Methanobacterium veterum sp. nov., from ancient Siberian permafrost

Kirill V. Krivushin,¹ Viktoria A. Shcherbakova,² Lada E. Petrovskaya³ and Elizaveta M. Rivkina¹

Correspondence

Kirill V. Krivushin
krivushin@bk.ru

¹Institute of Physicochemical and Biological Problems in Soil Sciences, Russian Academy of Sciences, Pushchino, 142290 Moscow Region, Russian Federation

²Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, 142290 Moscow Region, Russian Federation

³Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, 117997 GSP, Moscow V-437, Russian Federation

A methanogenic archaeon, strain MK4^T, was isolated from ancient permafrost after long-term selective anaerobic cultivation. The cells were rods, 2.0–8.0 µm long and 0.40–0.45 µm wide, and stained Gram-negative. Optimal growth was observed at 28 °C and pH 7.0–7.2 and in 0.05 M NaCl. The isolate used H₂ plus CO₂, methylamine plus H₂ and methanol plus H₂ as sources for growth and methanogenesis. Phylogenetic analysis of the 16S rRNA gene sequence of the strain showed close affinity with *Methanobacterium bryantii* (similarity >99% to the type strain). On the basis of the level of DNA–DNA hybridization (62%) between strain MK4^T and *Methanobacterium bryantii* VKM B-1629^T and phenotypic and phylogenetic differences, strain MK4^T was assigned to a novel species of the genus *Methanobacterium*, *Methanobacterium veterum* sp. nov., with the type strain MK4^T (=DSM 19849^T =VKM B-2440^T).

Low-temperature ecosystems play an essential role in Earth's climate and the balance of greenhouse gases in the atmosphere (Corradi *et al.*, 2005). Arctic permafrost is a natural cryobank of both aerobic (Zvyagintsev, 1985; Gilichinsky *et al.*, 1989; Vorobyova *et al.*, 1997; Vishnivetskaya *et al.*, 2000) and anaerobic (Rivkina *et al.*, 1998; Shcherbakova *et al.*, 2005; Vatsurina *et al.*, 2008) micro-organisms.

To date, a few species of methanogen [*Methanococcoides burtonii* (Franzmann *et al.*, 1992), *Methanogenium frigidum* (Franzmann *et al.*, 1997), *Methanomethylovorans hollandica* (Lomans *et al.*, 1999), *Methanobacterium subterraneum* (Kotelnikova *et al.*, 1998) and *Methanobacterium aarhusense* (Shlimon *et al.*, 2004)] have been isolated from modern low-temperature terrestrial ecotopes. Previously, methanogenic archaea affiliated to the genera *Methanobacterium* and *Methanosarcina* have been described from Arctic permafrost sediments of different ages (Rivkina *et al.*, 2007). The goal of the present study was to describe novel methanogenic archaea from ancient permafrost sediments.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MK4^T is EF016285.

Micrographs of cells of MK4^T and graphs showing the effects of temperature, pH and NaCl concentration on growth are available as supplementary material with the online version of this paper.

Samples were selected from deep (28 m beneath the surface) late-Pliocene permafrost (Kolyma lowland; 70° 06' N 154° 04' E). The age of the sediments (3 million years) was estimated on the basis of palaeomagnetic, palynological and palaeontological analyses (Sher, 1974, 1997; Sher *et al.*, 2005; Virina, 1997; Zazhigin, 1997; Schirrmeister *et al.*, 2002). Climatic and geological evidence suggests that these sediments have remained frozen for at least 3 million years. The native temperature of the permafrost was –10 °C. Permafrost was sampled aseptically with a fluid-free drilling machine that prevented down-hole contamination (Shi *et al.*, 1997). In the field and during transportation to the laboratory, samples were stored in a thermostatic container at –10 °C.

An enrichment culture was obtained by adding permafrost samples (approx. 10 g) to 60 ml vials with 5 ml anaerobic medium (Hungate, 1969) of the following composition (l⁻¹): K₂HPO₄, 0.29 g; KH₂PO₄, 0.29 g; NaCl, 1.0 g; MgCl₂·6H₂O, 0.2 g; NH₄Cl, 1.0 g; CaCl₂·2H₂O, 0.1 g; cysteine hydrochloride, 0.5 g; Na₂S·9H₂O, 0.5 g; vitamin solution (Balch *et al.*, 1979), 5 ml; trace element solution (Balch *et al.*, 1979), 10 ml. The medium was adjusted to pH 7.2–7.4. H₂ and CO₂ (4:1, 150 kPa) were used as the sole carbon and energy source.

