Methanosarcina spelaei sp. nov., a methanogenic archaeon isolated from a floating biofilm of a subsurface sulphurous lake

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A novel methanogenic archaeon, strain MC-15^T, was isolated from a floating biofilm on a sulphurous subsurface lake in Movile Cave (Mangalia, Romania). Cells were non-motile sarcina-like cocci with a diameter of 2–4 μm, occurring in aggregates. The strain was able to grow autotrophically on H_2/CO_2. Additionally, acetate, methanol, monomethylamine, dimethylamine and trimethylamine were utilized, but not formate or dimethyl sulfide. Trypticase peptone and yeast extract were not required for growth. Optimal growth was observed at 33 °C, pH 6.5 and a salt concentration of 0.05 M NaCl. The predominant membrane lipids of MC-15^T were archaeol and hydroxyarchaeol phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol as well as hydroxyarchaeol phosphatidylserine and archaeol glycosaminyl phosphatidylinositol. The closely related species, *Methanosarcina vacuolata* and *Methanosarcina horonobensis*, had a similar composition of major membrane lipids to strain MC-15^T. The 16S rRNA gene sequence of strain MC-15^T was similar to those of *Methanosarcina vacuolata* DSM 1232^T (sequence similarity 99.3 %), *Methanosarcina horonobensis* HB-1^T (98.8 %), *Methanosarcina barkeri* DSM 800^T (98.7 %) and *Methanosarcina siciliae* T4/M^T (98.4 %). DNA–DNA hybridization revealed 43.3 % relatedness between strain MC-15^T and *Methanosarcina vacuolata* DSM 1232^T. The G+C content of the genomic DNA was 39.0 mol%. Based on physiological, phenotypic and genotypic differences, strain MC-15^T represents a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina spelaei* sp. nov. is proposed. The type strain is MC-15^T (=DSM 26047^T=JCM 18469^T).

The Movile Cave is a chemosynthetically driven groundwater ecosystem which is located close to the Black Sea coast (43° 49N′ 28° 33′ E) near the city of Mangalia, Romania. At a depth of about 23 m (0 m above sea level) a subsurface lake exists containing high levels of hydrogen sulphide (up to 10 mg l^{-1}), methane (up to 6 mg l^{-1}) and ammonium (up to 6 mg l^{-1}). The cave ecosystem is characterized by having an atmosphere of 7–10 % O_2, 2–3.5 % CO_2 and 1–2 % CH_4 (Sarbu & Kane, 1995; Sarbu *et al.*, 1996). The temperature of the cave atmosphere is constant all year round and since there are no openings other than the artificial access shaft, and mesothermal water is present, it is about 19 to 20 °C in the galleries and up to 22 °C in the airbells. This is much higher than the typical temperature of most caves in the temperate zone, which are at about 11 to 12 °C; the annual mean external temperature. The pH of the water is 7.4, while its temperature is between 22 and 24 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of MC-15^T is JF812257. The accession number of the partial sequence of the *mcrA* gene is KJ608060.

In spite of the conditions being apparently unsuitable for sustaining life, the Movile biocenosis contains about 50 species of invertebrate, 33 of these being endemic to this cave (Sarbu, 2000). This diverse and extremely rich biocenosis was isolated from the outside world about 5.5 million years ago, probably during the Messinian Salinity Crisis, and its existence has been proven to be almost exclusively based on chemosynthesis (Sarbu *et al.*, 1996; Falniowski *et al.*, 2008). Chemosynthetic processes (such as sulphur oxidation, sulphate reduction, methanotrophy and methanogenesis) are performed by microorganisms forming biofilms that float on the surface of the water and covering the ceiling of the galleries near the water.

Since the cave system was discovered in 1986, several microbiological investigations have been conducted (Sarbu *et al.*, 1994; Vlasceanu *et al.*, 1997; Rohwerder *et al.*, 2003; Hutchens *et al.*, 2004; Chen *et al.*, 2009). Recently, a novel species of methanogenic archaea, *Methanobacterium movilense* sp. nov., was isolated from an anoxic sediment of the subsurface lake (Schirmack *et al.*, 2014).

At the time of writing, the genus *Methanosarcina* comprises 11 species with validly published names, which have been isolated from various habitats such as an anaerobic sludge digester (Zinder *et al.*, 1985), marine and lacustrine sediments (Sowers *et al.*, 1984; Lyimo *et al.*, 2000; Simankova *et al.*, 2001; von Klein *et al.*, 2002), as well as the deep subsurface (Shimizu *et al.*, 2011) and the active layer of permafrost soils (Wagner *et al.*, 2013).

