**Methanobacterium arcticum** sp. nov., a methanogenic archaeon from Holocene Arctic permafrost

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A mesophilic, non-motile, hydrogenotrophic, rod-shaped methanogen, designated M2⁷, was isolated from Holocene permafrost sediments of the Kolyma lowland in the Russian Arctic. Cells were 3–6 µm long and 0.45–0.5 µm wide. Strain M2⁷ grew on H₂/CO₂ and formate. Optimum conditions for growth were 37 °C, pH 6.8–7.2 and 0.1 M NaCl. The DNA G+C content was 38.1 mol%. On the basis of 16S rRNA gene sequence comparison with known methanogens, strain M2⁷ was affiliated with the genus *Methanobacterium* and was most closely related to *Methanobacterium veterum* MK4⁷ and *Methanobacterium bryantii* DSM 863⁷ (both 99 % 16S rRNA gene sequence similarity). However, no significant DNA–DNA relatedness was observed between strain M2⁷ and these type strains. We propose that strain M2⁷ represents a novel species, with the name *Methanobacterium arcticum* sp. nov., with type strain M2⁷ (=DSM 19844⁷ =VKM B-2371⁷).

At the time of writing, the genus *Methanobacterium* comprises 24 species with validly published names, although a number of them have been reclassified (http://www.bacterio.cict.fr/m/methanobacterium.html). The remaining members of the genus are hydrogenotrophic mesophiles, and only *Methanobacterium subterraneum* (Kotelnikova et al., 1998) and *Methanobacterium aarhusense* (Shlimon et al., 2004) were isolated from permanently cold habitats.

One of the reasons for the presence of methane in permafrost is methane formation in sediments at above-zero temperatures followed by its conservation during freezing. At the same time, one cannot exclude the possibility of methane formation in permafrost at sub-zero temperatures. Previously, we isolated three strains of methanogenic archaea from samples of Pliocene and Holocene permafrost from Eastern Siberia (Rivkina et al., 2007). Strains M2⁷, MK3 and MK4⁷ were non-motile hydrogenotrophic rods. Strain MK4⁷ has been assigned to a novel species, *Methanobacterium veterum* (Krivushin et al., 2010). In the present study, we report the characterization of strain M2⁷.

Strain M2⁷ was isolated from a sample of deep (2.0 m) Holocene permafrost sediments of the Kolyma lowland, Russia (70°06' N 154°04' E), by long-term enrichment (Rivkina et al., 2007). The protocols for drilling and subsequent handling of cores ensured that the retrieved material remained uncontaminated (Shi et al., 1997). Purity of the culture of strain M2⁷ was confirmed using the chromosomal DNA restriction pattern. The polyphasic characterization used the minimal standards detailed by Boone & Whitman (1988). *M. veterum* MK4⁷ and *Methanobacterium bryantii* VKM B-1629⁷ were used as reference strains.

Gram-staining was performed following standard protocols (Smibert & Krieg, 1994) and cells of strain M2⁷ stained Gram-negative. Cell morphology was examined using phase-contrast microscopy (I-2; Lumam) at ×1350 magnification and electron microscopy (JEM-100; JEOL) with ultrathin sections as described previously (Shcherbakova et al., 2005). Cells of strain M2⁷ were non-motile, slightly curved rods (Fig. 1a), 0.45–0.50 µm wide and 3.0–6.0 µm long, often forming chains and filaments more than 30 µm long. Cells of strain M2⁷ divided by the formation of septa and exponentially growing cells were resistant to lysis by hypotonic solution (1 % SDS in distilled water). During stationary growth or long-term storage, strain M2⁷ formed cyst-like cocoid cells (Fig. 1b), in which the cytoplasm was more dense and differentiated surface layers were observed. The cell wall was 10–11 nm thick in vegetative cells and 50 nm thick in cyst-like cells. The formation of cyst-like...
cells in halophilic archaea has been shown during nutrient limitation (El’-Registan et al., 2006; Suzina et al., 2006). Subsequent investigation is needed to understand the conditions under which strain M2T forms cyst-like cells.

