Methanobacterium movilense sp. nov., a hydrogenotrophic, secondary-alcohol-utilizing methanogen from the anoxic sediment of a subsurface lake

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A novel strain of methanogenic archaea, designated MC-20^T, was isolated from the anoxic sediment of a subsurface lake in Movile Cave, Mangalia, Romania. Cells were non-motile, Gram-stain-negative rods 3.5–4.0 μm in length and 0.6–0.7 μm in width, and occurred either singly or in short chains. Strain MC-20^T was able to utilize H_2/CO_2, formate, 2-propanol and 2-butanol as substrate, but not acetate, methanol, ethanol, dimethyl sulfide, monomethylamine, dimethylamine or trimethylamine. Neither trypticase peptone nor yeast extract was required for growth. The major membrane lipids of strain MC-20^T were archaeol phosphatidylethanolamine and diglycosyl archaeol, while archaeol phosphatidylinositol and glycosyl archaeol were present only in minor amounts. Optimal growth was observed at 33 °C, pH 7.4 and 0.08 M NaCl. Based on phylogenetic analysis of 16S rRNA gene sequences, strain MC-20^T was closely affiliated with *Methanobacterium oryzae* FPi^T (similarity 97.1 %) and *Methanobacterium lacus* 17A1^T (97.0 %). The G+C content of the genomic DNA was 33.0 mol%. Based on phenotypic and genotypic differences, strain MC-20^T was assigned to a novel species of the genus *Methanobacterium* for which the name *Methanobacterium movilense* sp. nov. is proposed. The type strain is MC-20^T (=DSM 26032^T=JCM 18470^T).

Movile Cave is a groundwater system located close to the city of Mangalia, a few kilometres from the Black Sea coast of Romania. This karstic region is characterized by numerous thermal springs (Sarbu & Popa, 1992). The cave is developed in Sarmatian limestone on two levels, one of which is dry while the other is submerged and forms a siphon. Where the ceiling of the flooded gallery rises above the level of the water, sealed spaces, called air bells, form. Proven to be almost completely isolated from the surface (Sarbu *et al.*, 1996), one of the striking features of this system is the constancy of its physical and chemical parameters. Thus, the cave is known for its unique subsurface ecosystem with groundwater rich in hydrogen sulfide (up to 10 mg l^{-1}), methane (up to 6 mg l^{-1}) and ammonium (up to 6 mg l^{-1}), being anoxic from a depth of 5 cm, and an atmospheric composition in the air bells of 7–10 % O_2, 2–3.5 % CO_2 and 1–2 % CH_4 (Sarbu & Kane, 1995; Sarbu *et al.*, 1996). The pH of the water is 7.4 while its temperature is between 22 and 24 °C. In contrast to the majority of the caves from the temperate zone in this area, where temperatures match the mean annual external

Abbreviations: GDGT, glycerol diphytanyl glycerol tetraether; HPLC-ESI-MS, HPLC electrospray interface MS.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Methanobacterium movilense* MC-20^T is JF812256.

Two supplementary figures are available with the online version of this paper.

temperatures, namely around 10–12 °C, in Movile Cave, due to the presence of mesothermal water and its lack of openings, the temperature of the atmosphere reaches 20 °C in the dry passages and slightly higher in the air bells. Life in the cave has been separated from the external environment for the past 5.5 million years and it is based exclusively on chemosynthesis such as sulfur oxidation (Sarbu *et al.*, 1996; Falniowski *et al.*, 2008). This microbial primary production acts as a food base for invertebrates, such as leeches, spiders, scorpions and insects. In total, 48 novel species have been described from the cave, 33 of which are endemic (Sarbu, 2000).

Recently, the general diversity of bacteria and archaea was analysed by 16S rRNA gene sequence and functional gene analyses, showing a large diversity of bacteria and archaea in the cave (Chen *et al.*, 2009). More detailed investigations on microbial diversity were performed for the group of methanotrophic bacteria by stable isotope probing, which identified a diverse range of methanotrophs belonging to the classes *Alphaproteobacteria* and *Gammaproteobacteria* (Hutchens *et al.*, 2004). Although the microbial communities of Movile Cave have been of interest since the ecosystem was discovered in 1986, to our knowledge only one novel strain, *Thiobacillus thioparus* LV43, has been isolated so far (Vlasceanu *et al.*, 1997). This strain is abundant in the microbial mats and characterized by ribulose-1,5-biphosphate carboxylase activity (Sarbu *et al.*, 1994).

