Methanobacterium lacus sp. nov., isolated from the profundal sediment of a freshwater meromictic lake

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An autotrophic, hydrogenotrophic methanogen, designated strain 17A1^T, was isolated from the profundal sediment of the meromictic Lake Pavin, France. The cells of the novel strain, which were non-motile, Gram-staining-negative rods that measured 2–15 μm in length and 0.2–0.4 μm in width, grew as filaments. Strain 17A1^T grew in a mineral medium and its growth was stimulated by the addition of yeast extract, vitamins, acetate or rumen fluid. Penicillin, vancomycin and kanamycin reduced growth but did not completely inhibit it. Growth occurred at 14–41 °C (optimum 30 °C), at pH 5.0–8.5 (optimum pH 6.5) and with 0–0.4 M NaCl (optimum 0.1 M). The novel strain utilized H_2/CO_2 and methanol/H_2 as substrates but not formate, acetate, methylamine/H_2, isobutanol or 2-propanol. Its genomic DNA G+C content was 37.0 mol%. In phylogenetic analyses based on 16S rRNA gene sequences, strain 17A1^T appeared to be a member of the genus *Methanobacterium*, with *Methanobacterium beijingense* 8-2^T (96.3 % sequence similarity) identified as the most closely related established species. Based on phenotypic and phylogenetic data, strain 17A1^T represents a novel species of methanogen within the genus *Methanobacterium*, for which the name *Methanobacterium lacus* sp. nov. is proposed. The type strain is 17A1^T (=DSM 24406^T=JCM 17760^T).

Hydrogenotrophic methanogens appear to be responsible for a large proportion of the methane emissions from freshwater lakes (Conrad, 1999) – emissions that represent a significant proportion (6–16 %) of the global natural emissions of methane (Bastviken *et al.*, 2004). To date, however, only a few methanogens have been isolated from freshwater lake sediments and characterized. These include *Methanomethylovorans hollandica*, which degrades dimethyl sulphide and methanethiol (Lomans *et al.*, 1999), and *Methanosarcina lacustris*, which can produce methane from methanol, methylamine or H_2/CO_2 (Simankova *et al.*, 2001). Lake Pavin in France (GPS coordinates 45° 29.74′ N 2° 53.28′ E) is a natural and deep (92 m) freshwater lake that is unusual because the water column has been stratified for a very long period without mixing (Michard *et al.*, 1994). The sediment is covered by a 30 m-thick, cold (5 °C), anoxic water column that is in a steady state (Assayag *et al.*, 2008). A molecular ecological study (Lehours *et al.*, 2007) revealed that the methanogenic community in this water column consisted mainly of acetoclastic members of the genus *Methanosaeta* and species belonging to the order *Methanomicrobiales* (putative hydrogenotrophs). During a study on methanogens in the sediment of Lake Pavin, several hydrogenotrophic strains were isolated from enrichments containing unidentified species of the genera *Methanosarcina*, *Methanosphaerula* and *Methanobacterium*. We describe here the taxonomic investigation of one of these hydrogenotrophic methanogens, which was designated strain 17A1^T.

In December 2007, samples of sediment from Lake Pavin were collected with a gravity-corer sampler and transported to the research laboratory under anaerobic conditions and at 4 °C in the dark. As soon as the samples reached the laboratory, 1 g sediment was used to inoculate 5 ml medium Mc, under an O_2-free N_2 headspace, in each of several Hungate tubes. Medium Mc, which was also used for the isolation and maintenance of strain 17A1^T, contained (l^{-1}): 0.15 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 0.6 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.024 g $CaCl_2.2H_2O$, 0.20 g NaCl, 0.01 g $FeSO_4.7H_2O$, 0.2 g yeast extract, 0.001 g resazurin, 2.0 g $NaHCO_3$, 0.5 g cysteine-HCl, 5 ml vitamin solution and 10 ml trace element solution. The vitamin solution contained (l^{-1}): 2.0 mg biotin, 2.0 mg

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 17A1^T is HQ110085.

folic acid, 10.0 mg pyridoxine-HCl, 5.0 mg thiamine-HCl . $2H_2O$, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg calcium D-pantothenate, 0.10 mg vitamin B_{12}, 5.0 mg *p*-aminobenzoic acid and 5.0 mg lipoic acid. The trace element solution contained (l^{-1}): 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 . 7H_2O$, 0.50 g $MnSO_4 . H_2O$, 1.0 g NaCl, 0.10 g $FeSO_4 . 7H_2O$, 0.18 g $CoSO_4 . 7H_2O$, 0.10 g $CaCl_2 . 2H_2O$, 0.18 g $ZnSO_4 . 7H_2O$, 0.01 g $CuSO_4 . 5H_2O$, 0.02 g $KAl(SO_4)_2 . 12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 . 2H_2O$, 0.03 g $NiCl_2 . 6H_2O$ and 0.30 mg $Na_2SeO_3 . 5H_2O$. The pH of the medium was adjusted to 6.8 and then the medium was boiled and cooled under O_2-free N_2/CO_2 (80 : 20, v/v) before being transferred to Hungate tubes under an O_2-free N_2/CO_2 (80 : 20, v/v) atmosphere. After inoculation, the headspace was pressurized to 2 atm with O_2-free H_2/CO_2 (80 : 20, v/v) and then the cultures were incubated, without shaking, in the dark at room temperature (about 22 °C). After 4 weeks, the cultures were examined for methane production and the presence of cells fluorescing at 420 nm (Mink & Dugan, 1977). Inocula from methane-positive cultures were serially diluted in fresh Mc medium containing vancomycin (0.2 g l^{-1}) and incubated under O_2-free H_2/CO_2 as before. Methanogen isolates were obtained by colony selection from Mc medium agar roll tubes (Hungate, 1969) inoculated with the most dilute methane-positive cultures. The dilution and colony selection process was repeated twice to yield a pure culture of strain 17A1^T. Cells of the novel strain fluoresced when examined at wavelengths of 340 or 420 nm. Only one cell type was observed in cultures of the strain under phase-contrast and epifluorescence microscopy and there was no growth of contaminating bacteria when medium Mc supplemented with glucose (5 mM), maltose (5 mM), cellobiose (5 mM), starch (5 mM) and yeast extract (0.5 %, w/v) was inoculated with strain 17A1^T.

