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GENERAL BIOLOGY

Discovery of Viable Methanotrophic Bacteria in Permafrost Sediments of Northeast Siberia

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Permafrost regions occupy some two thirds of Russia. It was found that fine-dispersion permafrost rocks are specific natural depositories of viable aerobic and anaerobic microbial communities [1, 2]. It should be noted that, the total count of microorganisms in permafrost sediments is only one order of magnitude less than in tundra soils. Moreover, there is evidence that because of the presence of unfrozen water strongly bound to soil particles, the metabolic activity of cold-adapted microorganisms is maintained at a sufficiently high level. For example, it was shown that metabolic activity of some microorganisms is observed at subzero temperatures as low as –17°C [3, 4]. The presence of carbon-containing gases, CH_4 and CO_2, in permafrost rocks is conventionally though to be due to the microbial activity in soil during the period preceding its freezing. If permafrosts were thawed as a result of global climate warming, viable microbial communities would be actively involved in various biogeochemical processes, including evolution and uptake of greenhouse gases (CH_4 and CO_2). The amount of greenhouse gases should depend on the ratio of methanogenic and methanotrophic components of paleomicrobial communities. However, neither the presence of methane oxidizing (methanotrophic) bacteria in permafrost nor their metabolic activity has been reported in the literature so far. The lack of reliable data induced us to carry out a special research using methods of molecular ecology.

We obtained the first experimental evidence for the existence of viable methanotrophic bacteria in permafrost sediments of northeast Siberia. Experimental samples were obtained from permafrost rocks by drilling. The drilling samples were handled meeting all necessary requirements intended to prevent contamination with foreign microflora [5]. The abilities to oxidize ^{14}C-methane to $^{14}CO_2$ and to assimilate radiocarbon were assayed. The methane concentration in the gas phase was 1 mM, and the specific radioactivity was 0.5 mCi/mmol. Flasks (15 ml) containing experimental samples (1 g) were stopped with rubber plugs and incubated at a constant temperature of +5°C or –5°C for 20 days. When incubation had been over, the radioactivity of the acid-labile and acid-resistant fractions was measured as described earlier [6].

In most samples studied in this work, the rate of $^{14}CO_2$ production from $^{14}CH_4$ was measured at an incubation temperature of +5°C. The maximum rate of methane oxidation was observed in sample 453/98 collected at a depth of 0.4 m (table). This layer is the upper permafrost boundary, and it is subjected to regular thawing in warm years. In contrast, the rate of methane oxidation in samples collected in deep permafrost layers (depth, 2–50 m; age, 10 000–200 000 years) was one to two orders of magnitude lower and was independent on soil layer depth or permafrost age within the ranges noted above. Incorporation of radiocarbon into acid-stable products of cells evidenced the process of methane assimilation. This process was observed only in four samples, and its rate was only 1/16 to 1/4 of the rate of methane oxidation.

It is interesting that eight samples oxidized methane at an incubation temperature of –5°C, with the methane oxidation rate by two samples being higher than that at an incubation temperature of +5°C. This finding can be regarded as evidence for the presence of methanotrophic bacteria capable of surviving in the overcooled state or the presence of unthawed intact cells.

The results of the study were compared with the polymerase chain reaction (PCR) assay of total DNA isolated from samples (1 g). The PCR analysis was performed using molecular markers of genes of membrane-bound (*pmoA*) and soluble (*mmoX*) forms of methane monooxygenase (MMO). The assay procedure described in [7] was used. The fact of the presence of these genes/enzymes in the majority of experimental samples supports the suggestion that the process of methaane oxidation by permafrost samples can be

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Methane consumption by permafrost samples from northeast Siberia and their PCR analysis