The genus *Methanobacterium* (WoRMS, 2013) comprises numerous recognized species isolated from different environments (e.g. *Methanobacterium oryzae*, rice field soil, Joulian *et al.*, 2000; *Methanobacterium aarhusense*, marine sediment, Shlimon *et al.*, 2004; *Methanobacterium veterum*, permafrost, Krivushin *et al.*, 2010; and *Methanobacterium lacus*, lake sediment, Borrel *et al.*, 2012), but not from a groundwater ecosystem such as Movile Cave. Some strains of the genus *Methanobacterium* are able to use secondary alcohols as a substrate, such as *Methanobacterium palustre* (Zellner *et al.*, 1988) and *Methanobacterium bryantii* (Balch *et al.*, 1979).

In this study, we describe the characteristics of a novel methanogenic archaeon, strain MC-20^T, which was enriched and isolated from anoxic sediment of the subsurface lake in Movile Cave, Mangalia, south-east Romania.

Strain MC-20^T was isolated from anoxic, grey-coloured sediment samples obtained in summer 2002. Enrichment of the strain was performed with 2 g fresh sediment added to 50 ml of sterile anoxic medium of the following composition (per litre): 0.15 g K_2HPO_4, 0.3 g KH_2PO_4, 0.6 g NaCl, 0.3 g $(NH_4)_2SO_4$, 0.12 g $MgSO_4.7H_2O$, 0.08 g $CaCl_2.2H_2O$, 40 g $NaHCO_3$, 0.5 g $Na_2S.3H_2O$, 10 ml Wolfe's trace element solution (per litre: 30 mg $MgSO_4.7H_2O$, 15 mg nitrilotriacetic acid, 5 mg $MnSO_4.4H_2O$, 1 mg $FeSO_4.7H_2O$, 1 mg $CoCl_2.6H_2O$, 1 mg $ZnSO_4.7H_2O$, 0.1 mg H_3BO_3, 1 mg $NaMoO_4.2H_2O$, 0.1 mg $CuSO_4.5H_2O$), 10 ml Wolfe's vitamin solution (per litre: 0.1 mg pyridoxine hydrochloride, 0.05 mg calcium D-(+)-pantothenate, 0.05 mg lipoic acid, 0.05 mg nicotinic acid, 0.05 mg *p*-aminobenzoic acid, 0.05 mg thiamine hydrochloride, 0.02 mg biotin, 0.02 mg folic acid, 0.001 mg vitamin B12) and 2 ml resazurin indicator solution. pH was adjusted to 7.2. The bottles were sealed with butyl rubber stoppers and secured with an aluminium crimp collar. After flushing the headspace with H_2/CO_2 (80 : 20, v/v, 100 kPa) the bottles were pressurized with N_2/CO_2 (80 : 20, v/v, 200 kPa) and incubated at 28 °C in the dark. After methane production was observed in the headspace, 5 ml of the culture were transferred to a new bottle with 50 ml of sterile anoxic medium, which was additionally supplemented with the antibiotics phosphomycin and erythromycin (each at 50 µl ml^{-1}) to suppress growth of non-methanogenic micro-organisms (Hilpert *et al.*, 1981). This procedure was repeated until a pure culture was obtained. All further incubations, including purity checks, were done without any antibiotics. The purity of the strain was confirmed by light microscope examination, the absence of growth in rich medium containing (per litre) 4 g glucose, 2 g yeast extract and 2 g peptone, and denaturing gradient gel electrophoresis analyses of DNA extracts obtained from the culture. The strain was maintained by 3 months of transfer into liquid medium. After regrowth at 28 °C the culture was stored at 5 °C. All preparation steps were done under strictly anaerobic conditions.

A light microscope (Axioskop 2; Zeiss) was used to perform phase-contrast microscopy of cells in the exponential growth phase. The results showed that cells of strain MC-20^T were short rods 3.5–4.0 µm in length and 0.6–0.7 µm in width (Fig. S1 available in IJSEM Online). Cells were non-motile and stained Gram-negative. Lysis of the cells was not observed in an SDS solution of up to 1 % (w/v).

