Kriosfera Zemli, 2011, vol. XV, No. 4, pp. 79–87 **http://www.izdatgeo.ru**

BIOGEOCHEMISTRY OF PERMAFROST IN CENTRAL YAKUTIA

A.V. Brouchkov[1], V.P. Melnikov[1], M.V. Schelchkova[2], G.I. Griva[3], V.E. Repin[4], E.V. Brenner[4], M. Tanaka[5]

[1] *Tyumen State Oil and Gas University, 56, Volodarskogo str., Tyumen, 625000, Russia; brouchkov@hotmail.com*
[2] *Ammosov North-East Federal University, 58, Belinskogo str., Yakutsk, 677000, Russia; rector-svfu@ysu.ru*
[3] *Tyumen Science Center, Siberian Branch of the Russian Academy of Sciences, 86, Malygina str., Tyumen, 625000, Russia; brouchkov@hotmail.com*
[4] *Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, 8, Akad. Lavrentieva, Novosibirsk, 630090, Russia; niboch@niboch.nsc.ru*
[5] *Hokkaido University, Kita 8, Nishi 5, Kita-ku, Sapporo, 060-0808, Japan; kouryu@general.hokudai.ac.jp*

Permafrost is widespread in the Northern Hemisphere and is as old as hundreds of thousands to millions of years. Frozen ground stores living microorganisms which remain unfrozen in the relatively warm environment (–2...–8 °C) but are immobilized and may be about the age of the host permafrost. A strain of *Bacillus* sp. was isolated from ~3 Ma permafrost and its 16S rDNA sequence was identified. A large group of microorganisms including fungi was isolated from the wedge ice. Permafrost deposits contain invertase, urease, katalase and dehydrogenase.

INTRODUCTION

Biochemical activity of permafrost and existence of microorganisms in it were discovered quite long ago [*Lozina-Lozinsky, 1972; Friedmann, 1994*]. In the late 1970s, bacteria, fungi, diatoms, and other organisms were discovered at the Antarctic Vostok station [*Abyzov et al., 1979*]. There have been reports of bacterial metabolism in low-temperature environments [*Clein and Schimel, 1995*] and of microbial life that survived at subzero temperatures in ancient deposits [*Ashcroft, 2000; Nicholson et al., 2000; Katayama et al., 2007*]. Microorganisms are stable against freezing and can bear it easily [*Lozina-Lozinsky, 1972*]. On the other hand, it is known that a part of water (over 10 %) in various materials can remain unfrozen at temperatures below –20 °C [*Brushkov, 1995*].

Bacteria can keep viable for a long time. The survival time of *Anthrax bacillus* spores was estimated at about 105 years [*Repin et al., 2008*]. Colonies were cultured from bacteria found in 40 Ma amber [*Greenblatt et al., 1999*]. However, the evidence these sporadic finds can provide for exceptional tenacity of microbial life is less reliable than that from purposive studies of permafrost. Permafrost occupies an enormous area worldwide and reaches 65 % of the territory in Russia. Its temperatures range most often between –2 and –8 °C and the age is locally as old as millions of years [*Ershov, 1988*].

At present there has been no proof that microorganisms would be capable of growing in permafrost where the growth is impeded by cell immobility, shor nutrients, and poor conductance of water films. Aging cultures stop growing even in laboratory. Water crystallization and arrest of metabolism reduces the growing capacity [*Lozina-Lozinsky, 1972*]. Unfrozen water in –2 and –4 °C permafrost exists in very thin (0.01–0.1 μm) films, far below the size of microorganisms. These channels are virtually unfit for life support, and any significant migration of cells is hardly possible. That is why bacteria in permafrost must be relict forms, their age being constrained by their stratigraphic position, freezing history, and data on optic aminoacid isomers; further implicit constraints are from biodiversity.

The tenacity of microorganisms in permafrost remains puzzling. Fossil DNA of mummies, mammoths, amber-entrapped insects, and other organisms turn out to be destroyed. The theory predicts that even small DNA fragments (100–500 nucleotides) can preserve no longer than 10 kyr in a normal climate and 1 myr the longest in cold areas [*Willerslev and Cooper, 2005*]. There may exist some mechanisms that prevent bacterial DNA from accumulation of defects. We describe some biogeochemical features of permafrost in Yakutia, mostly with the example of Mamontova Gora (Russian for *Mammoth Hill*) outcrop in Aldan and report some preliminary data on the discovered organisms.

MATERIALS AND METHODS

Samples for biogeochemical studies of frozen ground were collected from outcrops and underground structures at several sites. One is the Mamontova Gora (*Mammoth Hill*) site located on the left bank of the Aldan River, 325 km upstream of its inflow into the Lena.