Conditions for growth for strain M2T and M. bryantii VKM B-1629T (temperature, pH, NaCl concentration and substrates required for methanogenesis) were determined in MB medium [containing 1 l-l: 0.05 g sodium acetate trihydrate, 0.45 g (NH4)2SO4, 0.29 g K2HPO4, 0.18 g KH2PO4, 0.12 g MgSO4.7H2O, 0.06 g CaCl2.2H2O, 5.0 g NaCl, 10 ml vitamin solution (medium 141; DSMZ), 10 ml trace element solution (medium 141; DSMZ), 0.001 g resazurin, 0.25 g cysteine hydrochloride hydrate, 0.25 g Na2S.9H2O; H2/CO2 (80 : 20) at 200 kPa; Balch et al., 1979; Sowers & Noll, 1995] and for M. veterum MK4T (temperature and substrates required for methanogenesis) in DSMZ medium 506 with the same modification (Krivushin et al., 2010). All tests were performed twice and confirmed by two transfers. Growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The effect of temperature on growth was tested at –5, –2, 5, 10, 15, 24, 28, 37, 45, 50 and 55 °C, using a cryobath with ethylene glycol for the sub-zero temperatures. All strains were mesophiles (Morita, 1975): strain M2T grew at 15–45 °C (optimum 37 °C), M. bryantii VKM B-1629T grew at 20–50 °C (optimum 37 °C) and M. veterum MK4T grew at 10–50 °C (optimum 28 °C) (Supplementary Fig. S1, available in IJSEM Online). The pH of the basal medium was adjusted with sterile 1 M HCl, 10% (w/v) NaHCO3 or 8% (w/v) Na2CO3. At the end of exponential growth, pH was decreased by no more than 0.2–0.4 pH units. Strain M2T grew at pH 5.5–8.5 (optimum pH 6.8–7.2) and M. bryantii VKM B-1629T grew at pH 5.8–8.8 (optimum pH 6.9–7.0). The effect of NaCl on growth was tested with 0, 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl. Strain M2T grew with 0–0.3 M NaCl (optimum 0.1 M NaCl). Growth of M. bryantii VKM B-1629T was not affected by NaCl concentrations up to 0.3 M.

Growth with different carbon substrates was tested in the basal medium without sodium acetate as follows: H2/CO2, 20 mM methanol, 50 mM formate, 50 mM acetate, 20 mM ethanol, 20 mM 2-propanol, 20 mM 2-butanol, 50 mM methanol/H2, 20 mM methylamine/H2, 20 mM methanol and 20 mM trimethylamine. Strain M2T grew and produced methane with H2/CO2 (0.026 h–1) and formate (0.014 h–1) and M. bryantii VKM B-1629T grew with H2/CO2 (0.036 h–1) and produced methane without visible growth with 2-propanol and 2-butanol. Growth of strain M2T was not stimulated by the addition of acetate (1, 2, 5, 10, 20 mM), yeast extract (0.1 and 0.5 g l–1), CoM (25 mg l–1) or Casamino acids (1 g l–1). Growth of M. bryantii VKM B-1629T was induced by the addition of acetate (1–10 mM) and yeast extract (0.1 and 0.5 g l–1) and growth of M. veterum MK4T was induced by addition of acetate (Krivushin et al., 2010).

The effect of antibiotics on strain M2T, M. veterum MK4T and M. bryantii VKM B-1629T was determined by transferring 5-ml aliquots of culture to fresh medium containing (l-l) chloramphenicol (10 mg), bacitracin (10 mg), polymyxin (10 mg), vancomycin (2000 mg), erythromycin (1000 mg), kanamycin (2000 mg) or penicillin G (2000 mg). Tests were performed in duplicate with a non-antibiotic control for 1 week at the optimum temperature. Strain M2T, M. veterum MK4T and M. bryantii VKM B-1629T were sensitive to chloramphenicol and polymixin. Strain M2T was slightly sensitive to penicillin and bacitracin but M. veterum MK4T and M. bryantii VKM B-1629T were sensitive to bacitracin and resistant to penicillin. Bacitracin inhibits the formation of lipid-bound murein precursors in members of the domain Bacteria (Whitman et al., 2006) and it may have the same effect on methanogens of the family Methanobacteriaceae in the domain Archaea, which have cell walls composed of pseudomurein (Konig, 1995). All strains were resistant to vancomycin, erythromycin and kanamycin.

**Fig. 1.** Thin sections of cells of strain M2T showing a rod-shaped cell (a) and a cyst-like cell (b). CM, Cytoplasmic membrane; CW, cell wall; IL, inner layer of cell wall; N, nucleoid; OL, outer layer of cell wall. Bars, 100 nm.
Isolation of genomic DNA was carried out according to Marmur (1961). DNA–DNA hybridizations (four replicates) were performed as described by De Ley et al. (1970) and modified by Huß et al. (1983), using a Pye Unicam SP 1800 spectrophotometer equipped with a thermostregulator and hermetically sealed thermocuvettes. Standard deviations of hybridization experiments were between 5.0 and 10.5 %. DNA–DNA relatedness was 37 and 40 % between strain M2T and M. bryantii VKM B-1629T and M. veterum MK4T, respectively, and 62 % between M. bryantii VKM B-1629T and M. veterum MK4T. The DNA G+C contents of strain M2T, M. bryantii VKM B-1629T and M. veterum MK4T were 38.1 ± 0.3, 35.2 ± 0.5 and 33.8 ± 0.3 mol%, respectively, which were within the limits defined for the genus Methanobacterium.