Samples were incubated at 6 and 20 °C. Methane emission dynamics was observed by means of a Pye-Unicam 304 gas–liquid chromatograph with flame-ionization detector

as described previously (Kotelnikova *et al.*, 1993). Active methanogenesis was detected after 12 months of cultivation at 20 °C. After the methane concentration reached 40 %, the entire contents of the flask were transferred into a Balch tube (25 ml) supplied with 5 ml basal medium with a gas mixture of H₂ and CO₂. Pure cultures were obtained by subsequent 10-fold dilutions in roll-tubes. Cell morphology was examined using a Lumam I-2 phase-contrast microscope with 90×15 magnification and a JEM100 electron microscope with ultrathin sections as described by Shcherbakova *et al.* (2005). The cells were non-motile, curved rods, 2.0–8.0 µm long and 0.40–0.45 µm wide (Supplementary Fig. S1a, available in IJSEM Online). They stained Gram-negative but contained a complex, multi-layered, Gram-positive-type cell wall (Supplementary Fig. S1b). Cells occurred singly and as aggregates in old cultures. Filamentous cells (up to 30 µm long) were also frequently observed (not shown).

Methanobacterium bryantii VKM B-1629^T was used in comparative studies. DSMZ medium 506 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium506.pdf) with a few modifications [l⁻: sodium acetate trihydrate, 0.05 g; (NH₄)₂SO₄, 0.45 g; K₂HPO₄, 0.29 g; KH₂PO₄, 0.18 g; MgSO₄·7H₂O, 0.12 g; CaCl₂·2H₂O, 0.06 g; NaCl, 5.0 g; vitamin solution, 10 ml; trace element solution, 10 ml; resazurin, 0.001 g; cysteine hydrochloride monohydrate, 0.25 g; Na₂S·9H₂O, 0.25 g] was used for detection of the growth ranges of strain MK4^T and *Methanobacterium bryantii* VKM B-1629^T at different pH, temperature and NaCl concentrations. The compositions of the vitamin and trace element solutions are available at http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf. All tests were performed twice and confirmed by two transfers. Growth was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). Growth of strain MK4^T was observed at 10–46 °C (optimum 28 °C) and *Methanobacterium bryantii* VKM B-1629^T grew at 20–50 °C (optimum 37 °C) (Supplementary Fig. S2a). Both strains were mesophiles according to the classification of Morita (1975), but strain MK4^T grew over a wider range of temperature. The effect of pH on growth was measured in basal medium 506 with the addition of sterile 1 M HCl, 10 % (w/v) NaHCO₃ or 8 % (w/v) Na₂CO₃ solutions to obtain the required final pH. A decrease in the pH of the medium of no more than 0.2–0.4 pH units was observed at the end of the exponential phase. Strain MK4^T grew at pH 5.2–9.4 (optimum pH 7.0–7.2), whereas optimum growth of *Methanobacterium bryantii* VKM B-1629^T was observed at pH 6.9–7.0 (range pH 5.8–8.8) (Supplementary Fig. S2b). The effect of NaCl was examined in basal medium containing 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl. The strain grew at NaCl concentrations of 0–0.3 M, with optimum growth at 0.05 M NaCl. NaCl concentrations up to 0.35 M did not affect growth of *Methanobacterium bryantii* VKM B-1629^T (Supplementary Fig. S2c). In addition to H₂ plus CO₂, the following substrates were tested for growth of strain MK4^T and *Methanobacterium bryantii* VKM

B-1629^T: formate (50 mM), acetate (50 mM), methanol (50 mM) plus H₂, methylamine (20 mM) plus H₂, ethanol (10 mM), isopropanol (10 mM), isobutanol (10 mM), methylamine (20 mM) and trimethylamine (20 mM). H₂ plus CO₂ (growth rate 0.026 h⁻¹), methanol plus H₂ (0.014 h⁻¹) and methylamine plus H₂ (0.012 h⁻¹) supported growth and methanogenesis of strain MK4^T, while *Methanobacterium bryantii* VKM B-1629^T grew on H₂ plus CO₂ (0.031 h⁻¹) and used isobutanol for methane production without visible growth. Although growth of *Methanobacterium bryantii* VKM B-1629^T on isopropanol was reported previously (Zellner & Winter, 1987), it was not observed in the present study. Examination of the effects of the addition of acetate (1, 2, 5, 10 and 20 mM), yeast extract (0.1 and 0.5 g l⁻¹), coenzyme M (25 mg l⁻¹) and Casamino acids (1 g l⁻¹) on growth of strain MK4^T showed stimulation of growth by acetate only, at concentrations of 5 and 10 mM.