The river incision exposes Neogene-Pleistocene (from 16 Ma to a few kyr) alluvium deposited during Pleistocene glaciations. Neogene sand is frozen; permafrost is shallow or exposed at eroded surfaces.

Judging by the cryostructure, Neogene sediments froze up after their deposition and lithification. Middle Pleistocene sand shows signs of syngenetic (syndepositional) freezing: ice and soil veins at different levels. Therefore, permafrost may have existed continuously through the Pleistocene and, hence, the microorganisms found there must be very old. Neogene-Middle Pleistocene sand lies under up to 7–10 m thick Upper Pleistocene loam and silt which enclose wedge ice.

Sampling was from upright bluff walls, at depths 0.9–1.0 m below the active layer (which in the conditions of continuous erosion was no thicker than a few centimeters). Given that the outcrop is being rapidly eroded by the river (at least several meters annually), the sampled sediments appear to have been perennially frozen. Yearly spring floods take the slumped ground away, which prevents sediments from obstruction, deformation, and repeated freezing. The sediments, consisting of Middle Miocene (10–12 Ma) fine sand [*Baranova et al., 1976*], appear to have been cooled and frozen up in the latest Pliocene, about 3–3.5 Ma ago [*Bakulina and Spektor, 2000*] and never thawed later on because of the cold Yakutian climate. According to regional paleoclimate reconstructions, mean annual air temperatures through the Pleistocene were from –12 to –32 °C in winter and from +12 to +16 °C in summer [*Bakulina and Spektor, 2000*]. Thus, the age of permafrost at the Mammoth Hill site may reach 3.5 Ma. More samples were collected from younger wedge ice in Yakutia, from an underground gallery of the Institute of Permafrost (Yakutsk), as well as from frozen ground on the left bank of the Lena at the Neleger observatory site.

Permafrost sampling was with all precaution possible in the field. The microstructure of frozen ground was examined in 4–5 kg monolith blocks, and the samples for microbiological studies were about 50 g. Sampling was performed with metal instruments sterilized in ethanol and flame. The collected samples were sterile packed and transported in thermostatic containers with coolants at –5 °C, i.e., close to the natural conditions.

The physicochemical properties of the samples were determined by means of common procedures for soils: Turin wet combustion for total organic content (TOC) and humus; gasometry for carbonates; potentometry for pH; gravimetry for field water content; and Kachinsky procedure for grain-size composition. The activities of soil hydrolase enzymes (invertase, urease, and phosphatase) and oxidoreductases (dehydrogenase and katalase) were measured following the standard procedures [*Schelchkova, 2009*]. Aliquots for analyses were from samples that were powdered in the frozen state and mixed. Organic remnants were not removed. The analyses were run in triplicate. See Table 1 for the list of samples analyzed for enzymatic activity and Table 2 for the physicochemical properties of sediments of the 50-m high Mammoth Hill terrace.

Diluted samples of different percent concentrations were placed, under sterile conditions, to Petri dishes with YPD, MRS, and NA environments, as well as in meat-extract broth, in anaerobic and aerobic conditions.

For the sequence analysis, DNA was extracted using a Fast DNA kit for soil (BIO 101 Inc., Vista, CA). The 16S rRNA genes were amplified by a polymerase chain reaction (PCR) with primer sets specific to bacteria. The PCR process was run in 20 μl using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA); the amplicons were subject to electrophoresis and cleaning with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison,

Table 1. **Description of samples. Dating according [*Markov, 1973*]**

Sample No.	Age	Description
2	Upper Pleistocene	Subaerial loam, 20 cm above ice wedge-bearing sediments. Dense loam with abundant plant roots and plant remnants, frozen, light brown, plastic on thawing. Wavy-bedded cryostructure; ice lenses 1–3 mm thick, exposed ice content about 50 %
3	Upper Pleistocene	Ice wedge-bearing sediments, ~1 m below loam base
5	Middle Pleistocene	About 8 m downslope from hill top, 10 m far from No. 6 sampling location. Exposed gray sand with light gray interbeds. Massive structure, with dark spots of organic matter. Approximate age 150 kyr BP
6	Middle Pleistocene	Left valley side where proximal outcrop is located, before proximal cirque; upper slope part, 10 m downslope from hill top. Gray loam, frozen, with necrons, including green stalks. Massive cryostructure, with ~ 1 cm thick ice lenses. A Middle Pleistocene ice vein, rather distinct on scraping, with a soil vein above it. Approximate age 150 kyr BP
7	Middle Pleistocene	Trench, middle terrace slope, below outcrop where Nos. 1–6 were sampled, about 25 m upslope from water table. Exposed gray medium-grained sand, with alternating plane and cross bedding, lying over a pebble bed. Age of sediments above pebbles 300 kyr BP
8	Miocene	Beginning of 50-m terrace, about 90 m far from the left-hand side of the Aan-Appa Brook mouth, downstream Aldan River. Exposed Neogene sand. Trenching about 25 m upslope from water table. Gray sand, interbedded dark gray and yellowish medium to coarse sand. Cross bedding mixed with plane bedding, abundant "unconformities". A pebble bed, up to 20 cm thick, with fossil wood material above it, wherefrom the sample was collected. Approximate age 12 Ma

