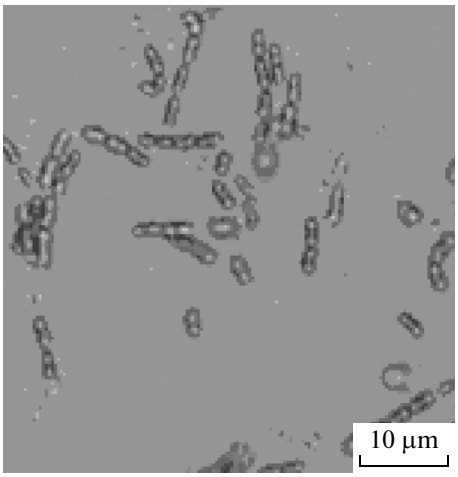

Fig. 1. The isolated strain of *Bacillus* sp., Gram staining.

$$k = e^R,$$

where G is the free energy of transition from the ordered to the random region, kcal/mole; T is the temperature, K; and R is the gas constant, ~0.001989 kcal/mol.

This expression can be used to estimate the approximate lifetime of biomolecules. For $G = 30$ kcal/mol, the lifetime of a molecule is about 300 years. For the period of temperature fluctuations 10^{-8} to 10^{-9} sec and $G = 20$ kcal/mole, their lifetimes are less than a year. The maximum known value of activation energy is about (usually less than) 45 kcal/mole [2]. The estimates presented are highly approximate, but they also demonstrate protein and DNA instability. The data on the heat instability of nucleic acids are presented in the work [15]. In this respect, there are interesting data on the lifetime of the natural smallpox virus, which is the most stable among viruses. Its lifetime was shown to coincide with the cited calculation data of several hundreds of years [7]. Of course, the microorganisms of particular concern are those surviving at low temperatures under natural conditions for a long time [11, 14]. It is possible to deduce the possibility of combinatory transformations predicted previously [7]. In this context, one cannot but mention the existence of biocatalysts such as ribozymes. In our case, it may be important that ribozymes are stable and active at temperatures below 0°C. The great diversity of relict microorganisms isolated from the ice vein aged 25–40 thousand years [14] is probably accounted for by the mobile genetic structures such as plasmids coding for longevity.

Thus, it is difficult to explain the long-term existence of microorganisms by inhibition of vital functions during anabiosis. If bacteria form spores, where metabolism is practically stopped [14], the foregoing calculations on denaturing even DNA only are all the more appropriate. The diversity of the mechanisms of DNA damage (methylation, deamination, depurina-

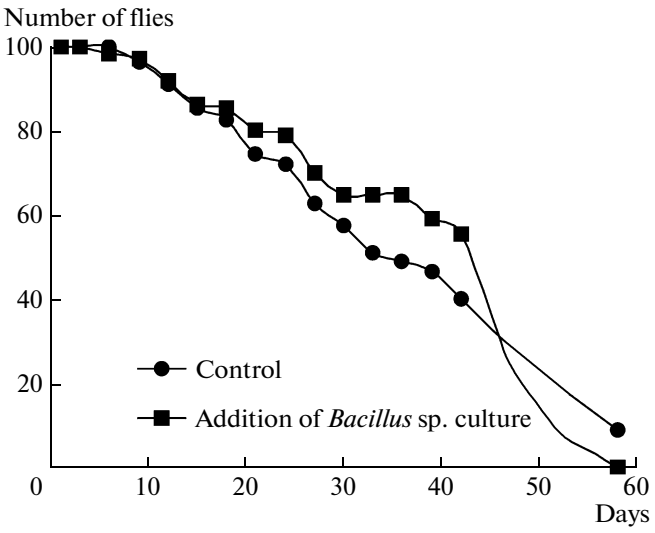

Fig. 2. Influence of *Bacillus* sp. culture (strain 3M) on the lifespan of *Drosophila melanogaster*.

tion, formation of thiamine dimers, cross-links, and breaks) in the context of this hypothesis leads to reestimation of the lifetime of macrobiomolecules, reducing its duration still more. Obviously, survival is conditioned by the presence of special repair or conservation mechanisms.

TESTING BACILLARY CULTURES IN HIGHER ORGANISMS

Testing in Drosophila melanogaster

The experiment was carried out in *Drosophila melanogaster* flies of the same age (24 h). Five pairs were placed in test tubes with a nutrient medium (5–7 ml). The volume of sampling was 100 flies for each group. Flies were selected for the experiment by etherization; the dead and surviving flies were counted every 3 days. The experiment was carried out with a 24-h culture of *Bacillus* sp. (strain 3M) grown in a meat–peptone broth. The culture (20 μl) was added to the test tube with the experimental group. In the control group, the flies were kept in the medium with yeasts; in the experimental group, the flies were kept in the medium with yeasts for the first 5 days and then in the medium with the bacillus for 24 h (alternated during the whole period of observation). To determine fertility, virgin flies were taken on the day of emergence. Female and male flies were separated and kept for 5 days until maximum fertility was reached. Then the files were put in pairs into test tubes with detachable caps. The bottoms of the caps were covered with Confectionery Agar with an addition of sugar. Fertility was recorded daily for 6 days by counting the egg mass and, in 24 h, the undeveloped eggs.