Cells of strain MK4^T from exponentially growing cultures were resistant to lysis by 1 % SDS and distilled water as a hypotonic solution.

Genomic DNA was isolated as described previously (Sambrook *et al.*, 1989). The DNA G+C contents, determined by the thermal denaturation method (four replications) using a Pye-Unicam SP1800 spectrophotometer, were 33.8±0.3 and 35.2±0.3 mol% for MK4^T and *Methanobacterium bryantii* VKM B-1629^T, respectively.

The 16S rRNA gene was amplified by PCR using an Eppendorf Mastercycler Personal Thermal Cycler. The gene was amplified in a 50 µl reaction containing 2 µl (approx. 1–5 ng) template DNA, 1 µl (20 pmol) each primer (Evrogen) and PCR kit reagent (Evrogen). The 16S rRNA gene was amplified with universal primers A8F (5'-TCCGTTGATCCTGCCGG-3') and 1492R (5'-ACGG-YTACCTTGTTACGACTT-3'). The PCR conditions were 94 °C for 5 min for primary denaturation followed by 30 cycles of 94 °C for 45 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 90 s (chain extension) and a final 5 min chain extension step at 72 °C. The obtained PCR product was purified by electrophoresis in 1 % agarose, excised from the gel and eluted with a MiniElute gel extraction kit (Qiagen). Sequencing reactions were performed using the ABI PRISM BigDye Terminator kit version 3.1 and an ABI PRISM 3100-Avant automatic DNA sequencer according to the protocols provided by the manufacturer. The sequence obtained (1347 bp) was subjected to phylogenetic analyses using MEGA4 (Tamura *et al.*, 2007), and a neighbour-joining tree is shown in Fig. 1.

The isolate was phenotypically similar to other species of the genus *Methanobacterium* (Table 1). Like *Methanobacterium beijingense*, *Methanobacterium congolense* and *Methanobacterium alcaliphilum*, the cells stained Gram-negative. Along with *Methanobacterium subterraneum* and *Methanobacterium aarhusense*, strain MK4^T demonstrated a wide growth temperature range. The most important differences from these and other species of the

Fig. 1. Phylogenetic dendrogram of 16S rRNA gene sequences showing the position of strain MK4^T relative to other species of the genus *Methanobacterium*. Evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). The sum of branch lengths is 0.13724225. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (from 1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004) and are in units of numbers of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There was a total of 1259 positions in the final dataset. Bar, 0.005 substitutions per nucleotide position.

genus *Methanobacterium* with validly published names are its low optimum growth temperature (28 °C;

Supplementary Fig. S2a) and ability to use H₂ plus methanol and H₂ plus methylamine for growth and methanogenesis. The main substrates for growth of MK4^T were H₂ and CO₂, as for other *Methanobacterium* species. Utilization of only H₂ plus methanol is typical of *Methanosphaera* species, and has also been shown for several other methylotrophic methanogens (Müller *et al.*, 1986). Strain MK4^T is the first reported member of the genus *Methanobacterium* that is capable of utilizing these substrates for growth and methanogenesis. The expanded range of utilizable substrates possibly increases the chances of survival under the nutrient-limited conditions of the permafrost.

Although high 16S rRNA gene sequence similarity was observed between the isolate and *Methanobacterium bryantii* VKM B-1629^T (99%), the results of DNA–DNA hybridization with the thermal reassociation method (De Ley *et al.*, 1970) indicated 62 ± 6% genomic relatedness (four replications). According to the minimal standards for the description of new taxa of methanogens (Boone & Whitman, 1988) and based on phylogenetic and phenotypic characters, a novel species of the genus *Methanobacterium* is proposed, *Methanobacterium veterum* sp. nov.

Description of *Methanobacterium veterum* sp. nov.