Table 2. **Physicochemical properties of 50-m terrace deposits at Mammoth Hill site**

Sample	pH aqu.	TOC, %	Humus, %	$CaCO_3$, %	Field water content, %	Hygroscopic moisture, %	Specific weight	Number of particles of size (mm)					Total	
								1–0.25	0.25–0.05	0.05–0.01	0.01–0.005	0.005–0.001	<0.001 mm	<0.01 mm
2	8.02	1.66	2.86	4.76	60.26	0.24	2.58	0.7	13.5	42.9	12.2	13.5	17.2	42.9
3	8.14	1.47	2.53	2.20	72.83	2.38	2.48	0.6	7.5	44.5	15.5	13.5	18.4	47.4
5	7.83	0.69	1.19	0	24.97	0.22	2.62	54.7	22.8	8.5	1.7	1.6	10.7	14.0
6	8.10	1.27	2.19	0	75.12	1.81	2.50	7.8	9.7	33.0	12.3	14.7	22.5	49.5
7	7.63	0.04	0.07	0	29.79	0.11	2.70	71.0	26.1	1.0	0.4	0.6	0.9	1.9
8	4.84	1.25	2.16	0	32.60	0.08	2.63	69.3	21.0	3.5	1.2	2.3	2.7	6.2

USA). The purified amplicons were cloned into the pCR2.1 vector and *E. coli* culture with the TA cloning kit (Invitrogen) following the recommendations of the manufacturer. A 16S rDNA-bearing plasmid DNA was prepared from an overnight culture with the Mini prep spin kit (Quiagen, Crawley, UK). Purified plasmid DNA was sequenced on an ABI PRISM 3100 Genetic Analyzer with the Big Dye Terminator cycle-sequencing kit (Applied Biosystems). The sequence length reached 1488 bp. The sequence was compared with similar sequences of reference organisms by BLAST search. A phylogenetic tree was constructed with CLUSTAL W.

SPORE-POLLEN ANALYSIS

The frozen Pleistocene deposits of the 50-m terrace of Mammoth Hill contain abundant tree and shrub pollen assemblages (50.8 %) with almost equal proportions of conifer (26.0 %) and small-leaved (24.8 %) plants. Among gymnosperms, highest percentages are of *Larix* (15.6 %) while other species have progressively lesser percentages: 6.8 % *Pinus sylvestris*, 2.0 % *Pinus pumila* and 1.4 % *Picea* (*P. obovata* and *P.* cf. *ajanensis*). The small-leaved plants are mostly shrubs: 13.0 % *Alnaster*, 4.2 % *Betula exilis*, and 1.4 % *Salix*. Tree birch pollen is within 6.2 % (*Betula* sect. *Albae*, *B. platyphylla*). Grass-subshrub assemblages constitute 48.6 % of pollen, with highest abundances of *Artemisia* (41.1 %), a typical representative of dry steppe environments. Generally, comparison between fossil and extant spore-pollen spectra has been ambiguous. Some samples record deposition in a drier and, possibly, colder climate while others appear to be deposited in a warmer and wetter environment relative to the present one.

ENZYMATIC ACTIVITY

The enzymatic activity of ancient permafrost was investigated in comparison with that of present cryosols, for nine common soil types of the middle-taiga subzone of Central Yakutia [*Schelchkova, 2009*] including forest (lessive) and meadow (chernozem-like, sod-gley, and alas) cryosols.

The present soils possess the entire hydrolase and oxidoreductase range we studied (Table 3). The presence of hydrolyzing enzymes of carbohydrate, nitrogen, and phosphoric metabolism is evidence of digestion and enrichment in simple sugars and mineral compounds of phosphorus and nitrogen. The topsoil (A1) horizons of cryosols are rich in invertase. Urease activity being more variable, the frozen soils may be of low, medium, or high urease enrichment. Unlike the hydrolytic enzymes, dehydrogenase, is involved in redox reactions of biogenesis in humus, along with phenoloxidase enzymes, and in a way reflects the intensity of humification in soils. Dehydrogenase in the cryotic soils of Central Yakutia is of low or medium contents. Thus, the present soils show signs of all main reactions of organic compounds (breakdown and humification) typical of soil formation processes.

