Biogeochemistry of methane and methanogenic archaea in permafrost

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Abstract

This study summarizes the findings of our research on the genesis of methane, its content and distribution in permafrost horizons of different age and origin. Supported by reliable data from a broad geographical sweep, these findings confirm the presence of methane in permanently frozen fine-grained sediments. In contrast to the omnipresence of carbon dioxide in permafrost, methane-containing horizons (up to 40.0 mL kg^{-1}) alternate with strata free of methane. Discrete methane-containing horizons representing over tens of thousands of years are indicative of the absence of methane diffusion through the frozen layers. Along with the isotopic composition of CH_4 carbon ($\delta^{13}C$ $-64‰$ to $-99‰$), this confirms its biological origin and points to *in situ* formation of this biogenic gas. Using ^{14}C-labeled substrates, the possibility of methane formation within permafrost was experimentally shown, as confirmed by $\delta^{13}C$ values. Extremely low values (near $-99‰$) indicate that the process of CH_4 formation is accompanied by the substantial fractionation of carbon isotopes. For the first time, cultures of methane-forming archaea, *Methanosarcina mazei* strain JL01 VKM B-2370, *Methanobacterium* sp. strain M2 VKM B-2371 and *Methanobacterium* sp. strain MK4 VKM B-2440 from permafrost, were isolated and described.

Introduction

In the framework of global change studies in the last few decades, attention has been given to methane emission from high-latitude ecosystems. Biogenic methane is formed through a series of biochemical reactions performed by a specific group of strictly anaerobic methanogenic archaea (Ferry, 1993, 2001; Zinder, 1993). Their natural habitat in Arctic regions is tundra with its humid reducing conditions and a high content of organic matter favorable to anaerobic processes. Methane formation accompanied by emission from the surface is observed in modern tundra soils (Whalen & Reeburgh, 1990; Kotsyurbenko *et al.*, 1996, 2004; Wagner *et al.*, 2003) and bog-lake bottom deposits (Zimov *et al.*, 1997; Nozhevnikova *et al.*, 2001, 2003; Walter *et al.*, 2006). The rate of emission, ranging from 0 to 250 mg $C(CH_4)$ m^{-2}, depends on soil humidity, temperature and vegetation type (Christensen, 1993; Samarkin *et al.*, 1994; Reeburgh *et al.*, 1998; Worthy *et al.*, 2000) and provides for up to 7% of the global contribution of CH_4 to the atmosphere (Mathews & Fung, 1987; Cicerone & Oremland, 1988).

However, significant amounts of methane have been isolated from the biogeochemical cycle and are conserved below the seasonally thawed layer in the permafrost (Rivkina *et al.*, 1992; Gilichinsky *et al.*, 1997; Wright *et al.*, 1998), at an average depth of several hundred meters. A huge deposit of methane in permafrost is, consequently, a tremendous potential source of ancient CH_4. This methane occurs in discrete layers at various near-surface depths with negligible lithostatic pressure. Discontinuities in the methane distribution provide a record of changing conditions, favorable for anaerobic microbial activity, and indicate that methane is present in a bound nondiffusible form (Rivkina *et al.*, 2001).

Besides methane, viable anaerobic microorganisms, including methane-producing archaea, are also preserved in the permafrost (Rivkina *et al.* 1998)

Using radiolabeled substrates, $NaH^{14}CO_3$ and $Na^{14}CH_3CO_2$, it was shown that methane formation in Holocene frozen deposits may occur at subzero temperatures down to

−16.5 °C (Rivkina *et al.*, 2002, 2004). Deep below the surface (several hundred meters), methane is found in the clathrate form (Dallimore & Colett, 1995) and low temperatures and high pressures are believed to be required for its formation (Kvenvolden, 1993). In the present paper, we report on our studies on the methane found in near-surface permafrost contrasting with these thermobaric conditions. Unlike deep high-pressured methane hydrates, this methane could be liberated easily into the atmosphere and reinvolved in present-day turnover, should the permafrost degrade as a result of global warming.

Considering methane biogeochemistry in the permafrost, our specific goals were: (1) to establish patterns of methane distribution in the permafrost; (2) to determine the isotopic composition of methane carbon, i.e. the origin of the methane; (3) to isolate methane-producing archaea; and (4) to investigate the effect of long-term preservation of the methane-producing community in the permafrost on its metabolic activity.

Materials and methods

Sites and cores

Biogeochemical investigations were conducted in the north-eastern Arctic tundra (125–162°E, 68–72°N) between the Lena delta and the mouth of the Kolyma river (Fig. 1), located outside Russia's oil and gas basins. These are coastal lowlands, where summers are mild (average July temperature is 10 °C) and winters are extremely cold (temperature falls below −40 °C). Permafrost occurs throughout the area to depths of *c.* 600–800 m. In this study, we consider the upper 100 m of the late Cenozoic permafrost. Most of the studied horizons comprise terrigenous (alluvial, lacustrine and boggy) hydrocarbonate-calcium fine-grained deposits: sands, loams and sandy-loams with pH close to neutral. The marine layers are of the same textural composition and NaCl salinity.

The late Cenozoic section formed under severe climate conditions in the last few million years, when sedimentation occurred concurrently with freezing from below. It is generally composed of syngenetically frozen (syncryogenic) layers, whose permafrost age is taken to be equal to the age of the sediments. During this period, this permafrost reacted to climatic oscillations by aggradations in cold periods and thawing in warm periods. Such conditions alternated through the entire Pleistocene (Shvetsov & Dostovalov, 1959; Washburn, 1980). Warming followed by cooling resulted in refreezing of the thawed parts downward from the top. Thus, a monolithic frozen sequence was formed, firmly bound by ice, containing refrozen (epigenetically frozen) layers, in which the age of the permafrost is younger than that of the sediments and the never-thawed syncryogenic part.

In this cross-section, the following strata, differing in origin and age, determined by radiocarbon, paleomagnetic, palynological and paleontological analyses (Sher, 1974, 1997; Virina, 1997; Zazhigin, 1997; Schirrmeister *et al.*, 2002; Sher *et al.*, 2005) were studied:

(1) The lowermost sediments date back to the second half of the Pliocene, ~2–3 Ma.

(2) The overlying 50–60 m is of late Pliocene to early Pleistocene age, 0.6–1.8 Ma.

(3) Near the present-day coastline is a 20-m thickness of marine sediments, frozen after sea-level dropped at the end of the mid Pleistocene 120 kyr ago.

(4) Further inland, mid Pleistocene sediments, 200–600 kyr old, are spread sporadically and comprise syngenetically or epigenetically frozen terrigenous horizons.