When 75 μl of the strain 3M in normal saline solution (1 billion cells/ml) was applied to the surface of the nutrient medium, fertility was observed to decrease five times compared to the control group. The average fertility of a female fly was 58.1 ± 8.61 in the control group and 10.2 ± 3.44 in the experimental group. When the bacterial culture was added after keeping the flies on yeasts, the portion of survived flies increased on days 24–42 of the experiment compared to the control group in spite of the death of flies in both groups after 50 days (Fig. 2).

Testing in Laboratory Mice

Bacterial culture was prepared by analogy with the testing in Drosophilas using a 24-h culture of *Bacillus* sp. (strain 3M), but with its freezing–thawing prior to introduction. Experiments were carried out in F1 CBA/Black-6 mice (15 animals per group on average). In the first series of experiments, the effects of culture doses on immune system parameters were studied in young animals (aged 3–4 months). Two groups of animals were used: one was used as a control, and animals in the other group received a saline solution. The bacterial culture was introduced once intraperitoneally: 5000; 50000; 500000; 5000000; and 50000000 microbial objects (m.o.) per animal. In the second series of experiments, the effect of the bacterial culture on physiological and behavioral reactions was estimated in "senior" mice (aged 17 months); the culture was introduced once intraperitoneally at a dose of 5000 m.o. The control group consisted of animals of the same age. The animals were euthanized by dislocation of the cervical vertebrae. The following standard methods were used for estimations: index of the organ (the ratio of organ weight to body weight, %) for morphophysiological activity of thymus and spleen, levels of phagocytic (PC, %) and metabolic (NST, %) activities of spleen macrophages for the activity of nonspecific immune resistance factors, the reaction of a delayed hypersensitivity (DTH) in vivo for cell-mediated immunity, the number of antibody-forming cells in 1 million nucleus-containing cells in the spleen for the activity of humoral immunity, the load-lifting test for the muscle strength of animals, and the "Open Field" test for behavioral responses; the lifespan was estimated using standard methods.

It was shown that *Bacillus* sp. (strain 3M) at a dose of 5000 m.o. favored an increase in the thymus and spleen indices by 60–80%. The bacillary culture stimulates the phagocytic activity of spleen macrophages at a small dose (5000 m.o.) and inhibits it at average doses (500000 and 5000000 m.o.). The bacillary culture almost at all doses enhances the activity of humoral immunity; the dose of 5000 m.o. favors the increase in functional activity of both cell-mediated and humoral immunity.

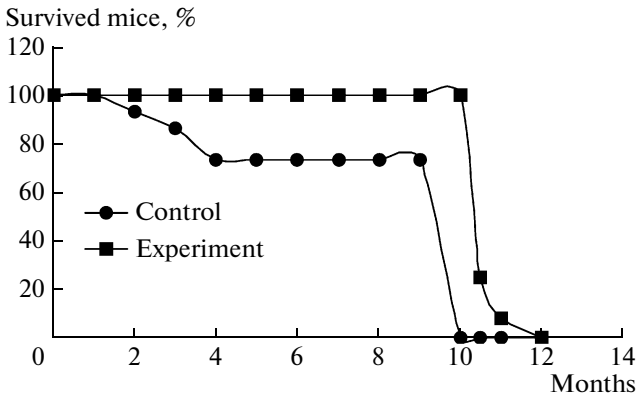

Fig. 3. Influence of *Bacillus* sp. culture (strain 3M) during parenteral introduction of 5000 cells on the lifespan of laboratory mice aged 17 months.

In this context, the dose of 5000 m.o. was chosen for studying the culture influence on life-span. The minimal and maximal lifespans of mice from the control group were 589 days and 833 days, respectively. The minimal and maximal lifespans of mice from the experimental group were 836 and 897 days, respectively (Fig. 3). Two 2 months after the culture was introduced, animals from the experimental group showed a higher body weight compared to animals from the control group. The muscle strength of the experimental animals increased by about 60% compared to their coevals from the control. The enhanced spatial intellect and exploratory activity of animals was evidenced by their more frequent visits to internal sectors of the experimental field, an increase in the number of upright postures, and visits to burrows. It seems that intraperitoneal introduction of the bacterial culture stimulates the immune system and improves the emotional stability of laboratory animals. The enhanced lifespan of mice is evidence of possible presence of geroprotectors in the bacterial culture of *Bacillus* sp. We emphasize that investigations into the properties of cultures of relict microorganisms should be considered as very preliminary in respect to both their scope and performance.

CONCLUSIONS

Isolation and identification of relict microorganisms from permafrost is an independent task of microbial ecology and systematics due to an abundance of novel species. The nature of their longevity at temperatures below –2...–8°C requires investigation.

The strain *Bacillus* sp. 3M has been isolated from permafrost of the Mamontova Gora aged about 3.5 million years and identified by the 16S rDNA. The younger permafrost (25–40 thousand years) contains a large group of microorganisms including fungi.

The decrease in mortality of *Drosophila melanogaster* flies and laboratory mice in some experiments, together with the stimulation of immune system and improvement of physical state of the mice, suggest that the studies of relict microorganisms are promising for gerontology.

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