Catalase activity is an indicator of biological activity in soils. Soil catalase, as well as the intracellular one, is important as it decomposes hydrogen peroxide which is toxic for living organisms. Catalase activity in soils normally depends on the total organic content (TOC) and amount of microorganisms. Cryosols most often have low or medium catalase enrichment, and the enzymatic activity decreases downward proportionally to progressive decrease in TOC and biogenic components. Invertase, urease, and catalase are present throughout the soil profile while phosphatase and dehydrogenase activity is restricted to A1 topsoil (Table 3). As to dehydrogenase, the latter fact characterizes its function and indicates that humification reactions are especially active in the A1 horizon. The presence of phosphatase in A1 only is evidence of slow phosphoric metabolism. Cryosols are generally poor in mineral phosphorus, and in Central Yakutia they have low or medium enrichment in hydrolytic and redox enzymes, which are most active in topsoil.

The data we obtained were used to calculate enzymatic activity statistics for three horizons (A, B and BC (C)) in cryosols: arithmetic means, variance limits, and confidence intervals (Table 4). It is reasonable to use the statistics for comparing modern

Table 3. **Enzymatic activity of cryosols, Central Yakutia**

Horizon	Depth, cm	Invertase, mg glucose/(g·hr)	Urease, mg NH_4^+/(g·24 hr)	Phosphatase, mg FF/(g·hr)	Dehydro-genase, mg TFF/(10 g·24 hr)	Catalase, ml O_2/(g·min)
Lessive high-effervescent cryosol						
A	0–5(9)	0.57	1.36	0.576	3.860	1.3
B	28–38	0.25	0.30	0	1.244	1.1
BC_{ca}	50–70	0.27	0.01	0	0	0
Lessive solodic cryosol						
A	0–6	2.62	37.77	8.98	–	11.42
B	10–30	0.45	3.86	0.97	–	0.55
BC	50–100	0.03	1.58	0	–	0.14
Alas tussock-sapropel-gley soil						
A	0–22	0.55	10.81	14.91	–	5.04
LD_1	22–40	0.29	8.45	0	–	0.81
LD_2	40–68	0.26	7.66	0	–	1.09
Alas tussock-meadow soil						
LD_1	0–23	1.34	13.45	0.81	–	3.07
LD_2	23–40	0.09	9.19	0	–	0.79
B_{ca}	50–110	0.33	0	0	–	0.33
Alas steppe soil						
LD_1	0–19	0.81	2.06	0.88	–	4.95
B_{ca}	19–103	0.42	0.77	0	–	0.07
Chernozem cryosol						
A	0–19	2.95	5.00	5.25	4.15	1.60
B	47–72	0.74	0.40	0	0	0.69
C	72–102	0.49	0.20	0	0	0.54
Meadow-chernozem cryosol						
A	0–12(14)	4.23	2.86	3.37	3.00	2.54
B_{ca}	45–80	0.81	0.22	0	0	0.57
C	80–100	0.55	0.10	0	0	0.13
Tussock-gley cryosol						
A	3–18	3.03	1.16	1.27	2.75	1.58
B_g	58–70	0.21	0.36	0.49	0	0.40
C	70–90	0.23	0.25	0.43	0	0.20
Meadow-to-forest transitional cryosol						
A	2–21	3.29	1.31	5.58	1.70	2.27
B_{ca}	37–78	0.65	0.15	0	0	0.10
BC	80–100	0.35	0.12	0	0	0.03

frozen soil and ancient permafrost in terms of enzymatic activity. The 30 kyr to 12 Ma frozen sediments of the 50-m terrace bear signature of activity for some enzymes we studied (Table 5). Invertase activity shows up in all samples being the highest in Upper and Middle Pleistocene sediments. Its magnitude is commensurate with that in mineral horizons B of modern cryosols (Tables 4, 5). Urease activity has been found in three out of six samples (Nos. 2, 3, 5) from the Upper and Middle Pleistocene section, with its magnitude fitting the confidence interval of urease activity of B and BC horizons in modern cryosols of Central Yakutia (Table 6). Dehydrogenase activity appears in two of the six samples (Middle Pleistocene): it is quite low in No. 5 and rather high in No. 6 (3.225 mg TFF/(10 g · 24 hr)) fitting the confidence interval of that in modern topsoil. Catalase activity is present in Upper Pleistocene samples and one of three Middle Pleistocene samples, and is as low as in mineral horizons BC and in soil-forming sediments of modern cryotic soils. As for phophotase activity, it has not been observed in the fossil permafrost samples from the Mammoth Hill site.

Thus, invertase is the most persistent enzyme surviving in permafrost. Its activity shows up in samples of all ages from Miocene to Late Pleistocene. Urease, dehydrogenase, and catalase are less preserved being restricted to Upper and Middle Pleistocene deposits of some samples, while phosphatase lacks from fossil permafrost.