(5) The upper part of the Cenozoic section consists of the 40-m-thick syngenetically frozen, never thawed late

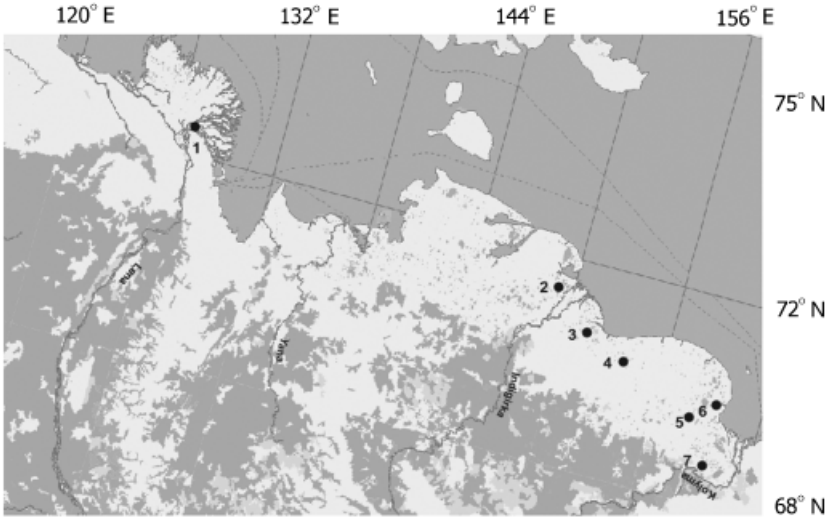

Fig. 1. North-east Arctic study sites: 1, Bykovskii peninsula, Lena river (Laptev Sea coast); 2, low-lying basin of Indigirka river; 3, Upper Khomus-Yuryakh river; 4, Alazeya river; 5, Chukochya river; 6, East Siberian Sea coast; 7, Khalarchinskaya tundra (sandy plain).

Pleistocene Icy Complex (12–60 ka), with deep polygonal ice wedges up to 10 m wide.

(6) Epigenetically frozen sands of the same age make up the river bed deposits.

(7) Holocene strata consist of 2- to 10-m-thick syngenetically frozen floodplain deposits (Holocene Icy Complex) and eolian sands, and permafrost sediments, comprising the top of the late Pleistocene Icy Complex, melted during the Holocene optimum, and refrozen 5–7 kyr ago. This 'covered' layer was formed by refreezing of the sediments that have melted and drained, and by a alas horizon consisting of similar deposits, overlain by bottom or peat soils.

Each of these strata has been investigated via boreholes over a wide area (Fig. 1). As each depth is represented by a single core sample, replication was achieved through the samples collected from the same geological strata in different boreholes, at different depths and at different sites. The data presented are based on ~100 boreholes and ~2000 gas-tested samples. The sampling step-interval in each borehole was 1.0 m.

Permafrost was sampled using a portable dry drilling rig that operates without fluids and prevents down-hole contamination. The strict protocols for drilling and the subsequent handling of cores are designed to ensure uncontaminated material is retrieved (Shi *et al.*, 1997). The corer (diameter 68–107 mm) cuts 30-cm-long cores. After removal from the corer, the surfaces of the cores were cleaned by shaving with an alcohol-sterilized knife. For microbiological studies the cores were split into *c.* 5-cm-long segments, placed into sterile aluminum boxes or plastic bags, stored in the field within a hole in the permafrost at a temperature of $-10\,^{\circ}C$, and transported frozen to the laboratory by air. During transportation, the samples were held in insulated containers with gel packs of Super Ice (Pelton Shepherd Industries) as refrigerant, maintaining them below freezing. In the laboratory, the samples were stored in a freezer at $-18\,^{\circ}C$ for 2–3 months until microbial analysis. At each step, the temperature was monitored by microloggers (StowAway XTI, Onset Computer Corp.). To monitor the possibility of contamination during the drilling process, several tests were carried out. Previous studies have employed fluidless drilling techniques combined with an exogenous bacterial tracer such as a pure culture of *Serratia marcescens*. In tests using the isolation techniques, *S. marcescens* was found only on the surface of the frozen sample, never inside the frozen cores (Shi *et al.*, 1997). Recently, this was confirmed by new methodology developed and tested in the Canadian High Arctic by Juck *et al.* (2005), using fluorescent microspheres and a green fluorescent protein-marked *Pseudomonas* strain. In the field seasons 2004–2006 we also used these microspheres to monitor sterility and did not find them in any cores.

Gas and physicochemical analyses

Gas samples were collected by degassing 50 g of frozen cores in a 150-mL syringe under nitrogen atmosphere. CH_4 concentration was measured by headspace-equilibration GC (Alperin & Reeburgh, 1985) using KhPM-4 (Russia) gas chromatographs with hydrogen-flame ionization detector and hydrogen as a carrier gas. In the same samples, the $\delta^{13}C$ of methane and carbon dioxide was analyzed on GC Combustion III Thermo Finnigan interface and Delta^{plus} XL mass spectrometer (Germany). Isotope ratios (‰) are given as δ values vs. the VPDB standard (Coplen, 1994): $\delta^{13}C(‰) = \left[\frac{(^{13}/^{12})\text{sample}}{(^{13}/^{12})\text{standart}} - 1\right] \times 1000$, where the $^{13}C/^{12}C$ absolute ratio is 0.0112372. The determination error of $\delta^{13}C$ was $\pm 0.2‰$.

To characterize the permafrost environment as a microbial habitat, along with sampling for microbial and gas investigations, the samples from the boreholes were collected for physicochemical (thermal state, ice content, textural structure and grading of soil, mineral and chemical composition) and geological analyses (dating, climatic and landscape reconstructions, etc.). After drilling, ground radiation and temperature measurements from top to bottom (1-m step-interval) were taken in the boreholes. The vertical temperature profile in the studied boreholes consisted of three layers:

(1) an active layer (0.5–0.8 m) that freezes in winter and thaws in summer;

(2) a layer of annual temperature fluctuations that diminished with depth to zero amplitude (~12–15 m);

(3) a layer with constant subzero temperature independent of season, i.e. mean annual temperature that varied in the studied area from -7 to $-8\,^{\circ}C$ in depressions to -11 to $-13\,^{\circ}C$ in watersheds.

Redox potential was measured in the field immediately after melting, using a 'Ecotest-120' (Russia) potentiometer with a platinum electrode and a silver chloride electrode as reference.

Estimation of methane production

To estimate hydrogenotrophic and acetoclastic methanogenesis, seven samples of different age (late Pliocene to Holocene) were aseptically pulverized using a knife; 5 g of frozen sample was placed into 20-mL sterile vials and purged with N_2. During this preparation, the temperature of the sample was raised to $0\,^{\circ}C$. Next, 100 μL of $NaH^{14}CO_3$ (18 Ci mol^{-1}) or $Na^{14}CH_3CO_2$ (40 Ci mol^{-1}) solution containing 10 μCi was injected into the vial. The vial was thoroughly shaken and placed in a refrigerated liquid bath (VMR Scientific Products) for incubation. Incubation temperature was -16.5, -10, -5, -1.8 and $5\,^{\circ}C$. The period of incubation was 3 weeks. Every sample was represented by seven replicate subsamples. After incubation, the sample was fixed

by injection of 10 mL of fixing solution: 200 g L^{-1} NaCl, 1 N KOH and 0.1 mL antifoam reagent (antifoam A concentrate, Sigma). All experiments for each temperature were carried out with five replicates of each subsample. Control measurements made in the first few hours after injection of the isotope allowed us to exclude the influence of defrosting and also the incorporation of ^{14}C in methane when the freezing condition had not yet been stabilized. A 'killed' control was created by injection of KOH (pH 12) directly after the labeled substrate was added. Counts per minute in the killed control did not exceed 250. Newly formed radioactive CH_4 was removed from the vials by an air flow (50 mL min^{-1}). It was passed through a drexel bottle with a fixing solution and combusted to $^{14}CO_2$ at 700–800 °C with cobalt oxide as catalyst (Laurinavichius & Belyaev, 1978). At the final stage, $^{14}CH_4$ oxidized to $^{14}CO_2$ was absorbed in the vial with a mixture of 2 mL β-phenylethylamine and 10 mL Universal LSC cocktail (Sigma). The complete removal time did not exceed 1 h. Vials were counted on an LS 5000 TD liquid scintillation counter (Beckman).

