**Methanobacterium veterum** sp. nov., from ancient Siberian permafrost

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A methanogenic archaean, strain MK4T, 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 MK4T and *Methanobacterium bryantii* VKM B-1629T and phenotypic and phylogenetic differences, strain MK4T was assigned to a novel species of the genus *Methanobacterium*, *Methanobacterium veterum* sp. nov., with the type strain MK4T (=DSM 19849T =VKM B-2440T).

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 aarhuense* (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.

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 (1−1): 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 a 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 x 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-1629T was used in comparative studies. DSMZ medium 506 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium506.pdf) with a few modifications [11]: 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 MK4T and *Methanobacterium bryantii* VKM B-1629T 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 MK4T was observed at 10–46 °C (optimum 28 °C) and *Methanobacterium bryantii* VKM B-1629T grew at 20–50 °C (optimum 37 °C) (Supplementary Fig. S2a). Both strains were mesophiles according to the classification of Morita (1975), but strain MK4T grew over a wider range of temperature. The effect of pH on growth was measured in basal medium 506 with the addition of 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 MK4T grew at pH 5.2–9.4 (optimum pH 7.0–7.2), whereas optimum growth of *Methanobacterium bryantii* VKM B-1629T 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-1629T (Supplementary Fig. S2c). In addition to H2 plus CO2, the following substrates were tested for growth of strain MK4T and *Methanobacterium bryantii* VKM B-1629T: 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), methylene (20 mM) and trimethylamine (20 mM). H2 plus CO2 (growth rate 0.26 h–1), methanol plus H2 (0.014 h–1) and methylene plus H2 (0.012 h–1) supported growth and methanogenesis of strain MK4T, while *Methanobacterium bryantii* VKM B-1629T 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-1629T 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 MK4T showed stimulation of growth by acetate only, at concentrations of 5 and 10 mM.

Cells of strain MK4T 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 MK4T and *Methanobacterium bryantii* VKM B-1629T, 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 8F (5'-TCCGTTGATCTGGCCG-3') and 1492R (5'-ACGGYTACCTTGTTACGACTT-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 MK4T demonstrated a wide growth temperature range. The most important differences from these and other species of the
Methanobacterium veterum sp. nov.

Fig. 1. Phylogenetic dendrogram of 16S rRNA gene sequences showing the position of strain MK4T 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 0.1372425. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (from 1000 replicates) are shown next to the branches which the associated taxa clustered together in the bootstrap test. Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in units of numbers of base substitutions per nucleotide position.

Discussion 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

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Cell width (µm)</td>
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<td>0.4–0.5</td>
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<td>Cell length (µm)</td>
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<td>Range</td>
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<td>Yeast extract</td>
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<td>Peptone</td>
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<td>Acetate, cysteine</td>
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<td>–</td>
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<td>DNA G + C content (mol%)†</td>
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<td>32.7 (Tm)</td>
<td>31 (LC)</td>
<td>38.9 (Tm)</td>
<td>39.5 (LC)</td>
<td>34</td>
<td>54.5 (Tm)</td>
<td>41–42 (Bd)</td>
<td>34 (Tm)</td>
<td>34.9 (LC)</td>
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*F, Formate; iB, isobutanol; iP, isopropanol; MeN, methylamine; MeOH, methanol.
†Determined by buoyant density analysis (Bd), HPLC analysis (LC) or melting point analysis (Tm).
The type strain, MK4T (DSM 19849T = VKM B-2440T), was isolated from ancient (3 million years old) permafrost sediments from the Kolyma lowland, Russia (70°06′ N 154°04′ E).

Acknowledgements

We thank Dr Nataliya Suzina (Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino, Russia) for help with the electron micrographs and Dr Anatoly Lyensenko (Winogradsky Institute of Microbiology, Moscow, Russia) for determination of G+C content. We are grateful to Dr Jean Ezubezhy for suggesting the species name. This work was supported by RFBR grants 08-05-00268 and 08-04-01004.

References