Table 4. **Statistics of enzymatic activity of cryosols in Central Yakutia**

Enzyme	Horizon	*n*	$M \pm m$	Lim	$M \pm tm$ ($p = 0.05$)
Invertase	A	9	2.535 ± 0.454	0.55–4.24	1.65–3.42
	B	9	0.499 ± 0.084	0.09–0.81	0.34–0.66
	BC (C)	8	0.314 ± 0.057	0.03–0.55	0.20–0.42
Urease	A	9	8.421 ± 3.959	1.16–37.77	0.66–16.18
	B	9	2.633 ± 1.233	0.15–8.45	0.22–5.05
	BC (C)	8	1.240 ± 0.935	0–7.66	0–3.07
Phosphatase	A	9	4.625 ± 1.598	0.58–14.91	1.49–7.94
	B	9	0.054 ± 0.054	0–0.49	0–0.16
	BC (C)	9	0.048 ± 0.048	0–0.43	0–0.09
Dehydrogenase	A	5	3.092 ± 0.435	1.70–4.15	2.24–3.94
	B	5	0.249 ± 0.242	0–1.24	0–0.74
	BC (C)	5	0 ± 0	0–0	0–0
Catalase	A	9	3.686 ± 1.085	0.70–11.42	1.56–5.81
	B	9	0.564 ± 0.055	0.07–1.10	0.36–0.77
	BC (C)	8	0.308 ± 0.127	0–1.09	0.06–0.56

Note. *n* – number of samples; $M \pm m$ – arithmetic mean; Lim – variance limits; $M \pm tm$ (at probability $p = 0.05$) – confidence interval.

Table 5. **Enzymatic activity of 50-m terrace deposits**

Sample No.	Geologic period	Invertase, mg glucose/(g·hr)	Urease, mg NH_4^+/(g·24 hr)	Phosphatase, mg FF/(g·hr)	Dehydro-genase, mg TFF/(10 g·24 hr)	Catalase, ml O_2/(g·min)
2	Upper Pleistocene	0.644	0.164	0	0	0.2
3	Upper Pleistocene	0.690	0.043	0	0	0.2
5	Middle Pleistocene	0.362	0.042	0	0.361	0
6	Middle Pleistocene	0.684	0	0	3.225	0.1
7	Middle Pleistocene	0.399	0	0	0	0
8	Miocene	0.155	0	0	0	0

In the stratigraphic profile of the 50-m terrace, the activity of invertase, urease, and catalase shows a generally decreasing depthward trend. Samples of fine grain sizes (loam) and rich in organic matter have higher enzymatic activity than in coarser sandy loam and sand (Table 6). There is high positive correlation between invertase and catalase activity and clay-silt fraction percentage (correlation coefficient r = = 0.769–0.911), as well as between catalase activity and TOC and humus contents ($r = 0.752$). Other positively associated attributes are invertase activity vs. humus and organic contents ($r = 0.445$–0.447), urease activity vs. TOC and silt fraction percentages ($r = 0.422$–0.525), and dehydrogenase activity vs. percentage of clay-silt fraction. This proportionality is due to the fact that enzymes in soils are most often immobilized, i.e., are bound with the surfaces of fine organic and mineral particles. Sorptive activity is the highest in mineral particles smaller than 0.001 mm (silt fraction and colloids, after Kachinsky) due to high dispersion and abundance of clay minerals and humus. Enzymes coming into soils are stabilized on soil minerals and organic matter by means of ion, hydrogen, and covalent bonds. The bonds with organic-mineral colloids can be very strong which, in turn, makes protein molecules resistant against unfavorable environment agents (e.g., microbial proteolysis) and maintains their long tenacity. Low temperatures in permafrost likewise arrest microbial activity and sustain the immobilized enzymes. According to our earlier thermodynamic studies of active invertase in buried soils from Upper Pleistocene deposits, inver-

Table 6. **Correlation relationships (r) of enzyme activities with some physicochemical properties of permafrost**

Parameter	Invertase	Urease	Dehydrogenase	Catalase
Clay-silt	0.910789*	0.422323***	0.478089***	0.882493*
Silt	0.872943*	0.376933	0.579961***	0.768905**
Fine silt	0.892061*	0.435522***	0.460784***	0.899808*
Humus	0.445122***	0.524052***	0.137730	0.751701**
TOC	0.447233***	0.525079***	0.137399	0.753424**

Note. r reliable to probability $*p \le 0.01$; $**p \le 0.05$; $***p \le 0.2$.

tase is either free or bound there, while the immobilized invertase has a high activation energy and thermal stability [*Ashcroft, 2000*]. Being a membrane-bound enzyme, dehydrogenase – unlike invertase, urease, and catalase – is not secreted by bacteria like extracellular hydrolases. Therefore, its presence in permafrost may be evidence of living or dead bacterial cells.