Microbial and phylogenetic analyses

Archaea were cultivated according the Hungate anaerobic technique (1969). A basal medium of the following composition was used (g L^{-1}): 0.29 K_2HPO_4, 0.29 KH_2PO_4, 1.0 NaCl, 0.2 $MgCl_2 \cdot 6H_2O$, 1.0 NH_4Cl, 0.1 $CaCl_2 \cdot 2H_2O$, 0.5 cysteine hydrochloride, plus 5 mL vitamin solution and 10 mL trace elements solution (Balch *et al.*, 1979). Single colonies were obtained by subsequent 10-fold dilutions in roll-tubes (Hungate, 1969) with basal medium containing 2 g L^{-1} Difco agar.

To obtain the enrichment cultures, ~10 g of Holocene and Pliocene permafrost samples were put into the 30-mL serum bottles, where 5 mL of basal mineral medium was added with H_2+CO_2 (4 : 1, v/v) or acetate (10 mM) as carbon and energy source. The Holocene sample (peaty-loam of lake-swamp origin), located at a depth of 80 cm below the permafrost table, is 2920 kyr old (Rivkina *et al.*, 2004). The late Pliocene samples (feldspathic sandy-loam of lake-alluvial genesis), located at a depth of 52 m from the day surface, are ~3 Myr old. The samples were incubated at temperatures of 6 and 20 °C. Methane production was measured weekly using chromatography. After methane concentration reached 40%, the entire content of the flask with the active methanogenesis was transferred into a Balch tube (25 mL), supplied with 5 mL of the basal medium with a gas mixture of H_2 and CO_2.

Pure cultures were obtained by subsequent 10-fold dilutions in roll-tubes. Single colonies were transferred into liquid medium. Pure culture of strain JL01 was maintained on the same mineral medium with casaminic acid (1 g L^{-1}). Media 141 and 506 (http://www.dsmz.de/microorganisms/media_list.php) were used for cultivation of strains M2 and MK4, respectively. CH_4 formation in the tubes was used for growth indication. Specific growth rate was calculated from the linear part of a half-logarithmic graph (CH_4 vs. time).

The growth of isolates was tested on acetate (50 mM), methanol (100–160 mM), monomethylamine (20 mM), dimethylamine (20 mM), trimethylamine (20 mM), formate (50 mM) and H_2+CO_2 (1 : 4). The effects of temperature on the growth rates of isolates were tested at −5, −2, 5, 10, 15, 24, 28, 37, 45 and 50 °C. Culturing at subzero temperatures was carried out in a cryobath with antifreeze (ethylene glycol).

Gram-staining was performed following standard protocols (Smibert & Krieg, 1994). Cell morphology was examined using ultrathin sections. Cells were prefixed with 1.5% glutaraldehyde in cacodylate buffer (pH 7.2) at 4 °C, washed three times in the same buffer, and refixed in a 1% solution of OsO_4 in the buffer at 20 °C. The sections were dehydrated in a series of alcohol solutions of increasing concentration (30%, 50% and 70%), embedded in Epson 812 epoxy resin, mounted on a grid, and contrasted for 30 min in a 3% solution of uranyl acetate in 70% ethanol and then with lead citrate (Reynolds, 1963) at 20 °C for 4–5 min. Ultrathin sections (500–700 Å thick) were examined in a JEM100 electron microscope (Japan).

Isolation of DNA from the biomass was carried out according to Marmur (1961). G+C content was assessed by thermal denaturation of DNA using a Pye Unicam SP1800 (UK) spectrophotometer. DNA–DNA hybridization was determined by reassociation (De Ley *et al.*, 1970).

Genomic DNA was extracted using the method of Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR using archaeal 16S rRNA gene primers 8F (5′-TCCGGTTGATCCTGCCGG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′). Additional primers for sequencing were 340F (5′-CCTACGGGRBGCASCAG-3′), 530F (5′-GTGCCAGCAGCCGCGG-3′) and A1041F (5′-GAGAGGWGGTGCATGGCC-3′). The PCR product was purified using a Wizard PCR Preps DNA Purificaton System (Promega). The sequencing reactions were performed using a CEQ Dye Terminator Cycle Sequencing kit and CEQ2000 XL (Beckman Coulter, USA) automatic DNA sequencer according to the protocols provided by the manufacturer. The nucleotide sequences of strains JL01 (1370 bp), M2 (1434 bp) and MK4 (1347 bp) have been deposited in the GenBank database under accession numbers AF519802, DQ517520 and EF016285, respectively.

The NCBI and RDP databases were used for phylogenetic analysis. Sequences were aligned using CLUSTAL X, version 1.64b (Thompson *et al.*, 1994). TREECON version 1.3b software (Van de Peer & De Wachter, 1997) was used to estimate evolutionary distances (Jukes & Cantor, 1969), and to build phylogenetic trees using the neighbor-joining method.

Results

Methane content and distribution

Figure 2 shows the methane distribution in the late Cenozoic strata of the north-eastern Arctic. Methane was found on the present-day floodplain bogs, Holocene 'covered' layer and alas horizons (sites 1–7), mid Pleistocene marine deposits (site 6), late Pliocene to early Pleistocene (sites 3–5 and 7) and Pliocene (sites 1, 3–6) suites. The lowest concentrations of methane (< 0.01 mL kg^{-1}) were found in the floodplains in virtually all river valleys in the area, in Holocene eolian and late Pleistocene river-bed sands (site 7), and in the Icy Complexes of both ages in different locations in the region (sites 1–2 and 4–6) with the exception of the many-tiered mid Pleistocene Icy Complex on the Khomus-Yuryakh river (site 3).

The late Pleistocene Icy Complex is found beneath the Holocene 'covered' layer or alas horizons. Methane was found in Holocene samples only, while underlying Pleistocene deposits and pure ice were either free of CH4 or contained methane at trace level concentrations. At the transition from the mid (Fig. 2, site 2) or late Pleistocene (Fig. 2, sites 1, 3–6) Icy Complexes to the underlying late Pliocene to early Pleistocene methane-rich suites of more than 50 m thickness, the CH4 concentration increased sharply and varied between 0.8 and 30.0 mL kg^{-1}.

The methane concentration also increased sharply from trace levels to 28.0 mL kg^{-1} at the transition to these suites from the late Pleistocene river-bed sands (Fig. 2, site 7) and from the Icy Complex to the marine sediments (Fig. 2, site 6). By contrast, when alas horizons overlay marine deposits or late Pliocene sands underlay late Pliocene to early Pleistocene or marine sediments (Fig. 2, sites 3–6), the methane concentration did not change and remained at 2–17 mL kg^{-1} in all layers. When Holocene floodplain sediments overlay the late Pleistocene Icy Complex or when the latter overlay the mid Pleistocene complex, the CH4 concentration was again continuous, and was at just trace level.