Here, we report on and describe a novel methanogenic archaeon, strain MC-15^T, belonging to the genus *Methanosarcina*, and isolated from the microbial mat floating on the subsurface lake of Movile Cave (Mangalia, south-east Romania).

Strain MC-15^T was isolated from a floating biofilm-like mat obtained from the surface of Movile Cave water in 2002. Pre-incubation for the enrichment of methanogenic archaea was carried out with a 1 g sample of biofilm in 50 ml sterile bicarbonate-buffered, anoxic OCM medium (Boone *et al.*, 1989). Acetate, methanol (each at a final concentration of 20 mM) or H_2/CO_2 (80 : 20, v/v, 1 bar) were used as substrates. Bottles were sealed with butyl rubber stoppers and secured with aluminium crimp collars. After flushing the headspace with H_2/CO_2, the bottles were pressurized with N_2/CO_2 (80 : 20, v/v, 2 bar) and incubated at 28 °C in the dark. After methane production was observed, 5 ml enrichment culture was transferred to new bottles containing 50 ml sterile anoxic medium, which was additionally supplemented with a mixture of the antibiotics phosphomycin and erythromycin (each at 50 μl ml^{-1}) to suppress the growth of non-archaeal microorganisms (Hilpert *et al.*, 1981).

For further isolation, cultivation and physiological tests the following medium was used (l^{-1}): 2.7 g $NaHCO_3$, 1.0 g NaCl, 0.5 g KCl, 0.4 g $MgCl_2 . 6\ H_2O$, 0.3 g KH_2PO_4, 0.3 g cysteine hydrochloride, 0.3 g Na_2S, 0.25 g NH_4Cl and 0.1 g $CaCl_2 . 2\ H_2O$. Additionally, 10 ml trace element solution (l^{-1}: 30 mg $MgSO_4 . 7H_2O$, 15 mg nitrilotriacetic acid, 5 mg $MnSO_4 . 4H_2O$, 1 mg $FeSO_4 . 7H_2O$, 1 mg $CoCl_2 . 6\ H_2O$, 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 vitamin solution (l^{-1}: 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 B_{12}) and 2 ml resazurin solution as redox indicator was added. The substrate was H_2/CO_2 (80 : 20, v/v), except when testing for growth on different substrates. The pH was adjusted to 7.0.

Isolation of strain MC-15^T was performed by serial dilution, up to 10^{-4}, in the medium described above, using the most dilute culture with positive growth as an inoculum for the next series of dilutions. This procedure was repeated until a pure culture was obtained. All further incubations, including purity checks, were performed without any added antibiotics. The purity of the strain was verified by light microscopy, denaturing gradient gel electrophoresis (DGGE; D-Code System, Bio-Rad) and by testing for the absence of growth in medium that contained (l^{-1}): 4 g glucose, 2 g yeast extract and 2 g peptone. Gram staining was carried out using a classic method described by Süssmuth *et al.* (1999). The strain was maintained by transferring every three months into liquid medium. After regrowth at 28 °C the culture was stored at 5 °C before inoculating again. Long-term storage was in 50 % (v/v) glycerol at −80 °C. All the preparation steps were performed 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 show that strain MC-15^T cells are sarcina-like cocci with a diameter of 2–4 μm that occur in aggregates (Fig. 1). Cells were non-motile and Gram-stain-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-15^T in the medium described above. Growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The methane concentration was measured by gas chromatography as described previously (Morozova & Wagner, 2007). All tests of growth 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. Strain MC-15^T was seen to grow at temperatures of between 0 and 54 °C, with an optimum at 33 °C (Fig. 2a). The salinity range for growth was determined in medium with 0.02–0.6 M NaCl present. Optimum growth was at 0.05 M NaCl (Fig. 2b). The pH range was adjusted with 1 M HCl and 1 M NaOH to initial pH values of between pH 4 and 10, respectively. No significant shift in pH was observed during growth. Growth was measured over the entire pH range tested with optimum growth occurring at