RESULTS: ISOLATION AND IDENTIFICATION OF MICROORGANISMS

Frozen Miocene sediments at the Mammoth Hill site contained a cultivable bacterium capable of both aerobic and anaerobic growth, the optimal growth temperature being +37 °C. It is a relatively large (1.0–1.5 × 3–6 μm) rod-shaped bacillus which develops chains in the culture (Fig. 1) and can form spores. It is immobile and gram-positive. It belongs to the *Bacillus* genus but is more likely a new species.

The 16S rRNA gene sequence of the bacillus reported in this study was deposited in DDBJ/EMBL/GenBank under accession number AB178889 and entry ID 20040510203204.24251. It is most closely related with *Bacillus simplex* and *B. macroides* which are 96–97 % homological with 16S rRNA.

Growth of bacteria at low temperatures was reported earlier [*Ashcroft, 2000*]. *Bacillus anthracis* is known to easily survive freezing [*Repin et al., 2008*], though the optimal temperature for its growth is rather high. Spores of *Bacillus* are especially resistant [*Nicholson et al., 2000*]. For instance, *B. thuringiensis* and *B. macroides* were found in amber with a radiometric age of 120 Ma [*Greenblatt et al., 1999*]. Therefore, the discovery of a viable bacillus in Miocene-Pleistocene permafrost at the Mammoth Hill site is not surprising. The capability of developing spores was known in gram-positive *Bacillus, Clostridium, Streptomyces*, etc. [*Nicholson et al., 2000*] and has been recently discovered also in gram-negative microorganisms. Thus, spore formation is a widespread survival mechanism which may involve horizontal gene transfer.

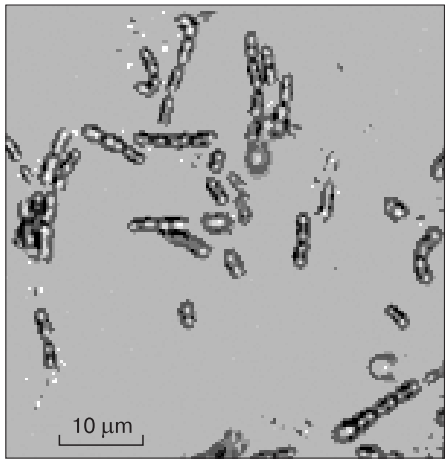

Fig. 1. Isolated strain *Bacillus* F (Gram coloration).

Furthermore, we cultured a number of other microorganisms from the Mammoth Hill permafrost (Fig. 2, 3) and extracted DNA from some cultures. The latter cultures were also exposed to the PCR process and 16S ribosomal DNA sequencing, whereby the following species were obtained: *Planococcus* sp. Tibet-IIVa1, *Arthrobacter sulfonivorans*, *Bacillus mojavensis*, *Jeotgalicoccus psychrophilus*, *Psychrobacter pulmonis*, etc. Some main biochemical properties of the isolates are listed in Table 7.

White mycelium has been found in an underground gallery of the Institute of Permafrost (Yakutsk) at a depth about 7 m below the ground surface. Similar mycelium was reported from the Fox Permafrost Tunnel in Alaska. The species (strain PF) was identified according to its morphology and 18S rRNA amplified gene sequence. It is closely related to *Penicillium echinulatum* and may be a new species. Samples of permafrost were pretreated, together with samples of strains *P. echinulatum* from the culture bank, and incubated at 25, 5, and –5 °C. The strains PF from permafrost and IFO 7760 and IFO 7753 *P. echinulatum* showed different patterns of spore formation and growth: PF was growing quite rapidly at –5 °C. Note that the isolates grew in Petri dishes at –5 °C, both in crystallized and supercooled (potato agar) media, more rapidly in the former. The strain *Penicillium echinulatum* isolated from permafrost beneath the Permafrost Institute in Yakutsk may be extant and brought from the surface, though being well adapted to the cold and feeding conditions of the underground gallery. Furthermore, this species can grow in aerobic conditions only, and its ability of growing in permafrost appears not very realistic.

Much younger (25–40 kyr) wedge ice samples from Yakutia and Alaska contained tens of microorganism species isolated with the same procedure [*Katayama et al., 2007*]. Most of those bacteria were gram-positive and closely related to *Arthrobacter* and *Micrococcus* spp. while mycelium was affiliated with *Geomyces* sp.