Growth and substrate utilization were determined by culturing strain MC-20^T in the medium described above and growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The methane concentration was measured by GC as described previously (Morozova & Wagner, 2007). All growth tests were performed in triplicate at 28 °C. The effect of temperature on growth was tested using H_2/CO_2 (80 : 20, v/v) as substrate at 0, 5, 10, 16, 22, 28, 33, 38, 44, 54 and 64 °C. Growth of strain MC-20^T was observed at 0–44 °C with optimum growth at 33 °C (Fig. S2a). The pH range for growth was adjusted to pH 4.1–9.9 with 1 M HCl or 1 M NaOH. Growth was observed between pH 6.2 and 9.9 with optimum growth at pH 7.4 (Fig. S2b). The salinity range was determined in medium with 0.02–0.6 M NaCl. Optimum growth was measured at 0.08 M and salt concentrations of up to 0.3 M were tolerated (Fig. S2c). The substrate spectrum of strain MC-20^T was determined by addition of the following carbon sources to the growth medium: H_2/CO_2 (80 : 20, v/v, 150 kPa), sodium formate (80 mM), sodium acetate (40 mM), methanol (20 mM), ethanol (20 mM), 2-propanol (10 mM), 2-butanol

(20 mM), dimethyl sulfide (20 mM), monomethylamine (20 mM), dimethylamine (20 mM) and trimethylamine (20 mM). Cultures were incubated at 28 °C for 10 weeks and growth was subsequently monitored through GC measurements of methane in the headspace and by visual analysis of increasing turbidity. Growth was observed with H_2/CO_2, formate, 2-propanol and 2-butanol, but not with acetate, methanol, ethanol, dimethyl sulfide, monomethylamine, dimethylamine or trimethylamine (Table 1). The generation time with H_2/CO_2 at 28 °C was 3.3 ± 0.2 days (mean ± SD).

Intact membrane lipids were examined for strain MC-20^T and its closest phylogenetic relatives, *Methanobacterium lacus* 17A1^T and *Methanobacterium oryzae* FPi^T (see below), using a method described by Zink & Mangelsdorf (2004). The intact lipids were detected with a HPLC electrospray interface MS (HPLC-ESI-MS) system. Strain MC-20^T contained a set of membrane diether lipids, detected in the HPLC-ESI-MS negative and positive ion mode. The major lipids were archaeol phosphatidylethanolamine (at m/z=774 $[M-H]^-$ or m/z=776 $[M+H]^+$) and diglycosyl archaeol (at m/z=975 [M−H]− or m/z=999

Table 1. Characteristics of strain MC-20^T and related species of the genus *Methanobacterium*

Strains: 1, MC-20^T (data from this study); 2, *Methanobacterium oryzae* FPi^T (Joulian *et al.*, 2000); 3, *Methanobacterium lacus* 17A1^T (Borrel *et al.*, 2012); 4, *Methanobacterium beijingense* 8-2^T (Ma *et al.*, 2005). +, Positive; −, negative; †, detected; ‡, moderately abundant; §, abundant; ND, not determined. ArPE, archaeol phosphatidylethanolamine; Ar-PI, archaeol phosphatidylinositol; Ar-Gly, glycosyl archaeol; Ar-diGly, diglycosyl archaeol; UK, unknown coumpound (diglycosyl-di-*O*-alkyl-glycerol); Gly-GDGT, glycosyl-glycerol diphythanyl glycerol tetraether; diGly-GDGT, diglycosyl-GDGT; GlyP-GDGT, glycosyl-phosphatidyl-GDGT; diGlyP-GDGT, diglycosyl-phosphatidyl-GDGT or glycosyl-phosphatidyl-GDGT-hexose; diGlyP-GDGT-Gly, diglycosyl-phosphatidyl-GDGT-hexose.