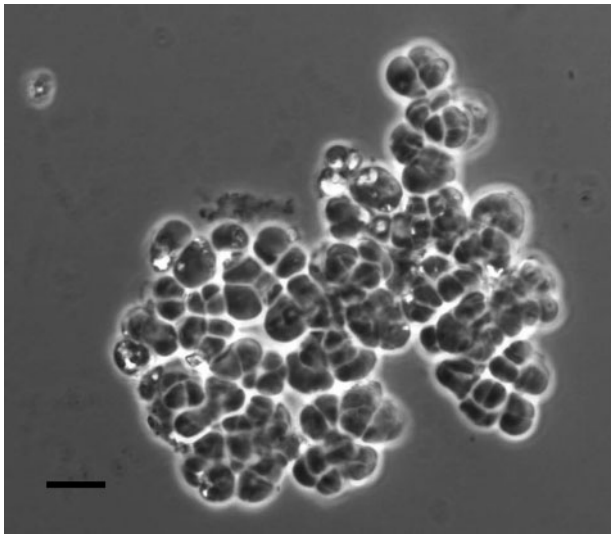

Fig. 1. Phase-contrast micrograph of cells of strain MC-15^T. Bar, 5 μm.

pH 6.5 (Fig. 2c). The substrate spectrum of strain MC-15^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), 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 by measuring methane in the headspace by gas chromatography and by visual analysis of the increase in turbidity. Growth was observed with H_2/CO_2, acetate, methanol, monomethylamine, dimethylamine and trimethylamine but not with dimethyl sulfide and formate (Table. 1). The addition of acetate, trypticase or yeast extract did not stimulate growth with H_2/CO_2. The generation time with H_2/CO_2 at 28 °C was 5.1 ± 0.3 days.

Intact membrane lipids of strain MC-15^T and of its closest phylogenetic relatives, *Methanosarcina vacuolata* DSM 1232^T (Zhilina & Zavarzin, 1987) and *Methanosarcina horonobensis* HB-1^T (Shimizu *et al.*, 2011) were examined, using the method described by Zink & Mangelsdorf (2004). The intact lipids were detected using an HPLC electrospray interface MS system (HPLC-ESI-MS). Strain MC-15^T contained a set of diether membrane lipids (Table 1). The major lipids were archaeol and hydroxyarchaeol phosphatidylethanolamine (ArPE, $[M\text{-}H]^-$ at *m*/*z* 774; ArOHPE, $[M\text{-}H]^-$ at *m*/*z* 790), phosphatidylglycerol (ArPG, $[M\text{-}H]^-$ at *m*/*z* 805; ArOHPG, $[M\text{-}H]^-$ at *m*/*z* 821) and phosphatidylinositol (ArPI, $[M\text{-}H]^-$ at *m*/*z* 893; ArOHPI, $[M\text{-}H]^-$ at *m*/*z* 909) as well as hydroxyarchaeol phosphatidylserine (ArOHPS, $[M\text{-}H]^-$ at *m*/*z* 834) and archaeol glycosaminyl phosphatidylinositol (ArGlcN-PI, $[M\text{-}H]^-$ at *m*/*z* 1054). Minor lipids were dihydroxyarchaeol phosphatidylglycerol (Ar-diOHPG, $[M\text{-}H]^-$ at *m*/*z* 837), archaeol phosphatidylserine