For amplification and sequencing of the 16S rRNA gene, DNA was extracted using the method described by Sambrook et al. (1989) and the 16S rRNA gene was amplified by PCR using the archaeal 16S rRNA gene primers 8F (5’-TCCGGTTGATCCTGCCGG-3’) and 1041R (5’-G GCCATGACCWCCCCTC-3’). The amplification products were purified and sequenced using a Wizard PCR Preps DNA Purification System kit (Promega), a CEQ Dye Terminator Cycle Sequencing kit and CEQ 2000 XL automatic DNA sequencer (Beckman Coulter), according to the manufacturers’ instructions. An almost-complete sequence (~1434 bp) of the 16S rRNA gene was obtained from strain M2T. Phylogenetic 16S rRNA gene sequence analyses were performed with the neighbour-joining method (Saitou & Nei, 1987) using the maximum-composite-likelihood model for estimating evolutionary distances between DNA sequences (Tamura et al., 2004). Bootstrap values based on 1000 replicates were estimated using MEGA4 (Tamura et al., 2007). The 16S rRNA gene sequence tree (Fig. 2) clearly demonstrated that strain M2T was a member of the family Methanobacteriaceae in the order Methanobacteriales and that its closest phylogenetic relatives were M. bryantii DSM 863T (99 % 16S rRNA gene sequence similarity) and M. veterum MK4T (99 %).

On the basis of the 16S rRNA gene sequence analysis and comparisons of characteristics of non-motile, rod-shaped, methanogenic strains that utilize H2/CO2 for growth and methane generation, strain M2T was related to the genus Methanobacterium (Boone & Whitman, 1988; Bonin & Boone, 2006). Strain M2T differed from M. bryantii VKM B-1629T and M. veterum MK4T by its ability to utilize formate as a sole carbon and energy source and other phenotypic differences (Table 1) and exhibited low DNA–DNA relatedness with its closest phylogenetic relatives.

![Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain M2T within the genus Methanobacterium. Bootstrap values (>50 %) based on 1000 replicates are shown at nodes. Bar, 5 substitutions per 1000 nucleotide positions.](image)

### Table 1. Differential characteristics of strain M2T and its closest phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Rods, cyst-like cells</td>
<td>Rods, clamps</td>
<td>Rods*</td>
</tr>
<tr>
<td>Cell dimensions (µm)</td>
<td></td>
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</tr>
<tr>
<td>Width</td>
<td>0.45–0.5</td>
<td>0.5–0.6</td>
<td>0.40–0.45*</td>
</tr>
<tr>
<td>Length</td>
<td>3.0–6.0</td>
<td>10–15</td>
<td>2.0–8.0*</td>
</tr>
<tr>
<td>Substrate(s)</td>
<td>Formate</td>
<td>2-Propanol, 2-butanol</td>
<td>Methylamine/H2, methanol/H2</td>
</tr>
<tr>
<td>Stimulatory factor(s)</td>
<td>None</td>
<td>Yeast extract, acetate</td>
<td></td>
</tr>
<tr>
<td>Temperature for growth (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>15–45</td>
<td>10–50</td>
<td>10–45</td>
</tr>
<tr>
<td>Optimum</td>
<td>37</td>
<td>37</td>
<td>28</td>
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<tr>
<td>pH for growth</td>
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<tr>
<td>Range</td>
<td>5.5–8.5</td>
<td>5.8–8.8</td>
<td>5.2–9.4*</td>
</tr>
<tr>
<td>Optimum</td>
<td>6.8–7.2</td>
<td>6.9–7.0</td>
<td>7.0–7.2*</td>
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<tr>
<td>NaCl concentration for growth (M)</td>
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<td></td>
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<tr>
<td>Range</td>
<td>0–0.3</td>
<td>0–0.3</td>
<td>0–0.3*</td>
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<tr>
<td>Optimum</td>
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<td>0–0.3</td>
<td>0.05*</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>38.1</td>
<td>35.2</td>
<td>33.8*</td>
</tr>
</tbody>
</table>

*Data from Krivushin et al. (2010).
Therefore, strain M2^T represents a novel species of the genus *Methanobacterium*, for which we propose the name *Methanobacterium arcticum* sp. nov.

**Description of Methanobacterium arcticum**

*Methanobacterium arcticum* (arc’ti.cum. L. neut. adj. arcticum northern, arctic, from the Arctic, referring to the isolation of the type strain from Arctic permafrost).

Cells are Gram-negative, non-motile, non-spore-forming, slightly curved rods, 0.45–0.50 μm wide and 3.0–6.0 μm long. Often forms filaments and cyst-like coccolid cells. Strictly anaerobic. Chemoautotrophic. Optimal growth at 37 °C, maximum temperature is 45 °C. Optimal growth at pH 6.8–7.2; no growth at pH 5.0 or 9.0. Utilizes H2/CO2 and formate for growth and methane generation, but not acetate, methanol, ethanol, 2-propanol, 2-butanol, methylamines, methanol/H2 or methyamine/H2. Growth is not stimulated by acetate. The DNA G+C content of the type strain is 38.1 mol%.

The type strain M2^T (=DSM 19844^T =VKM B-2371^T) was isolated from Holocene permafrost sediments from the Kolyma lowland, Russia (70°06’ N 154°04’ E).

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**References**