Characteristic	1	2	3	4
Source	Subsurface lake sediment	Rice field soil	Lake sediment	Anaerobic digester
Cell dimensions (μm)	0.6–0.7 × 3.5–4.0	0.3–0.4 × 3–10	0.2–0.4 × 2–15	0.4–0.5 × 3–5
Gram stain	−	ND	−	−
Temperature range (°C)	0–44	20–42	14–41	25–50
Optimum temperature (°C)	33	40	30	37
pH range	6.2–9.9	6.0–8.5	5–8.5	6.5–8.0
Optimum pH	7.4	7.0	6.5	7.2
Tolerance of NaCl (M)	0.02–0.6	0–0.4	0–0.4	0–0.5
Optimum NaCl for growth	0.08	0.08	0.1	ND
Utilization of:				
H_2/CO_2	+	+	+	+
Methanol	−	−*	(+H_2) +	−
Ethanol	−	−*	−*	−
2-Propanol	+	−	−	−
2-Butanol	+	−	−	−
Acetate	−	−*	−	−
Formate	+	+	−	+
Dimethyl sulfide	−	−*	−*	−*
Monomethylamine	−	−*	−*	−*
Dimethylamine	−	−*	−*	−*
Trimethylamine	−	−*	−	−
DNA G+C content (mol%)	33.0	31.0	37.0	38.9
ArPE	§	§	‡	ND
Ar-PI	†	†	†	ND
Ar-Gly	†	†	‡	ND
AR-diGly	§	†	§	ND
UK	−	−	§	ND
Gly-GDGT	−	−	†	ND
diGly-GDGT	−	−	†	ND
GlyP-GDGT	−	†	†	ND
diGlyP-GDGT	−	−	†	ND
diGlyP-GDGT-Gly	−	†	†	ND
Generation time (days) at 28 °C	3.3 ± 0.2	ND	0.9	ND

*Data obtained in this study.

$[M+Na]^+$), and the minor lipids were archaeol phosphatidylinositol (at m/z=893 $[M-H]^-$ or m/z=895 $[M+H]^+$) and glycosyl archaeol (at m/z=837 $[M+Na]^+$; Table 1). The two closest relatives, *Methanobacterium lacus* 17A1^T and *Methanobacterium oryzae* FPi^T, also contained these membrane lipids. However, the two species contain some additional tetraether membrane lipids (GDGT; glycerol diphytanyl glycerol tetraether) in small amounts (Table 1), which were not present in strain MC-20^T. *Methanobacterium lacus* 17A1^T additionally contained glycosyl-GDGT (m/z=1486 $[M+Na]^+$), diglycosyl-GDGT (m/z=1648 $[M+Na]^+$), glycosyl-phosphatidyl-GDGT (m/z=1544 $[M+H]^+$) and diglycosyl-phosphatidyl-GDGT (m/z=1728 $[M+Na]^+$) or glycosyl-phosphatidyl-GDGT-hexose (m/z=1728 $[M+Na]^+$) and diglycosyl-phosphatidyl-GDGT-hexose (m/z=1890 $[M+H]^+$). *Methanobacterium oryzae* FPi^T additionally contained only glycosyl-phosphatidyl-GDGT and diglycosyl-phosphatidyl-GDGT-hexose. Furthermore, *Methanobacterium lacus* 17A1^T had, in relatively high amounts, an as yet unknown diether lipid that is tentatively identified as diglycosyl-di-*O*-alkyl-glycerol (m/z=1208 $[M+Na]^+$). The mixture of mainly diethers (forming bilayers) with minor amounts of tetraethers (usually forming monolayers) points to a partly 'riveted' membrane bilayer in *Methanobacterium lacus* and *Methanobacterium oryzaee* as known also from other methanogens (De Rosa *et al.*, 1994).

For phylogenetic analysis, cells of strain MC-20^T were centrifuged and DNA was extracted using the UltraClean Soil DNA kit (MO BIO Laboratories) according to the manufacturer's protocol. Amplification of the 16S rRNA gene was carried out with primers ArUn4F (5′-TCYGGTTGATCCTGCCRG-3′; Hershberger *et al.*, 1996) and Arc1492R (5′-GGCTACCTTGTTACGACTT-3′; DeLong, 1992). PCR fragments were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced by GATC Biotech (Konstanz, Germany). Sequencing resulted in a 1347 bp gene product for strain MC-20^T that was automatically aligned with closely related sequences obtained from GenBank using the integrated SINA aligner (Pruesse *et al.*, 2007) from the ARB-SILVA website (www.arb-silva.de/aligner) and imported into ARB (Ludwig *et al.*, 2004). After manual refinement of the alignment, evolutionary distances were calculated and a phylogenetic tree (Fig. 1) was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) with a termini filter that is implemented in the ARB program. To evaluate the tree topologies, a bootstrap analysis with 1000 replications was performed. Strain MC-20^T showed highest 16S rRNA gene sequence similarity with the type strains of *Methanobacterium oryzae* FPi^T (97.1 %) and *Methanobacterium lacus* 17A1^T (97.0 %). DNA–DNA hybridization experiments were not carried out as the sequence similarities to the nearest relatives were clearly below 98.7 %, as recommended by Stackebrandt & Ebers (2006). The genomic DNA G+C content of the novel strain was determined through HPLC (Tamaoka & Komagata,