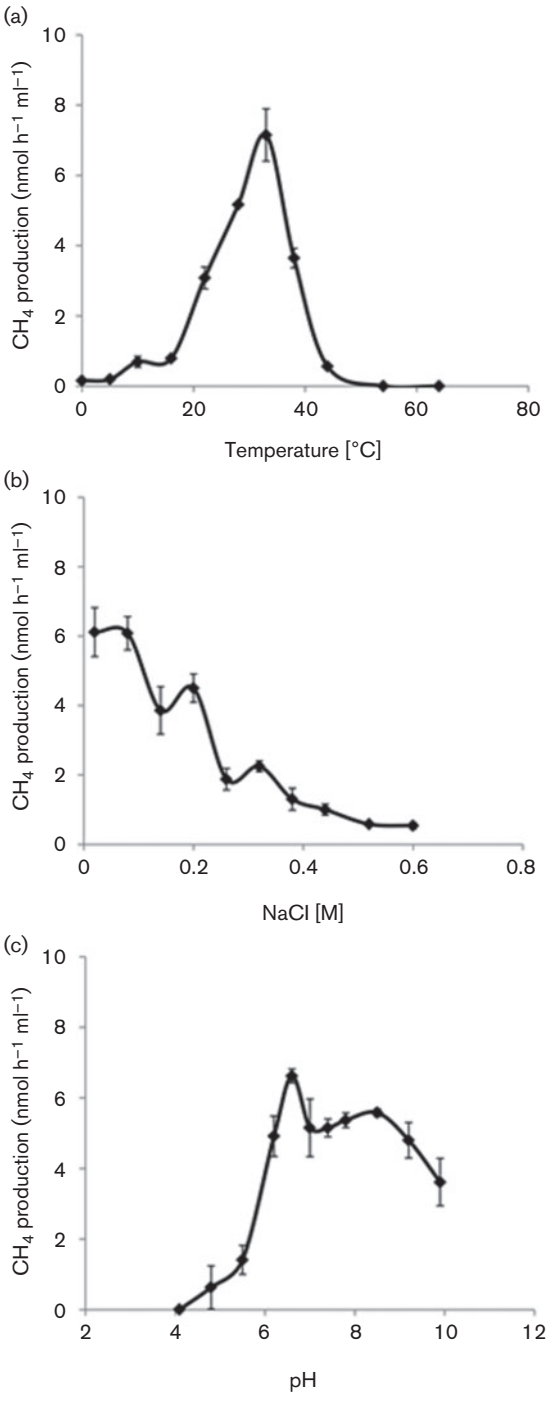

Fig. 2. Graphs showing the dependence of methane production of strain MC-15^T on temperature (a); salt (NaCl) concentration (b) and pH (c). Methane production was measured in the linear phase of growth. Data are means ± SE (n=3).

(ArPS, $[M\text{-}H]^-$ at *m*/*z* 818), hydroxyarchaeol glycosaminyl phosphatidylinositol (ArOHGlcN-PI, $[M\text{-}H]^-$ at *m*/*z* 1070), diglycosyl hydroxyarchaeol (ArOH-diGly, $[M\text{-}H]^-$ at *m*/*z* 991), hydroxyarchaeol phosphatidyl-L-threonine (ArOHPT,

Table 1. Characteristics of strain MC-15^T and related species of the genus *Methanosarcina*

Strains: 1 *Methanosarcina spelaei* sp. nov. MC-15^T (data from this study); 2 *Methanosarcina vacuolata* DSM 1232^T (Zhilina & Zavarzin, 1987); 3 *Methanosarcina horonobensis* HB-1^T (Shimizu *et al.*, 2011); 4 *Methanosarcina siciliae* T4/M^T (Ni & Boone, 1991). +, positive; –, negative; †, detected; ‡, moderately abundant; §, abundant; ND, not determined. ArPE, archaeol phosphatidylethanolamine; ArOHPE, hydroxyarchaeol phosphatidylethanolamine; Ar-diOHPE, dihydroxyarchaeol phosphatidylethanolamine; ArPG, archaeol phosphatidylglycerol; ArOHPG, hydroxyarchaeol phosphatidylglycerol; Ar-diOHPG, dihydroxyarchaeol phosphatidylglycerol; ArPS, archaeol phosphatidylserine; ArOHPS, hydroxyarchaeol phosphatidylserine; Ar-diOHPS, dihydroxyarchaeol phosphatidylserine; ArPI, archaeol phosphatidylinositol; ArOHPI, hydroxyarchaeol phosphatidylinositol; Ar-diOHPI, dihydroxyarchaeol phosphatidylinositol; ArGlcN-PI, archaeol glycosaminyl phosphatidylinositol; ArOHGlcN-PI, hydroxyarchaeol glycosaminyl phosphatidylinositol; Ar-diGly, diglycosyl archaeol; ArOH-diGly, diglycosyl hydroxyarchaeol; diGly-GDGT, Diglycosyl glyceroldibiphytanylglycerol tetraether; ArOHPT, hydroxyarchaeol phosphatidyl-l-threonine; ArPGP-Me, archaeol phosphatidylglycerophosphate methylester

