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SHORT COMMUNICATIONS

Thermophilic Bacteria of the Genus *Geobacillus* from Permafrost Volcanic Sedimentary Rocks

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Geothermal environments on Earth are unique habitats for thermophilic and hyperthermophilic microorganisms and occur in volcanically active regions. On the Antarctic continent and the adjacent islands, continuous volcanic activity occurred throughout the Cenozoic era. At present, volcanoes Erebus, Rittmann, and Melbourne show activity on the continent. The islands of the South Sandwich archipelago and Deception Island, which are adjacent to Antarctica are also of volcanic origin and are located in the zone of tectonic activity.

The conditions in the thermal ecosystems localized near a volcanic crater or an outlet of a fumarole sharply contrast with the environmental conditions on the continent, which are characterized by negative average annual temperatures, strong winds, and a high level of UV radiation in the summer period.

Such ecosystems, which are geothermal oases isolated from one another, have been studied in detail, including those on the Antarctic continent [1–6]. However, it is still unclear what occurs to microorganisms in the case of cessation of the geothermal activity and transfer of the ecosystems to the permafrost state. It may be suggested that if microorganisms from the geothermal niches of Antarctic volcanoes encounter cold conditions where the maximal temperature does not rise above 0°C, natural cryoconservation of the microbial community in permafrost volcanic ashes or cinder occurs.

The goal of the present work was to study the possibility of preservation of viable thermophilic microorganisms in permafrost volcanic rocks.

In the work, the permafrost volcanic rocks of the Deception Island situated in the Bransfield Strait near the northwestern coast of the Antarctic Peninsula were studied. The last series of volcanic eruptions was recorded in 1967–1970; however, the geothermal activity is still observed in the form of fumaroles opening onto the surface as emission of low-temperature (50–90°C) vapor and gases [7]. The samples were collected aseptically in the course of the joint Spanish–Russian Antarctic expedition in 2009 as described earlier [8] and delivered to the laboratory in the frozen state. A total of three wells were drilled for sampling on a plane ground (62°59′7.40″ S, 60°40′43.00″ W) near Crater Lake, and four samples from the depths of 1.0–1.8 m of the permafrost at least 180 years old were investigated [9].

Enumeration of thermophilic bacteria and their subsequent isolation were carried out on solid nutrient media TSA (Fluka) and R2A (Sigma) at 55°C, pH 7.0. The pure cultures were incubated under aerobic conditions in the 35–80°C range. The morphological, physiological, and biochemical features of the cells were studied using the generally accepted methods [10]. In order to determine the 16S rRNA gene sequence and the G+C composition, DNA was isolated according to Marmur [11]. Amplification of the 16S rRNA gene was carried out with the universal bacterial primers 21F and 1492R and the Encyclo PCR kit of reagents (Evrogen, Russia) on an Eppendorf MasterCycler. The DNA was sequenced at Evrogen. Preliminary analysis of the nucleotide sequences obtained was carried out using the BLAST software package; the MEGA5 package [12] was used for alignment and phylogenetic analysis. The nucleotide composition was determined using the thermal DNA denaturation method on the Beckman Coulter DU800 spectrophotometer with a temperature-controlled cell. The type strain *Geobacillus stearothermophilus* B-510^T was used as the standard.

Thermophilic bacteria incapable of growth at room temperature were revealed in all the permafrost samples examined. Their number was ~50 CFU/g in each of the samples. Two bacterial strains (D2355 and D4455) demonstrated stable growth at 55–60°C and were selected for further study. The isolated thermophilic bacteria formed small non-pigmented colonies on the agar surface. The cells of both strains were rods of varying size with rounded ends, forming oval spores.