Methanobacterium veterum (ve'te.rum. L. gen. pl. n. *veterum* of the old, of old things, of antiquity, referring to the isolation of the type strain from ancient permafrost).

Table 1. Characteristics of the type strains of *Methanobacterium* species

Strains: 1, MK4^T; 2, *M. bryantii* DSM 863^T (Bryant *et al.*, 1967; Boone, 1987); 3, *M. oryzae* DSM 11106^T (Joulain *et al.*, 2000); 4, *M. beijingense* DSM 15999^T (Ma *et al.*, 2005); 5, *M. congolense* DSM 7095^T (Cuzin *et al.*, 2001); 6, *M. alcaliphilum* DSM 3387^T (Worakit *et al.*, 1986); 7, *M. subterraneum* DSM 11074^T (Kotelnikova *et al.*, 1998); 8, *M. formicicum* DSM 1535^T (Bryant & Boone, 1987); 9, *M. palustre* DSM 3108^T (Zellner *et al.*, 1989); 10, *M. aarhusense* DSM 15219^T (Shlimon *et al.*, 2004). Cells of all strains are rods. ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Gram staining	–	Variable	ND	–	+	–	+	+	+	+
Cell width (µm)	0.4–0.45	0.5–1.0	0.3–0.4	0.4–0.5	0.4–0.5	0.5–0.6	0.1–0.15	0.4–0.8	0.5	0.7
Cell length (µm)	2.0–8.0	10.0–15.0	3.0–10.0	3.0–5.0	2.0–10.0	2.0–25.0	0.6–1.2	2.0–15.0	2.5–5.0	5.0–18.0
Temperature for growth (°C)										
Range	10–46	20–50	20–42	25–50	25–50	ND	3–45	ND	20–45	5–48
Optimum	28	37	40	37	37–42	37	20–40	37–45	33–37	45
pH for growth										
Range	5.2–9.4	5.8–8.8	6.0–8.5	6.5–8.6	5.9–8.2	7.0–9.9	6.5–9.2	ND	ND	5–9
Optimum	7.2–7.4	6.9–7.0	7.0	7.2–7.7	7.2	8.1–9.1	7.8–8.8	6.6–7.8	7.0	ND
NaCl range (M)	0–0.3	0–0.3	0–0.4	0–0.5	ND	ND	0.2–1.2	ND	0–0.3	ND
Substrates used*	H ₂ /CO ₂ , MeN/ H ₂ , MeOH/H ₂	H ₂ /CO ₂ , iB, iP	H ₂ /CO ₂ , F	H ₂ /CO ₂ , F	H ₂ /CO ₂	H ₂ /CO ₂	H ₂ /CO ₂ , F	H ₂ /CO ₂ , F	H ₂ /CO ₂ , iP, iB	H ₂ /CO ₂
Growth factors required	–	Acetate, cysteine, vitamin B	Yeast extract	Yeast extract	–	Peptone	ND	Acetate, cysteine	ND	–
DNA G+C content (mol%)†	33.8 (T _m)	32.7 (T _m)	31 (LC)	38.9 (T _m)	39.5 (LC)	34	54.5 (T _m)	41–42 (Bd)	34 (T _m)	34.9 (LC)

*F, Formate; iB, isobutanol; iP, isopropanol; MeN, methylamine; MeOH, methanol.

†Determined by buoyant density analysis (Bd), HPLC analysis (LC) or melting point analysis (T_m).

Gram-negative-staining, non-motile, non-spore-forming, anaerobic, chemoautotrophic rods. Cells are slightly crooked, 2.0–8.0 µm long and 0.40–0.45 µm wide, occur singly or in chains (up to 30 µm long) and show epifluorescence at 420 nm. Cells divide through septum formation. Uses H₂ plus CO₂, methanol plus H₂ and methylamine plus H₂ as sources for growth and methane production. It does not grow on formate, acetate, ethanol, isopropanol, isobutanol, methylamine or trimethylamine. Growth is stimulated by acetate. Optimum growth conditions are 28 °C, pH 7.0–7.2 and 0.05 M NaCl. The G+C content of the DNA of the type strain is 33.8 ± 0.3 mol%.

The type strain, MK4^T (=DSM 19849^T =VKM B-2440^T), was isolated from ancient (3 million years old) permafrost sediments from the Kolyma lowland, Russia (70° 06' N 154° 04' E).

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