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

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A mesophilic, non-motile, hydrogenotrophic, rod-shaped methanogen, designated M2^T, 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 M2^T 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 M2^T was affiliated with the genus *Methanobacterium* and was most closely related to *Methanobacterium veterum* MK4^T and *Methanobacterium bryantii* DSM 863^T (both 99% 16S rRNA gene sequence similarity). However, no significant DNA–DNA relatedness was observed between strain M2^T and these type strains. We propose that strain M2^T represents a novel species, with the name *Methanobacterium arcticum* sp. nov., with type strain M2^T (=DSM 19844^T =VKM B-2371^T).

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 M2^T, MK3 and MK4^T were non-motile hydrogenotrophic rods. Strain MK4^T has been assigned to a novel species, *Methanobacterium veterum* (Krivushin *et al.*, 2010). In the present study, we report the characterization of strain M2^T.

Strain M2^T 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 M2^T was confirmed using the chromosomal DNA restriction pattern. The polyphasic characterization used the minimal standards detailed by Boone & Whitman (1988). *M. veterum* MK4^T and *Methanobacterium bryantii* VKM B-1629^T were used as reference strains.

Gram-staining was performed following standard protocols (Smibert & Krieg, 1994) and cells of strain M2^T 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 M2^T 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 M2^T 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 M2^T formed cyst-like coccoid 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M2^T is DQ517520.

A supplementary figure is available with the online version of this paper.

Fig. 1. Thin sections of cells of strain M2^T 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.

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 M2^T forms cyst-like cells.

Conditions for growth for strain M2^T and *M. bryantii* VKM B-1629^T (temperature, pH, NaCl concentration and substrates required for methanogenesis) were determined in MB medium [containing l⁻¹: 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* MK4^T (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 M2^T grew at 15–45 °C (optimum 37 °C), *M. bryantii* VKM B-1629^T grew at 20–50 °C (optimum 37 °C) and *M. veterum* MK4^T 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 M2^T grew at pH 5.5–8.5 (optimum pH 6.8–7.2) and *M. bryantii* VKM B-1629^T 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 M2^T grew with 0–0.3 M NaCl (optimum 0.1 M NaCl). Growth of *M. bryantii* VKM B-1629^T 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 methylamine and 20 mM trimethylamine. Strain M2^T grew and produced methane with H₂/CO₂ (0.026 h⁻¹) and formate (0.014 h⁻¹) and *M. bryantii* VKM B-1629^T grew with H₂/CO₂ (0.036 h⁻¹) and produced methane without visible growth with 2-propanol and 2-butanol. Growth of strain M2^T 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-1629^T 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* MK4^T was induced by addition of acetate (Krivushin *et al.*, 2010).

The effect of antibiotics on strain M2^T, *M. veterum* MK4^T and *M. bryantii* VKM B-1629^T was determined by transferring 5-ml aliquots of culture to fresh medium containing (l⁻¹) 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 M2^T, *M. veterum* MK4^T and *M. bryantii* VKM B-1629^T were sensitive to chloramphenicol and polymyxin. Strain M2^T was slightly sensitive to penicillin and bacitracin but *M. veterum* MK4^T and *M. bryantii* VKM B-1629^T 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. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain M2^T within the genus *Methanobacterium*. Bootstrap values (>50 %) based on 1000 replicates are shown at nodes. Bar, 5 substitutions per 1000 nucleotide positions.

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 thermoprogrammer 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 M2^T and *M. bryantii* VKM B-1629^T and *M. veterum* MK4^T, respectively, and 62 % between *M. bryantii* VKM B-1629^T and *M. veterum* MK4^T. The DNA G+C contents of strain M2^T, *M. bryantii* VKM B-1629^T and *M. veterum* MK4^T 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'-GGCCATGCACCWCCTCTC-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 M2^T. 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 M2^T was a member of the family *Methanobacteriaceae* in the order *Methanobacteriales* and that its closest phylogenetic relatives were *M. bryantii* DSM 863^T (99 % 16S rRNA gene sequence similarity) and *M. veterum* MK4^T (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 M2^T was related to the genus *Methanobacterium* (Boone & Whitman, 1988; Bonin & Boone, 2006). Strain M2^T differed from *M. bryantii* VKM B-1629^T and *M. veterum* MK4^T 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.

Table 1. Differential characteristics of strain M2^T and its closest phylogenetic relatives

Strains: 1, *Methanobacterium arcticum* sp. nov. M2^T; 2, *M. bryantii* VKM B-1629^T; 3, *M. veterum* MK4^T. Data were obtained in this study unless otherwise indicated. All strains grew with H₂/CO₂ and formed filaments.

Characteristic	1	2	3
Cell shape	Rods, cyst-like cells	Rods, clamps	Rods*
Cell dimensions (µm)			
Width	0.45–0.5	0.5–0.6	0.40–0.45*
Length	3.0–6.0	10–15	2.0–8.0*
Substrate(s)	Formate	2-Propanol, 2-butanol	Methylamine/H ₂ , methanol/H ₂
Stimulatory factor(s)	None	Yeast extract, acetate	Acetate
Temperature for growth (°C)			
Range	15–45	10–50	10–45
Optimum	37	37	28
pH for growth			
Range	5.5–8.5	5.8–8.8	5.2–9.4*
Optimum	6.8–7.2	6.9–7.0	7.0–7.2*
NaCl concentration for growth (M)			
Range	0–0.3	0–0.3	0–0.3*
Optimum	0.1	0–0.3	0.05*
DNA G+C content (mol%)	38.1	35.2	33.8*

*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* sp. nov.

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 coccoid 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 methylamine/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).

Acknowledgements

We thank Dr Anatoliy Lysenko (Vinogradsky Institute of Microbiology, Moscow, Russia) for the determination of G+C content and Dr Ekaterina Gavrish for help with restriction analysis. We are grateful to Dr Jean Euzéby for suggesting the species name. This work was supported by the RFBR (grant numbers 08-05-00268 and 08-04-01004).

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