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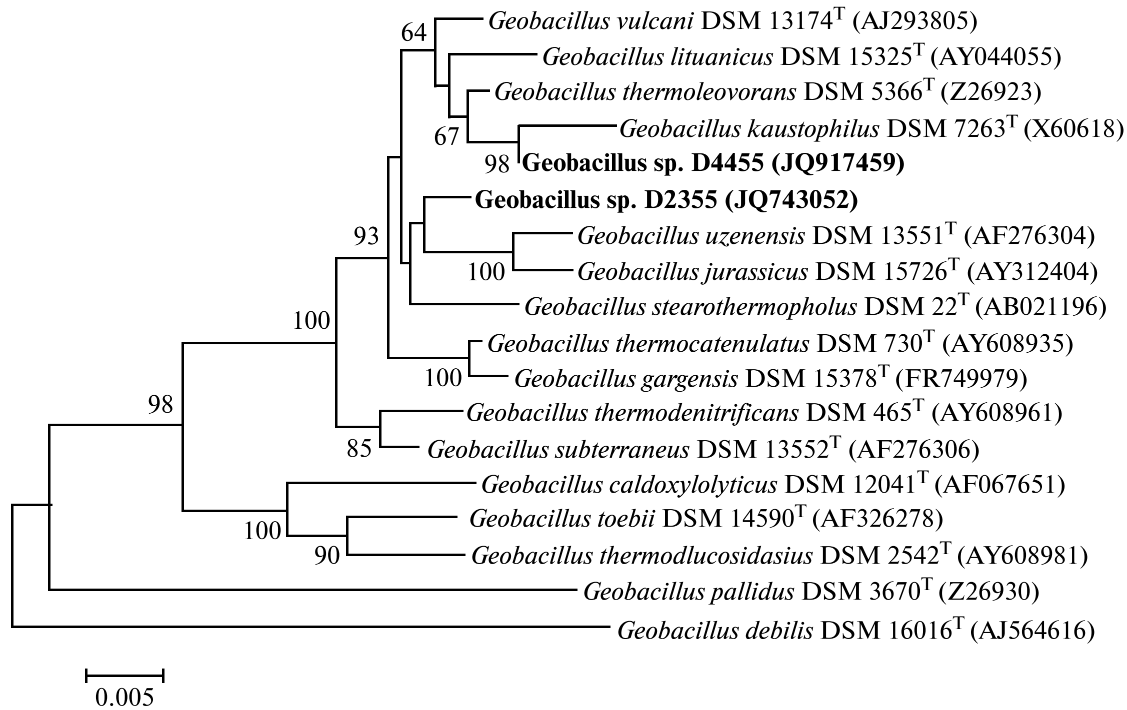

Fig. 1. The rootless phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of the representatives of the genus *Geobacillus* and the strains D2355 and D4455 isolated from permafrost rocks. The type strains of the *Geobacillus* species were used for phylogenetic analysis; the GenBank accession numbers are shown in parentheses.

The D2355 cells were motile. Analysis of ultrathin sections showed that the cells of both strains possessed the cell wall of gram-positive type, which contained the S layer (data not shown).

In order to specify the taxonomic position of the isolates D4455 and D2355, the 16S rRNA gene sequences (1417 and 1422 nucleotides, respectively) were determined. The sequences were deposited in the NCBI database under accession nos. JQ917459 and JQ743052. Phylogenetic analysis of the sequences showed that both strains belonged to the genus *Geobacillus*: the strain D2355 was the most closely related to the species *G. stearothermophilus* (99.1% similarity), while the strain D4455 was closest to *G. thermoleovorans* (99.7% similarity). The dendrogram constructed by the neighbor-joining method is shown on Fig. 1. The genus *Geobacillus* includes thermophilic gram-positive bacteria, aerobes or facultative anaerobes [13], which are capable of growth within the range of temperatures from 37 to 78°C. The strains D2355 and D4455 grew in the 40– 75°C temperature range with the optima at 68 and 65°C, respectively (Fig. 2). Comparison between certain physiological and biochemical characteristics of the Antarctic isolates and the representatives of the most closely related species (table) revealed that the isolates had a number of phenotypic characteristics differentiating them from the most closely related known species of the genus *Geobacillus*. Isolated strains D2355 and D4455 were deposited in the All-Russian Collection of Microorganisms-VKM under accession numbers B-2785 and B-2786, respectively.