Hole no.	Depth, m	Age (thousand years)	Oxidation/assimilation of CH_4 (pmol per g soil per day)		PCR amplification with group-specific primers							
			+5°C	–5°C	*Methylomicrobium*	*Methylomonas*	*Methylococcus*	*Methylobacter*	*Methylosphaera*	*Methylocaldum*	*Methylocella*	*Methylosinus*/*Methylocystis*
453/98	0.4	up to 1	398/9	238/51	–	+	+	+	–	+	+	–
6/91	4.8	10	12/0	n.d.	+	–	–	+	–	+	+	–
″	6.3	10	0/0	n.d.	+	–	–	+	–	+	–	+
″	11.8	10	4/0	46/0	+	+	–	+	–	+	+	–
7/90	2.3	15–20	2/0	0/0	–	–	+	–	–	+	+	–
17/91	12.35	30	32/2	n.d.	+	–	–	+	–	–	–	–
14/99	8.4	20–30	30/2	30/0	+	+	+	+	+	+	–	+
″	26.7	100	61/15	27/3	+	+	+	+	–	+	+	–
15/99	6.7	20–30	6/0	0/0	+	+	+	+	–	+	+	–
″	14.2	100	8/0	11/0	+	+	+	+	–	–	–	–
″	23.4	100	12/0	5/0	+	+	+	+	–	+	–	–
16/99	10.9	100	5/0	0/0	+	+	+	+	–	+	+	+
1/98	16.0	1800–3000	n.d.	0/0	+	–	+	+	–	–	+	+
2/94	32	1800–3000	n.d.	55/0	+	–	+	+	–	–	–	–
″	50.3	1800–3000	n.d.	38/0	+	–	+	+	–	–	+	–

Note: Primers of functional genes *pmoA* and *mmoX* gave positive signals in all samples tested; n. d. not determined.

attributed to activity of methanotrophic bacteria. However, MMO genes were not found in rocks stored in permafrost for 1.8–3 million years. Perhaps, this was due to a significantly lower count of methanotrophic bacteria in these samples. The PCR amplification products of the corresponding sizes were obtained in these samples only after 40 days of incubation under aerobic conditions in the presence of methane (20% of the gas phase volume) at a temperature of +5°C and with addition of specially developed mineral medium of the following composition (g/l): NaH_2PO_4, 0.01; $MgSO_4$, 0.2; $CaCl_2$, 0.2; NaCl, 0.5; $NaHCO_3$, 0.3; KNO_3, 0.1; and 0.1 ml of the standard solution of microelements [8].

The qualitative composition of populations of methanotrophic bacteria in permafrost rocks was determined using the markers of genera of methanotrophic bacteria as primers for PCR amplification [7, 9]. Representatives of the genera *Methylomicrobium*, *Methylobacter*, and *Methylomonas*, belonging to type I, were found in almost all furrows of various ages and depths of deposition. Oligonucleotide markers specific to the genera *Methylosinus* and *Methylocystis* gave positive results only in a few samples. The lack of these bacteria, belonging to type II, in the majority of samples was quite surprising, because they are widely believed to be the most viable group of methanotrophs. The use of genetic markers of the genus *Methylocella* (acidophilic psychrotrophic methanotrophs [10]) gave positive results in 9 out of 15 samples tested. The primer specific to the methanotrophic bacteria of the genus *Methylosphaera* was amplified only with one DNA sample isolated from permafrost sample 14/99 (depth, 8.4 m; age, 20000–30000 years). Although bacteria of this genus are psychrophilic, they have been found so far only in Antarctic marine ecosystems [11], tundra soils, and taiga soils [7].

The use of molecular markers of DNA of thermotolerant methanotrophic bacteria of the genera *Methylococcus* and *Methylocaldum* gave positive result of PCR amplification in the majority of permafrost samples tested in this work [12]. This fact can be regarded as evidence for a high degree of preservation of bacterial cells under permafrost conditions. Because no genes typical of the genus *Methylocaldum* were found in DNA samples stored in permafrost for 1.8–3 million years, it should be concluded that the degree of preservation depends both on the properties of bacterial cells and on the duration of their cryoconservation.

It should be noted in conclusion that two enrichment cultures of psychrophilic methanotrophic bacteria

capable of growing at +5°C under methane atmosphere were isolated from permafrost samples 6/91 and 7/90. Light microscopy showed that chains of rodlike cells similar to *Methylobacter* and *Methylomicrobium* were dominant in one sample, whereas small coccoids (perhaps, *Methylococcus*) were dominant in another sample. These data were supported by the results of PCR assay.

Thus, the results of this study demonstrated that even after long-term storage in permafrost (from 1 thousand to 1.8–3 million years) methanotrophic bacteria are able to oxidize and assimilate methane not only at positive but also at negative temperatures. Therefore, methanotrophic bacteria are potentially active in permafrost ecosystems. In addition, studies of bulk permafrost revealed a broad range of diversity of methanotrophic bacteria, because representatives of almost all known genera of methanotrophic bacteria (including mesophilic, psychrophilic/psychrotrophic, and thermotolerant forms) were found in these samples. Additional molecular genetic and cultivation studies of methanotrophic communities of the cryosphere are required to clarify the taxonomic status and phylogeny of paleomethanotrophs. The mechanisms of their adaptation and survival under the conditions of long-term cryoconservation should also be studied by these methods.

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