The methane concentration did not appear to be correlated with any textural or chemical properties, organic matter content or sediment age. The methane content did

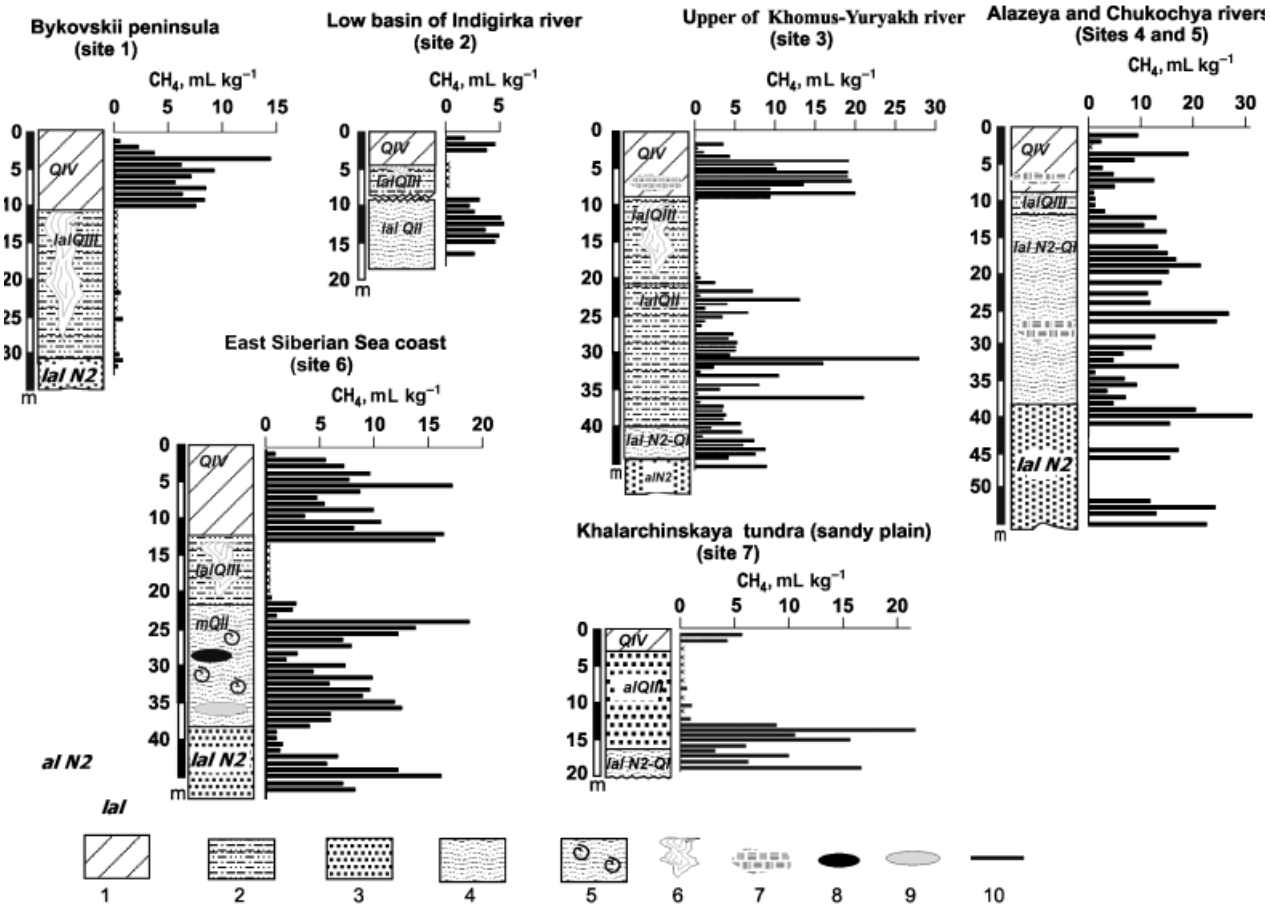

Fig. 2. Methane content in permafrost: 1, loam; 2, sandy loam; 3, sand; 4, loamy sands; 5, marine sediments; 6, ice wedges; 7, peat; 8, methane hydrate; 9, lenses of cryopegs; 10, methane concentration.

not exhibit any systematic trend with depth that would suggest a deep subsurface source or penetration downward from the surface. The CH4 distribution did, however, show a distinct and alternating pattern (Fig. 2, sites 1–7). In a generalized geological cross-section, in which the strata appear in chronological order, methane-containing layers are sandwiched between the layers free of methane (generally, the Icy Complexes).

Sedimentation was under reducing conditions, as indicating by the low redox potential (Table 1). The syncryogenic late Pleistocene Icy Complex free of methane differs by higher Eh values compared with the epicryogenic methane-containing layers.

Methane formation and composition

In the late Pleistocene Icy Complex samples, methane formation was not observed on either of the two substrates used and at either of the temperatures tested (above and below zero). Figure 3 shows the results for methane formation in two late Cenozoic samples of different age (Table 2).

In the late Holocene peaty loam sample (Fig. 3a), as incubation temperature decreased from 5 to $-16.5\,^{\circ}C$, metabolic activity also decreased from 15 to 0.03 μL $CH_4\,kg^{-1}$, and from 0.27 to 0.011 μL $CH_4\,kg^{-1}\,day^{-1}$ with radiolabeled bicarbonate and acetate as substrates, respectively. By contrast, in the ancient late Pliocene sandy loam sample (Fig. 3b), rates of acetate and bicarbonate methanogenesis were similar (about 0.026 μL $CH_4\,kg^{-1}\,day^{-1}$) and did not depend on the incubation temperature (-1.5 to $-16.5\,^{\circ}C$).

Table 1. Redox potential of permafrost sediments

Horizons	Eh (mV)
Holocene lake and swamp sediments, lQIV	48–228
Late Pleistocene Icy Complex, lalQIII2-4	192–294
Mid Pleistocene Icy Complex, lalQII	90–212
Mid Pleistocene, marine sediments, mQII	−40–120
Late Pliocene to early Pleistocene sediments, N2-lalQI	30–151

The isotopic composition of CH_4 carbon is shown in Fig. 4. Methane-containing layers can be separated into two groups: a main group containing horizons of different origin and age, from late Pliocene to Holocene, with $\delta^{13}C$ values ranging $-64‰$ to $-80‰$; and a minor group represented by mid Pleistocene epigenetically frozen terrigenous sediments on the lower Indigirka river (Fig. 2, site 2) with $\delta^{13}C$ values varying from $-90‰$ to $-100‰$.

Methanogenic isolates from permafrost

Active methanogenic enrichment cultures (40% of CH_4 in headspace) for Holocene and Pliocene samples were obtained after 6 and 12 months of incubation, respectively, only on H_2+CO_2 at 20 °C, although trace amounts of methane were also detected on acetate.

Three strains were isolated in pure cultures: strains JL01 and M2 from Holocene and MK4 from Pliocene sediments. Although CO_2+H_2 served as a favorable substrate for all enrichments, strain JL01 used only acetate (50 mM), methanol (160 mM), monomethylamine (20 mM), dimethylamine (20 mM) and trimethylamine (20 mM) as carbon sources, while the other two, strains M2 and MK4, grew exclusively on CO_2+H_2.