In this respect it is noteworthy that the life span of the most stable variola virus agrees with the estimates we obtained (several hundreds of years) [*Repin et al., 2008*]. Of special interest are obviously the microorganisms that long remain preserved in cold natural environments [*Friedmann, 1994; Katayama et al., 2007*]. Their longevity may be maintained by combinatorial transformations predicted before [*Repin et al., 2008*], possibly, somehow with participation of biocatalytic ribozymes which are stable and active at subzero temperatures. One may explain the great diversity of relict microorganisms isolated from the 25–40 kyr wedge ice [*Katayama et al., 2007*] in terms

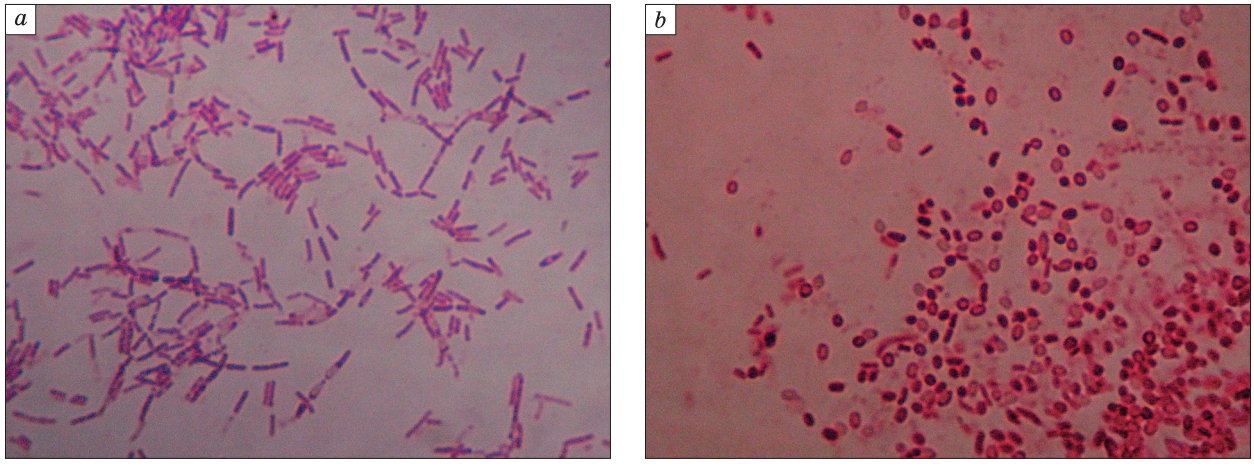

Fig. 2. Cell morphology of bacterial strains 17 (*a*) and 40 (*b*) isolated from frozen Neogene sand at Mammoth Hill (Gram coloration, magn. ×1500).

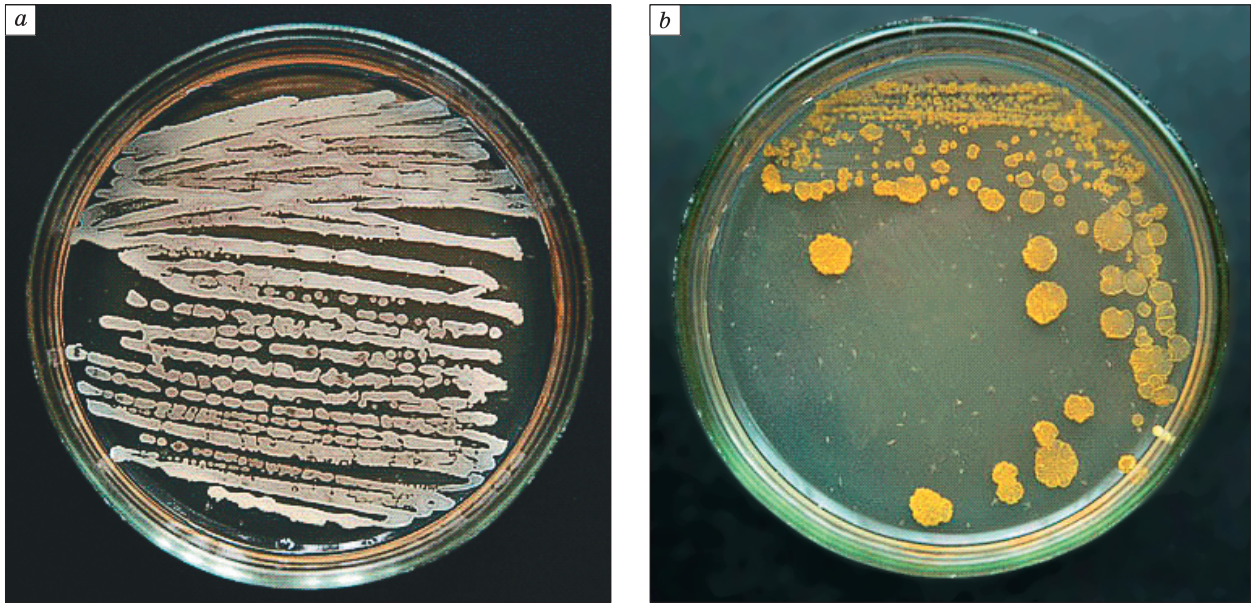

Fig. 3. Colonies of bacterial strains 6 (*a*) and 27 (*b*) isolated from frozen Neogene sand at Mammoth Hill.

of viability coding in plasmid-like mobile genetic structures.