Characteristic	1	2	3	4
Source	Floating mat, lake	Anaerobic digestor	Groundwater	Marine sediment
Cell dimension (μm)	2.0–4.0	1.0–2.0	1.4–2.9	1.5–3.0
Gram stain	–	+	–	–
Temperature range for growth (°C)	0–54	18−42	20−42	>20−48
Optimum temperature (°C)	33	37−40	37	37−40
pH range for growth	4.1– >9.9	6.0–8.0	6.0–7.8	5.8–7.2
Optimum pH	6.6	7.5	7.0−7.3	6.5−6.8
Tolerance of NaCl (M)	0.02–>0.6	0.1−0.6	0−0.35	0−>1.7
Optimum NaCl conc. (M)	0.02	0.1−0.2	0.1	0.4−0.6
Utilization of:				
H_2/CO_2	+	+	–	–
Methanol	+	+	+	+
Acetate	+	+	+	–
Formate	–	–	–	–
Dimethyl sulfide	–	–*	+*	+
Monomethylamine	+	+	–	ND
Dimethylamine	+	+	+*	ND
Trimethylamine	+	+	+*	+
DNA G+C content (mol%)	39.0	36.3	41.4	41.5−43
Membrane lipids				
ArPE	‡*	‡*	‡*	ND
ArOHPE	‡*	‡*	‡*	ND
Ar-diOHPE	–	–	†*	ND
ArPG	‡*	‡*	‡*	ND
ArOHPG	§*	§*	§*	ND
Ar-diOHPG	†*	†*	†*	ND
ArPS	†*	†*	–	ND
ArOHPS	‡*	‡*	‡*	ND
Ar-diOHPS	–	–	†*	ND
ArPI	‡*	‡*	‡*	ND
ArOHPI	‡*	‡*	‡*	ND
Ar-diOHPI	–	–	†*	ND
ArGlcN-PI	‡*	‡*	†*	ND
ArOHGlcN-PI	†*	†*	†*	ND
Ar-diGly	–	‡*	–	ND
ArOH-diGly	†*	‡*	–	ND
diGly-GDGT	–	–	†*	ND
ArOHPT	†*	†*	‡*	ND
ArPGP-Me	†*	–	†*	ND
Generation time at 28 °C (days)	5.1±0,3	ND	ND	ND

*Data obtained in this study.

$[M-H]^-$ at m/z 848) and archaeol phosphatidylglycerophosphate methyl ester (ArPGP-Me, $[M-H]^-$ at m/z 899). The lipid inventory of the close relatives, *Methanosarcina vacuolata and Methanosarcina horonobensis*, largely corresponded with the lipids found in MC-15^T. However, *Methanosarcina vacuolata* did not contain ArPGP-Me, but additionally did contain diglycosyl archaeol (Ar-diGly, $[M-H]^-$ at m/z 975) in significant amounts. *Methanosarcina horonobensis* did not contain ArPS and ArOH-diGly, but had dihydroxyarchaeol phosphatidylethanolamine (Ar-diOHPE, $[M-H]^-$ at m/z 806), phosphatidylserine (Ar-diOHPS, $[M-H]^-$ at m/z 850) and phosphatidylinositol (Ar-diOHPI, $[M-H]^-$ at m/z 925) as well as diglycosyl glyceroldibiphytanylglycerol tetraether (diGly-GDGT, $[M-H]^-$ at m/z 1624) in minor amounts.

Isolation of genomic DNA from strain MC-15^T was carried out using a microbial DNA isolation kit (MoBio) according to the manufacturer's protocol. For 16S rRNA gene amplification, general archaeal primers ArUn4F (5′-TCYGGTTGATCCTGCCRG-3′) (Hershberger *et al.*, 1996) and Arc1492R (5′-GGCTACCTTGTTACGACTT-3′) (Delong, 1992) were used. Sequencing was performed by GATC Biotech (Konstanz, Germany) and resulted in a 1346 bp gene product. Alignments were carried out with all isolates of the genus *Methanosarcina* with validly published names. Sequences were obtained from GenBank and aligned using the integrated SINA alignment tool from the ARB-SILVA website (Pruesse *et al.*, 2007). Sequences were checked manually using the ARB program (Ludwig *et al.*, 2004) and evolutionary distances were calculated based on neighbour-joining. Reconstruction of a phylogenetic tree was done by using the neighbour-joining method (Saitou & Nei, 1987; Fig. 3) and a termini filter implemented in the ARB program. To evaluate the tree topologies, bootstrap analysis with 1000 replications was performed. The 16S rRNA sequence of strain MC-15^T was closely related to that of *Methanosarcina vacuolata* DSM 1232^T (pairwise sequence similarity 99.3 %), *Methanosarcina horonobensis* HB-1^T (98.8 %), *Methanosarcina barkeri* DSM 800^T (98.7 %) and *Methanosarcina siciliae* T4/M^T (98.4 %). Additionally, the partial sequence of the methyl coenzyme-M reductase gene (*mcrA*) was provided. The primer sets mlas/mcrA-rev (Steinberg & Regan, 2008) and ME1/ME2 (Hales *et al.*, 1996) were used to achieve a 736 bp fragment of the *mcrA* gene, which was sequenced as described above. At the DNA level the *mcrA* sequence was related to *Methanosarcina horonobensis* HB-1^T (95.8 %), *Methanosarcina mazei* strain NBRC 101201 (95.1 %) and *Methanosarcina barkeri* strain Fusaro (93.8 %). At the amino acid level the most closely related database entries were from *Methanosarcina mazei* strain MT (98.4 %) and *Methanosarcina horonobensis* HB-1^T (98.0 %).