Strain JL01 always formed small aggregates in liquid medium (Fig. 5a). Microscopic observation has shown that

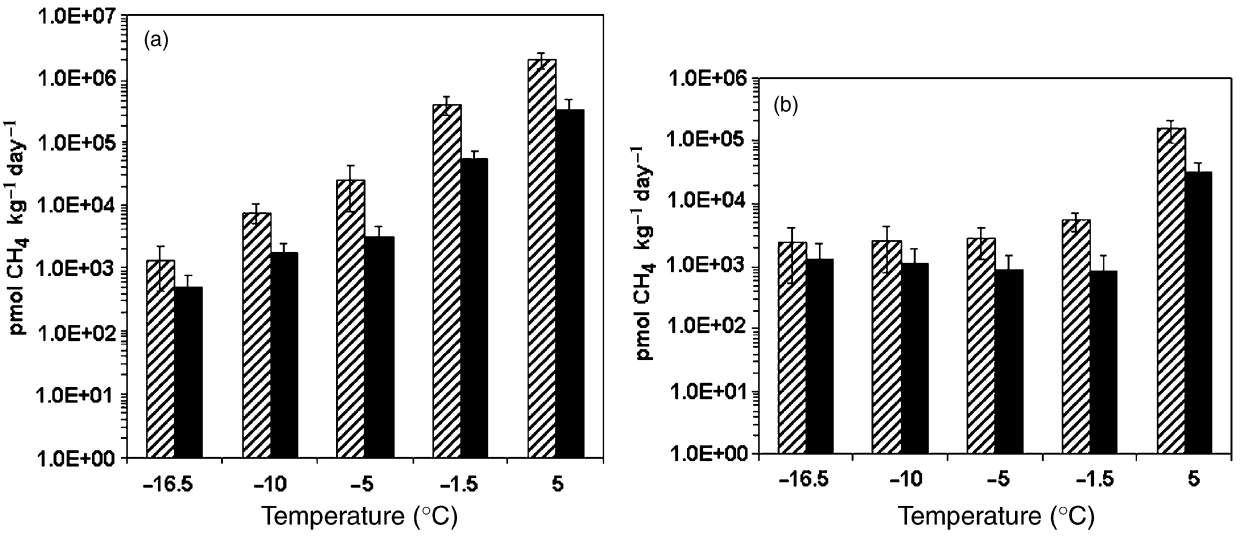

Fig. 3. Methane generation from $H^{14}CO_3^-$ (bars with diagonal lines) and $^{14}CH_3CO_2^-$ (black bars) at different temperatures in samples of Holocene (a) and early Pleistocene age (b); dotted line, killed control; error bar = SE.

this strain is similar to known methanosarcina like-cells (Fig. 5b). The cells stained Gram-positive. Electron-dense inclusions, possibly polyphosphates, which have often been observed in methanosarcinas (Mah, 1980; Mah & Boone, 1987; Sprott & Beveridge, 1993), were found in many cells. Table 3 shows growth rate results for strain JL01 on different substrates. Strain JL01 did not grow on the mixture of H_2 and CO_2, and the maximum growth rate was observed on methanol (0.079 h^{-1}) and on trimethylamine (0.067 h^{-1}).

Methanogenesis was observed at temperatures ranging from 10 to 37 °C with optimal growth at 24–28 °C (Fig. 6a). Optimal pH was 6.8–7.3, similar to that of sediment pore water (Fig. 6b). Optimal growth was observed at 0.45–0.60% NaCl concentration in the medium.

Phylogenetic analysis (Fig. 7a) showed that the isolate shared 99% homology to several *Methanosarcina mazei* strains, including the type strain of this species. DNA–DNA hybridization of JL01 and *Methanosarcina mazei* strain S-6 (DSM 2053) revealed a homology of 72% and, thus strain JL01 can be classified as a representative of *Methanosarcina mazei*. The DNA G+C content was 39.2 mol%, which is within the limits defined for strains of *Methanosarcina mazei* and *Methanosarcina barkeri* (38.8–43.9 mol%).

Strain M2 cells were nonmotile, slightly bent rods, 0.45–0.50 μm in diameter and 3.0–6.0 μm in length, often forming chains of rods and filaments more than 30 μm long. Cells divided through septum formation and stained Gram-negative. Often, cyst-like coccoid forms (Fig. 5c and d) with thickened cell walls were formed. Strain M2 used H_2–CO_2 and formate for growth and methane production (Table 3). Methanogenesis was observed at temperatures ranging from 15 to 45 °C (optimum at 37 °C, Fig. 6a), pH values of 5.5–9.0 (optimum at pH 7.0–7.2, Fig. 6b), and medium salinity (0.1–1.0%; optimum at 0.5%). The DNA G+C content of strain M2 was 40.0 mol%, which is within the limits defined for *Methanobacterium* sp. Phylogenetic analysis (Fig. 7b) showed that the new isolate shared 99% homology with *Methanobacterium bryantii* (AY196657) and 98% homology with *Methanobacterium ivanovii* (AF095261).

Cells of MK4 strain were nonmotile, crooked rods (Fig. 5e and f) with acuminate ends. Cells stained Gram-negative. The cells were 0.3–0.4 μm wide and 4–6 μm long. Strain MK4 produced methane only from a mixture of H_2 and CO_2 and was mesophilic. Growth and methanogenesis occurred at temperatures of 10–45 °C (Fig. 6a) with the optimum at 28 °C. Optimal pH values were at 7.2–7.4 (data not shown). Methanogenesis occurred in the NaCl concentrations of between 0.001% and 1.0% with the optimal concentration of 0.05–0.2%. The 16S rRNA gene sequence of strain MK4 was a stretch 1347 bp. Sequence similarity calculations after a neighbor-joining analysis indicated that the closest relatives of strain MK4 were *Methanobacterium* sp. strain M2 (99%) and *Methanobacterium bryantii* DSM 863^T (98%). The DNA G+C content of strain MK4 was 33.8 mol%.

The new methanogenic strains JL01, M2 and MK4 were deposited in Russian collection of microorganisms (VKM) under numbers VKM B-2370, VKM B-2371 and VKM B-2440, respectively.

Discussion

This study summarizes the methane content and distribution in permafrost layers of different age (from a few thousand to few million years old), origin (marine and terrestrial), depth (from the permafrost table at 0.5 m–100 m) and location, as well as indicates methane genesis using $\delta^{13}C$ isotopic composition. On the basis of ~2000 gas-tested samples collected from a broad geographical sweep

Table 2. Characteristics of permafrost sediments investigated for methane generation

Sample	Age ($\times 10^3$ years)	Ice content (%)	pH	C_{org} (%)	CH_4 (mL kg^{-1})	HCO_3^- (μmol kg^{-1})
(a) Peaty loam, QIV	2.9	164	5.1	9.5	5.6	100
(b) Sandy loam, N2-QI	2000	29	6.9	1.9	10.8	2200

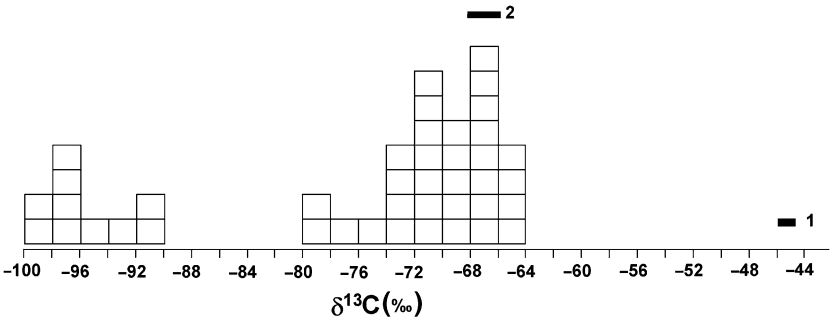

Fig. 4. $\delta^{13}C$ of methane from permafrost. Each small square presents one investigated sample: 1, atmospheric methane; 2, methane hydrate from permafrost in Makenthy delta (Dallimore & Colett, 1995), shaded squares, methane from terrigenous mid Pleistocene sediments (site 2, see Fig. 2); open squares, methane from horizons of different age and origin (sites 2, 3, see Fig. 2).