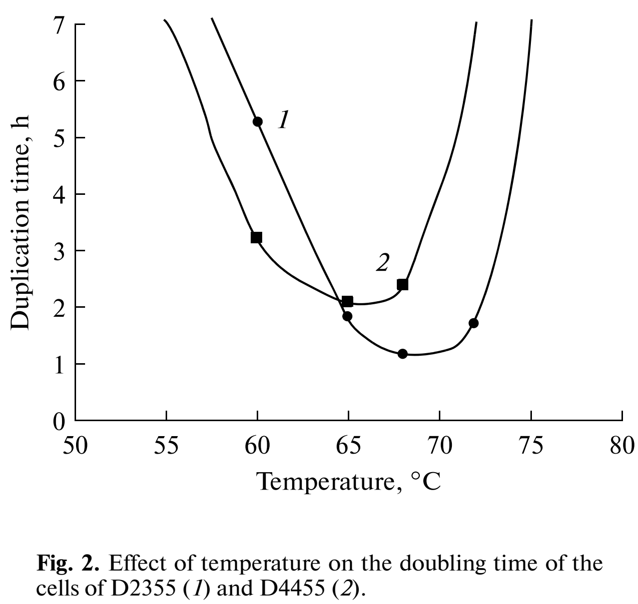

Fig. 2. Effect of temperature on the doubling time of the cells of D2355 (*1*) and D4455 (*2*).

According to the results of the studies of the geothermal oases of Antarctica, thermophilic and hyperthermophilic microorganisms were revealed in geothermally heated soils, fumaroles, and hot springs [5, 14, 15]. They were also revealed as a minor component of the community outside the thermal ecosystems

Differentiating characteristics of the Antarctic strains and the most closely related *Geobacillus* species

Characteristic	*Geobacillus* sp. D4455	*Geobacillus* sp. D2355	*G. stearothermophilus* DSM 22^{T}*	*G. thermoleovorans* DSM 5366^{T}*
Size, µm	0.6–0.8 × 2.0–3.0	0.4–0.5 × 1.5–2.2	0.6–1.0 × 2.0–3.5	0.9 × 6.0–8.0
Motility	–	+	+	+
Temperature, °C				
Range	40–75	40–75	37–65	35–78
Optimum	65	68	55–60	60–68
NO_2^- reduction	–	–/+	–	+
Gelatin hydrolysis	+	+	–/+	–
Casein hydrolysis	–	+	–/+	ND
Starch hydrolysis	–	+	+	–
Citrate utilization	–	–	–/+	+
Glucose fermentation	–	–	–/+	+
G+C, mol %	51.9	51.9	51.9	52–58

Note: * The data of [22, 23] were used; "+", "–", and "–/+" stand for positive, negative, and variable, respectively. ND stands for not determined.

[16]; however, they have never been found previously in permafrost rocks. In the present work, thermophilic bacterial strains of the genus *Geobacillus* capable of growing at temperatures from 40 to 75°C were isolated from the permafrost rocks containing volcanic ashes. In the investigated area, high temperatures were observed in fumaroles and geothermally heated soils occurring all over the island [7]. These geothermal oases are probably the source of *Geobacillus* sp. cells for the surrounding permafrost rocks. This work is the first investigation of the microorganisms of ancient thermal ecosystems preserved in permafrost sedimentary rocks. The results obtained indicate that thermophilic microorganisms of the geothermal communities are able to survive for considerable periods of time in permafrost rocks; however, the maximal duration of such cryopreservation is to be determined.

The most ancient permafrost volcanic sediments discovered on Earth (4–15 Ma) [17, 18] are an analogue of the most recent volcanic sediments on Mars, which are also represented by permafrost volcanic rocks (1–10 Ma). Permafrost volcanic sedimentary rocks seem to be the most promising objects for search for living organisms on Mars [20, 21], which stirs up interest in studying the microbiota of their terrestrial counterparts.

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