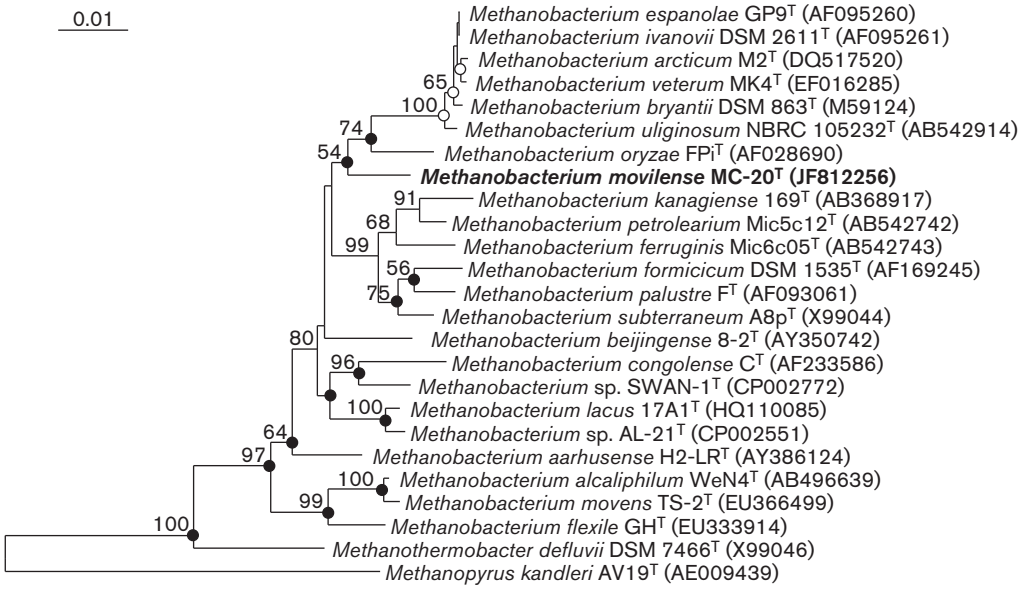

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain MC-20^T within the genus *Methanobacterium* (and *Methanopyrus kandleri* AV19^T as outgroup). Branches marked with open circles were also found in the maximum-parsimony tree (Felsenstein, 1981) whereas branches with filled circles were also found in both maximum-parsimony and maximum-likelihood trees (Fitch, 1971). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values $\geqslant$50 % are shown. Bar, 0.01 substitutions per nucleotide position.

1984) and calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah *et al.* (1989). The DNA G+C content of strain MC-20^T was 33.0 mol%.

Based on the phylogenetic and physiological characteristics determined according to the minimal standards for the description of new taxa of prokaryotic strains (Tindall *et al.*, 2010), strain MC-20^T is considered to represent a novel species of the genus *Methanobacterium*, for which the name *Methanobacterium movilense* sp. nov. is proposed.

Description of *Methanobacterium movilense* sp. nov.

Methanobacterium movilense (mo.vil.en′se. N.L. neut. adj. *movilense* of Movile, as a reference to Movile Cave, the source of the type strain).

Cells are strictly anaerobic, Gram-stain-negative, non-motile rods 0.6–0.7 μm in width and 3.5–4.0 μm in length, that occur either as single cells or short chains. Cells grow on H_2/CO_2, formate, 2-butanol and 2-propanol, but not on acetate, methanol, monomethylamine, dimethylamine, trimethylamine or dimethyl sulphide. Optimal growth occurs at 33 °C (range 0–44 °C), pH 7.4 (pH 6.2–9.9) and 0.08 M NaCl (0.02–0.6 M). The major cell membrane lipids are archaeol phosphatidylethanolamine and diglycosyl archaeol, with smaller amounts of archaeol phosphatidylinositol and glycosyl archaeol. The generation time is 3.3±0.2 days at 28 °C with hydrogen as substrate.

The type strain is MC-20^T (=DSM 26032^T=JCM 18470^T), isolated from sediment of the subsurface lake from Movile Cave, near the city of Mangalia, Romania. The G+C content of the genomic DNA of the type strain is 33.0 mol%.

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