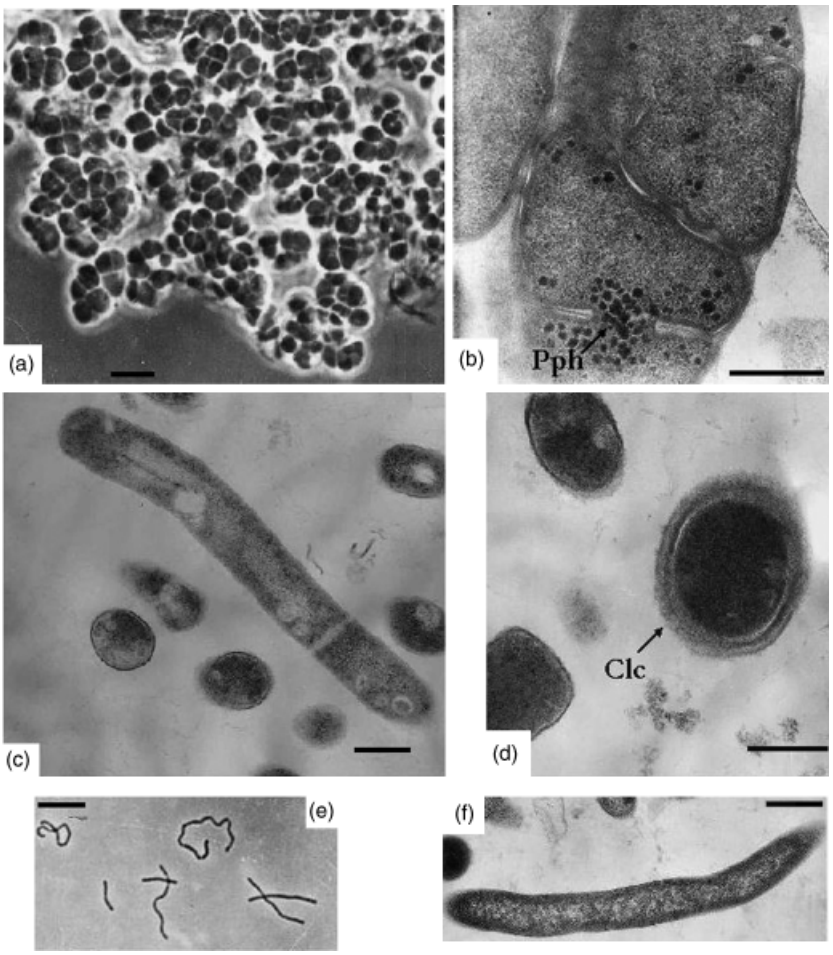

Fig. 5. Micrographs of methanogenic permafrost isolates. *Methanosarcina mazei* strain JL01: (a) phase contrast image, bar 10 μm; (b) ultrathin section, bar 0.5 μm. *Methanobacterium* sp. strain M2: (c) phase contrast image, bar 10 μm; (d) ultrathin section, bar 0.5 μm. *Methanobacterium* sp. strain MK4: (e) phase contrast image, bar 10 μm; (f) ultrathin section, bar 0.5 μm. Pph, polyphosphate inclusions; Clc, cyst-like cells.

(2000×500 km) from ~100 boreholes with a sampling step-interval in each borehole of 1.0 m these data confirm the presence of methane in permanently frozen fine-grained sediments. In contrast to the omnipresence of carbon dioxide in permafrost (Rivkina *et al*., 2005), all methane-containing samples (up to 40.0 mL kg^{-1}) represent epicryogenic horizons, while in alternating syncryogenic strata all samples were free of methane or contained methane at only trace levels. Discrete beds of CH_4 over many thousands of years are indicative of the absence of methane diffusion through the frozen layers. The absence of diffusion (calculated diffusion coefficient of 10^{-13} m^2 c^{-1}) allows us to suggest that methane is located in closed pores or exists in clathrate form. One possible way to reconcile methane hydrate formation at negligible lithostatic pressure is to assume that within the pore spaces of the permafrost high pressures are created by the freezing process (Rivkina *et al*., 2001). The possibility that methane-hydrate may exist in fine-grained sediments of the upper permafrost horizons was confirmed experimentally (Ershov *et al*., 1991; Chuvilin *et al*., 2005).

Table 3. Growth rate of strains J101 and M2 on different substrates

	Growth rate (h^{-1})	
Substrate	Strain JL01	Strain M2
Methanol	0.079	–
Acetate	0.036	–
Methylamine	0.025	–
Dimethylamine	0.034	–
Trimethylamine	0.067	–
H_2+CO_2	–	0.032
Formate	–	0.019

The vital functions of CH_4-forming archaea are possible in ecosystems with low redox potential and free of oxygen. These conditions are realized in natural systems where the

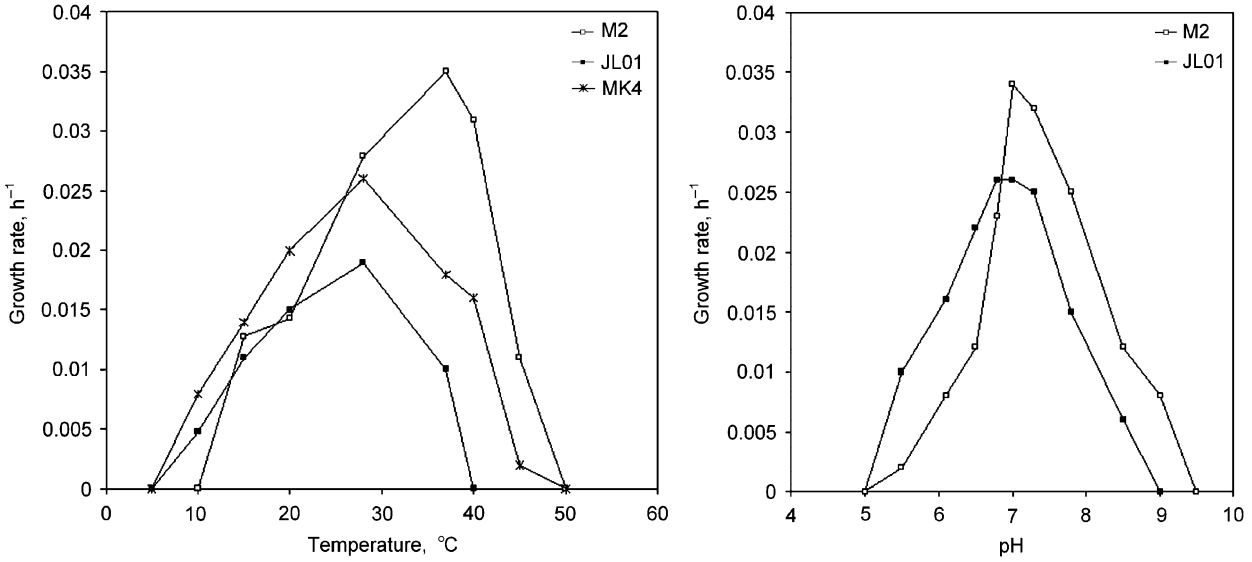

Fig. 6. Temperature and pH effect on growth of the new isolates.

rate of oxygen diffusion is smaller than the rate of organic matter oxidation. An example of such a system is provided by Arctic landscapes that provide conditions for summer production of methane, which is partially oxidized and partially released to the atmosphere. By the time of fall freezing, methane has been formed in the active layer, and fully removed or partially remaining in the lower part. Freezing from the bottom changes this lower layer into a permanently frozen state without (first type) or with methane (second type). Thus, methane is distributed in syngenetically frozen and never-thawed later horizons. The first type is found largely in Icy Complexes (Fig. 2, sites 1, 2, 4–6), which are free of methane. The mid Pleistocene Icy Complex (Fig. 2, site 3), where methane concentration reached 20 mL kg^{-1}, represents the second type.