DNA–DNA hybridization between strain MC-15^T and its closest relative *Methanosarcina vacuolata* DSM 1232^T revealed a reassociation value of 42.6 % ($\pm$0.7 %; two replicates). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostated 6×6 multicell changer and a temperature controller with an *in-situ* temperature probe (Varian). Determination of the genomic DNA G+C content was performed by HPLC according to the method of Tamaoka & Komagata (1984) and calculated from the ratio of deoxyguanosine and thymidine (Mesbah *et al.*, 1989).

Based on the phylogenetic and physiological characteristics and in accordance with the minimal standards for the description of new taxa of prokaryotic strains (Tindall *et al.*, 2010), strain MC-15^T is considered to represent a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina spelaei* sp. nov. is proposed.

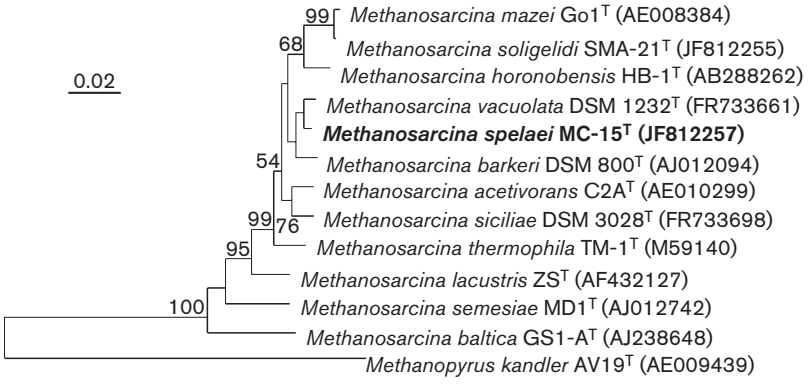

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences of the novel strain MC-15^T within the genus *Methanosarcina* (with *Methanopyrus kandleri* AV19^T as outgroup). The tree was reconstructed by the neighbour-joining algorithm, but all branches were also found in maximum-likelihood (Fitch, 1971) and maximum-parsimony trees (Felsenstein, 1981). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on neighbour-joining analysis of 1000 replications; only values $\geqslant$50 % are shown. Sequence accession numbers of the respective strain are in parentheses. Bar, 0.02 substitutions per nucleotide position.

Description of *Methanosarcina spelaei* sp. nov.

Methanosarcina spelaei (spe.lae′i. L. gen. n. *spelaei* from a cave, with reference to a cave as source of the type strain).

Cells are irregular sarcina-like Gram-stain-negative cocci with a diameter of 2.0–4.0 μm, which appear in aggregates. They are strictly anaerobic and grow autotrophically on H_2/CO_2. Additionally, acetate, methanol, monomethylamine, dimethylamine and trimethylamine are utilized, but not dimethyl sulfide or formate. Trypticase peptone and yeast extract are not required for growth. Optimal growth occurs at 33 °C (range 0–54 °C), pH 6.5 (range pH 4–10) and with 0.05 M NaCl. The generation time is 5.1 ± 0.3 days at 28 °C with H_2/CO_2 as substrate. Main membrane lipids are archaeol and hydroxyarchaeol phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol, as well as hydroxyarchaeol phosphatidylserine and archaeol glycosaminyl phosphatidylinositol. Minor lipids are dihydroxyarchaeol phosphatidylglycerol, archaeol phosphatidylserine, hydroxyarchaeol glycosaminyl phosphatidylinositol, diglycosyl hydroxyarchaeol, hydroxyarchaeol phosphatidyl-L-threonine and archaeol phosphatidylglycerophosphate methyl ester.

The type strain, MC-15^T (=DSM 26047^T=JCM 18469^T), was isolated from a floating mat of a subsurface lake of Movile Cave (Mangalia, Romania). The DNA G+C content of the type strain is 39.0 mol%.

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