Thus, the tenacity of microorganisms is hard to explain by retardation of life activity in anabiosis. If bacteria can form spores with almost arrested metabolism [*Nicholson, 2000*], the above calculations for denaturation, at least DNA, are especially relevant. The diversity of DNA damage mechanisms (metylation, desamination, apurinization, formation of thymine dimmers, cross-links, and breaks) implies, within the limits of the given hypothesis, revision to the assumed life time of biological macromolecules which must be ever shorter. The survival must be maintained by some special repair or conservation mechanisms.

CONCLUSIONS

1. A strain of *Bacillus* sp. was isolated from ~3 Ma permafrost at the Mammoth Hill site in Yakutia and its 16S rDNA sequence was identified; the same permafrost samples contained also other microorganisms. Younger permafrost (25–40 kyr) contained a large group of microorganisms including mycelium.

2. Low temperatures in permafrost arrest microbial activity and sustain immobilized enzymes. Permafrost deposits store invertase, urease, catalase, and dehydrogenase. The latter is a membrane-bound enzyme, is not secreted by bacteria into the environment, and its presence in permafrost may be evidence of living or dead bacterial cells.

Table 7. **Biochemical activity of strains isolated from fossil permafrost at Mammoth Hill site**

Parameters		Strains													
		6	13	15	17	20	27	29	30	32	33	34	37	40	F
Gram coloration		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pigmentation		yellow	–	–	–	–	–	–	–	yellow	–	yellow	–	–	–
Spore:	shape	–	S, E	S, E	E	E	E	E	E	–	–	–	–	E	E
	position	–	C, T	C, T	C	C	C	C	C	–	–	–	–	C	C
	inflated sporangium	–	+/–	+/–	–	–	–	–	–	–	–	–	–	–	–
Mobility		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth		+	–	–	–	+	+	–	–	+	–	+	+	+	+
Catalase activity		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase activity		+	+	+	+	+wk	+wk	+wk	+	+	+wk	+wk	+wk	–	+wk
Foges–Proskauer test		–	+	+	+	–	–	+	+	–	+	–	–	–	–
Citrate use		–	+	+	+	+	–	+	+	–	+	–	–	+	–
Nitrate reduction		+	+gas	+gas	+gas	+	+	+gas	+gas	+gas	+gas	+gas	+gas	+	+gas
Hydrolisis	casein	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	gelatine	–	+	+	+	+	+	+	+	+	+	–	–	+	+
	amylum	–	–	–	–	–	–	–	–	+	+	+	–	–	–
Acid formation from	glucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	mannite	–	+	+	+	–	–	+	+	–	+	–	–	–	–
	arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	lactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	mannose	–	+	+	+	+wk	+	+	+	–	–	+wk	–	+	+
	sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at	2 °C	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	8 °C	+	+	+	+	+	+	+	+	+	+	–	+	+	+
	43 °C	+	+	+	+	+	+	+	+	–	+	–	–	+	+
	6.5 % NaCl	+wk	+wk	+	+	–	–	+	+	–	+	–	–	+	+wk
	10 % NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	15 % NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	pH 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	pH 5	–	+wk	+wk	+wk	–	–	+wk	–	+	–	–	–	+wk	+wk
	pH 5.5	+	+	+	+	+	+	+	+	+	+	+	–	+	+
	pH 8.5	+	+	+	+	+	+	+	+	–	+	+	+	+	+
	pH 9	+	+	+	+	+	+	+	+	–	+	+	+	+	+
	pH 10	–	+	+	+	+	+	+	+	–	+	–	–	+	+
	pH 10.5	–	+	+	+	+	+wk	+	+	–	+wk	–	–	+	+
	pH 11	–	+	+	+	+	+wk	+	+	–	+wk	–	–	+wk	+wk
BEA formation:	ammonia	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	indole	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H_2S	+	–	+	+	+	–	+	+	+	+	+	+	–	+

Note. S – spherical spore; E – elliptic spore; C – centric position of spore; T – terminal position of spore; minus – negative reaction, plus – positive reaction, +wk – weakly positive reaction.

We wish to thank academician V.V. Vlasov (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk) for overall scientific support and discussions. Thanks are extended to people from the Ministry of Environment of Sakha Republic (Yakutia) who helped us with the field work.

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Received
10 February 2011