Epicryogenic units, where CH_4 was found at concentrations up to 40 mL kg^{-1} (Fig. 2, sites 1–7), comprise the main methane reservoir in permafrost. Methane was formed in nonfrozen sediments and subsequent freezing from the top removed it from the carbon cycle. In typical epicryogenic layers, such as marine sediments, methane was formed in the bottom sediments. After the regression of the Polar Ocean, these sediments became frozen and methane conservation took place. On the land, methane was formed in a similar fashion in the lake bottom layers and became preserved due their freezing following drainage The river-bed deposits also froze epigenetically as a result of channel meandering. Because the sedimentation of the deposits occurred under automorphic conditions, which are not favorable for anaerobiosis, they are free of methane (Fig. 2, site 7). The most favorable conditions for microbial activity concurred with periods of permafrost thawing, when within the thawed part methane formation took place. The following refreezing buried methane in the permafrost. Most of the methane preserved in late Cenozoic permafrost was formed in this way. In the present-day cryolithosphere, the methane distribution in alternating syngenetically and epigenetically frozen horizons along the cross-section helps to reconstruct the vertical permafrost dynamics (Gilichinsky *et al.*, 1997). To calculate the amount of potential emission of bound methane, it is necessary to estimate its concentrations in the main horizons of the Arctic sedimentary cover.

The subzero temperatures, themselves, are not a prohibitive factor for metabolic activity (Gilichinsky *et al.*, 1993). In recent years it has been shown that microorganisms are metabolically active in ice and permafrost at temperatures close to their natural habitats. It was established that new DNA formation, i.e. bacterial reproduction in Antarctic ice, occurred down to temperatures of -15 and $-17\,^{\circ}C$ (Carpenter *et al.*, 2000; Christner, 2002), and experiments with ^{14}C-labeled acetate ($CH_3^{14}COO_2^-$) confirmed the formation of microbial lipids in permafrost at temperatures down to $-20\,^{\circ}C$ (Rivkina *et al.*, 2000). The main ecological niches providing conditions for preservation and metabolism at subzero temperatures are salt water veins in ice (Price, 2000) and unfrozen water films in permafrost (Gilichinsky *et al.*, 1993; Gilichinsky, 2002). Summarizing the results of studies of microbial metabolism at subzero temperatures, it can be concluded that microbial cells may be able to grow at low temperatures and metabolize at as low as $-40\,^{\circ}C$ (Price & Sowers, 2004).

However, because at subzero temperatures the rates of biochemical reactions and biological processes become extremely low, the microbiological methods are not

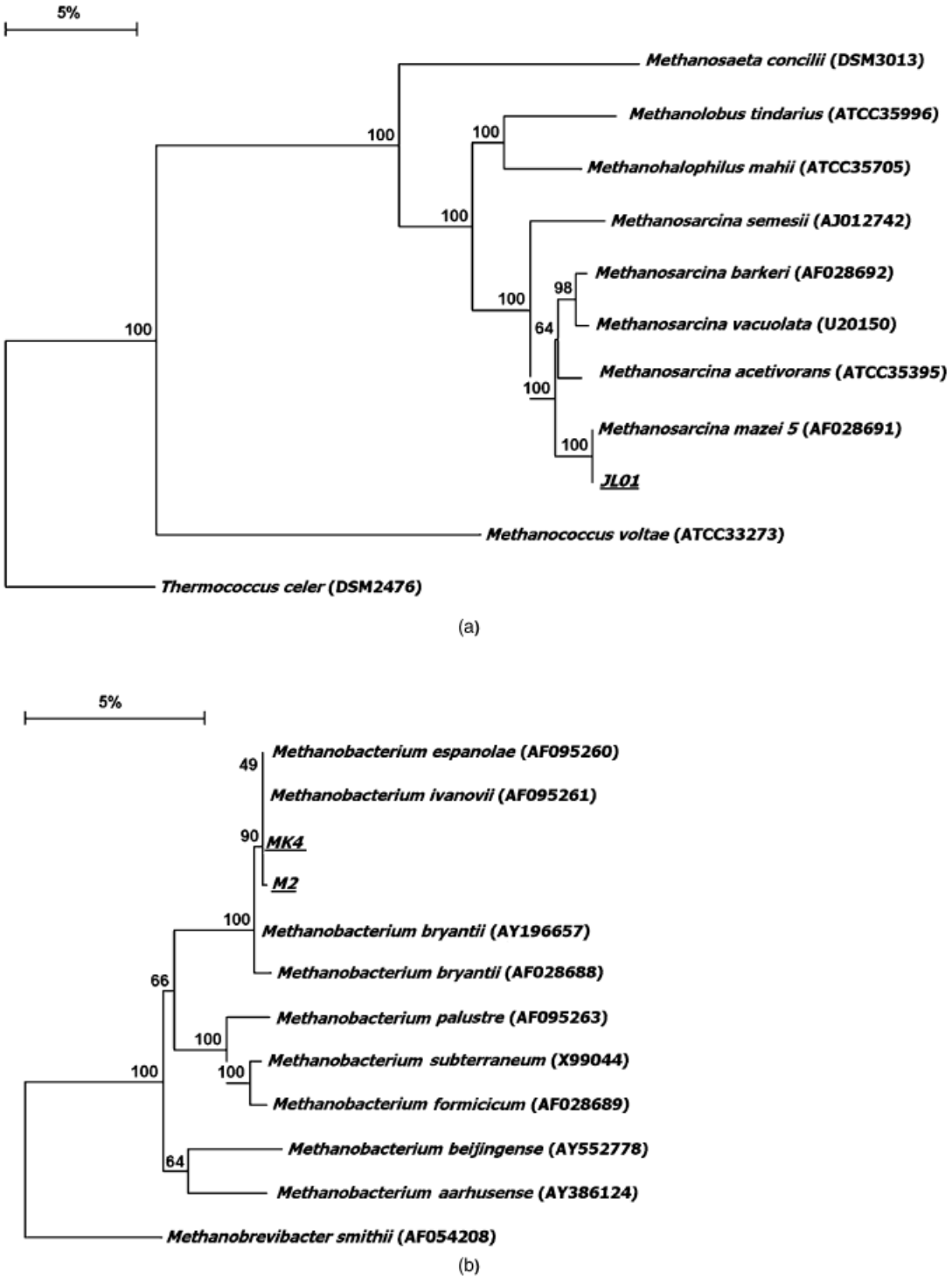

Fig. 7. Phylogenetic position of strains JL01 (a) and M2 and MK4 (b) among closely related species of the genera *Methanosarcina* and *Methanobacterium*, respectively, based on 16S rRNA gene sequence analysis. Accession numbers of type strains are shown in parentheses. The tree was built with the neighbor-joined method using the Jukes–Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. Bar, 5% estimated substitutions per nucleotide position.

sufficiently sensitive to detect cell activity. This explains why the possibility of methane formation at subzero temperatures as low as $-16.5\,^{\circ}C$ was shown experimentally only using radiolabeled substrates (Rivkina *et al.*, 2004). It is necessary to stress that experimental data provide only a demonstration of the potential ability of such a process, given that they do not fully model the natural conditions (Rivkina *et al.*, 2005) and that measured methane production rates at subzero temperatures represent the potential production rates when substrate supply is not limiting.

The experiments with radiolabeled substrates presented here showed that methanogenic archaea not only preserve their viability in the permafrost, but also are able to realize metabolic reactions at subzero temperatures. At these temperatures, microorganisms from both the relatively young Holocene sediments and the old Pliocene deposits are metabolically active. The fact that methane formation in these samples, unlike samples from the Holocene, took place at an equal rate in all studied spectra of subzero temperatures (Fig. 3) indicates that the residence time in permafrost promotes the adaptation and significant psychrophilization of Pliocene microbial communities compared with Holocene communities.

The extremely long lag-phase of incubation (6 months to 1 year) was probably required to repair damage accumulated in the cells through the period of existence in subzero temperature over a long period of geological time, and for metabolism reconstruction as a response to new conditions. Rapid activation of methane emission at the end of the lag-phase suggests that prior to that, active processes of adaptation and cell reparation of methanogenic archaea took place. These processes are necessary for the subsequent biomass accumulation during the chemoautotrophic and chemoorganotrophic growth at above zero temperatures.

The isotopic composition of methane, produced by methanogenic bacteria in natural ecosystems, lies in the range -50‰ to -70‰, while abiogenic methane substantively enriched in ^{13}C has $\delta^{13}C$ values in the range -45‰ to -50 ‰ (Fig. 4). Analysis of the isotopic composition of methane, formed from different substrates, revealed that more light methane is formed from CO_2+H_2. The rate of reaction also has an important effect on the isotopic composition of methane. The slower the methane-forming processes, the greater is fractionation (Zyakun, 1996) and the lighter is the methane isotopic composition. The isotopic composition of CH_4 carbon ($\delta^{13}C$ -64‰ to -99‰) in permafrost confirms its biological origin and, along with discrete bedding, points to *in situ* formation of this biogenic gas. The extremely low value of $\delta^{13}C$ methane in some samples (-90‰ to -99 ‰) allows us to conclude that methane mainly formed as a result of CO_2 reduction; the role of methane oxidation in the total balance was not significant, although viable methane-oxidizing bacteria are present in permafrost (Khmelenina *et al.*, 2002); part of the methane could be formed at subzero temperatures, which was accompanied by significant fractionating of carbon isotopes.

An argument in favor of a biogenic origin of methane and survival of methane-forming archaea over geological significant period of time is the fact that for the first time enrichments of methanogenic archaea, and also pure cultures of methane-forming microorganisms (strains JL01, M2 and MK4, Fig. 6), isolated from permafrost of different age, were obtained. In recent years, a few of methanogenic archaea have been isolated from permanently cold lake and marine sediments (Franzmann *et al.*, 1992, 1997; Simankova *et al.*, 2001; Von Klein *et al.*, 2002; Singh *et al.*, 2005; Kendall *et al.*, 2007). The new methanogenic strain JL01 belongs to family *Methanosarcinaseae*, genus *Methanosarcina*, which represents acetotrophic methanogens that predominate in many anaerobic ecosystems, including arctic soils (Simankova *et al.*, 2003). Some of these organisms are the most versatile methanogens able to use H_2+CO_2, acetate and methyl compounds. *Methanosarcina mazei* strain MT (Simankova *et al.*, 2003) isolated from tundra soil was psychrotolerant. Our strains JL01 and MT showed differences in substrate utilization, and optimal and range of temperature for growth.

Phylogenetic analysis places the new hydrogen-consuming archaea, strains M2 and MK4, in the family *Methanobacteriaceae*, genus *Methanobacterium*. Most *Methanobacterium* spp. are mesophilic and have been isolated from various freshwater habitats (Boone, 2001): *Methanobacterium subterianum* was isolated from granitic ground water (Kotelnikova *et al.*, 1998) and *Methanobacterium aarhusense* from marine sediments (Shlimon *et al.*, 2004). Strains M2 and MK4 probably represent novel species of the genus *Methanobacterium*.

Sequences of the 16S rRNA gene related to the order *Methanosarcinales* are present in cold marine sediments (Vetriani & Jannash, 1999). Study of the deep marine sediments by PCR amplification and sequence analysis of 16S rRNA and methyl coenzyme M reductase (*mcrA*) genes suggested limited methanogenic diversity with only three gene clusters identified within the *Methanosarcinales* and *Methanobacteriales* (Newberry *et al.*, 2004).

Recent studies using culture-independent methods have demonstrated that the active layer of arctic soils is a natural habitat of a diverse archaeal community (Kotsyurbenko *et al.*, 2004; Hoj *et al.*, 2005, 2006; Ganzert *et al.*, 2007). The active layer is exposed to seasonal variations in temperature and humidity, while the permafrost is characterized by constant subzero temperatures, which means permafrost microorganisms survive in an environment characterized by extremely low water activity and rates of nutrient exchange (Gilichinsky, 2002). These factors determine the

diversity and species composition in permafrost. The first results of culture-independent studies of archaeal communities in permanently frozen soil (Tiedje *et al.*, 1998; Steven *et al.*, 2006, 2007) justify this statement. Unfortunately, there are too few data regarding the presence of methanogens within observed archaea in these studies. Results of archaeal diversity within permafrost by molecular methods should be conducted with samples containing methane because both enrichment and pure cultures of methanogens can be obtained from such samples.

Conclusion

Permafrost is a huge reservoir of biogenic methane that is excluded from biogeochemical circulation. Unlike hypogene methane, ancient methane in the upper horizons of the cryolithosphere could be easily liberated into the atmosphere, should the permafrost degrade (this process can be observed sites of coastal thermal abrasion in the Arctic). Furthermore, one can expect that upon permafrost thawing, the paleomicrobial community will be actively reinvolved in present-day biogeochemical processes. This also includes production of greenhouse gases due to accessibility of organic matter or oxidation of buried methane by CH_4-oxidizing bacteria. Further investigations are clearly needed in this regard.

The mechanism of biogenic methane presence in permafrost includes original methane formation in sediments at temperatures above zero followed by its conservation during freezing. At the same time, one cannot exclude the possibility of methane formation within permafrost at subzero temperatures. This would depend on the ability of methanogens to not only survive and adapt in the permafrost but also to carry out metabolic reactions. Discovery of viable methanogens in ancient permafrost sediments provides significant evidence of the stability of these microbial populations through extremely long existence at subzero temperatures. The comparison of ancient isolates with modern methanogens provides a means to understand their adaptation strategy, which is the goal of our future studies.

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