Growth was measured by turbidimetry (absorbance at 535 nm) and methane was measured by gas chromatography. The temperature range for growth was determined by incubating at 7, 10, 14, 18, 22, 26, 30, 34, 37, 41 and 46 °C, while the pH range for growth was determined in medium Mc pre-equilibrated, by addition of mixtures of sodium acetate, acid acetic, $NaHCO_3$, Na_2CO_3, HCl and NaOH, to give pH values of 4.6, 5, 5.7, 6.5, 7.1, 7.5, 7.9, 8.2, 8.6, 9 and 9.5. Whatever the initial pH value, the pH levels measured before inoculation and 1, 2, 3 and 4 days after inoculation did not differ by more than 0.2 pH unit. The effect of salinity on growth was measured in Mc medium containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7 and 1 M NaCl. Cells for scanning electron microscopy and transmission electron microscopy were prepared by the methods described in Lehours *et al.* (2010) and Chavant *et al.* (2005), respectively.

The identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) used HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984) to determine the genomic DNA G+C content of the novel strain.

For the phylogenetic analysis, the 16S rRNA gene was amplified by PCR using the method described in Skillman *et al.* (2004) and the primers 21f (5′-TTCCGGTTGATCC-YGCCGGA-3′) (DeLong, 1992) and 1386r (5′-GCGGTGT-GTGCAAGGAGC-3′) (Skillman *et al.*, 2004). The PCR products were cloned and then sent to MWG Biotech (Germany) for sequencing. The 16S rRNA gene sequence of the novel strain (1351 bp) and the corresponding sequences of other strains were assessed with BLASTN (Altschul *et al.*, 1990), imported into MEGA4 (http://www.megasoftware.net/; Kitamura *et al.*, 2011) and aligned in CLUSTAL W (Thompson *et al.*, 1994). Alignments were manually checked and the phylogenetic position of strain 17A1^T established using a neighbour-joining tree generated in MEGA4. The robustness of the tree was evaluated using bootstrap analysis, with 1000 resamplings. In the tree, strain 17A1^T clustered with methanogens belonging to the genus *Methanobacterium* (Fig. 1). The 16S rRNA genes of the novel strain and cloned isolates from the sediments of the dystrophic Lake Grosse Fuchskuhle (Chan *et al.*, 2002; GenBank accession number AF481340) and the meromictic Lake Kinneret (Nüsslein *et al.*, 2001; AJ310872) were found to be similar (>97 % sequence similarity). The cultivated, recognized species that appeared most closely related to strain 17A1^T, with a 16S rRNA gene sequence similarity of 96.3 %, was *Methanobacterium beijingense* 8-2^T (Ma *et al.*, 2005). Since its 16S rRNA gene sequence showed <97 % similarity with the corresponding sequences of recognized species of the genus *Methanobacterium*, strain 17A1^T should be considered to represent a novel species (Stackebrandt & Goebel, 1994).

At the time of writing, the genus *Methanobacterium* comprised 19 species with validly published names (Bonin & Boone, 2006; Kitamura *et al.*, 2011; Krivushin *et al.*, 2010; Ma *et al.*, 2005; Mori & Harayama, 2011; Shcherbakova *et al.*, 2011; Shlimon *et al.*, 2004; Zhu *et al.*, 2011). These species usually grow by reducing CO_2 with H_2. The phenotypic characteristics of strain 17A1^T, when grown in medium Mc under H_2/CO_2, were consistent with those expected of members of the genus *Methanobacterium* (Table 1). Cells of the novel strain were Gram-staining-negative, non-motile rods (0.2–0.4 × 2–15 μm). The rods had rounded ends and varied in shape from straight or slightly curved during exponential growth to a bent shape during the stationary phase. Cells grew as filaments (Fig. 2) and exhibited fluorescence typical of methanogens when observed under ultra-violet light at 420 nm. Cells from an exponentially growing culture were resistant to lysis when they were transferred into distilled water (as a hypotonic solution) or 1 % (w/v) SDS. On agar under H_2/CO_2, strain 17A1^T grew as round greyish-white colonies with entire edges and diameters of approximately 1 mm.

Strain 17A1^T grew as a chemoautotroph in a modified medium Mc without vitamins and yeast extract. Growth was stimulated by the addition of acetate (5 mM), vitamins (5 ml l^{-1}), yeast extract (0.2 g l^{-1}) or rumen fluid (5 ml l^{-1}). Strain 17A1^T utilized H_2/CO_2 and methanol

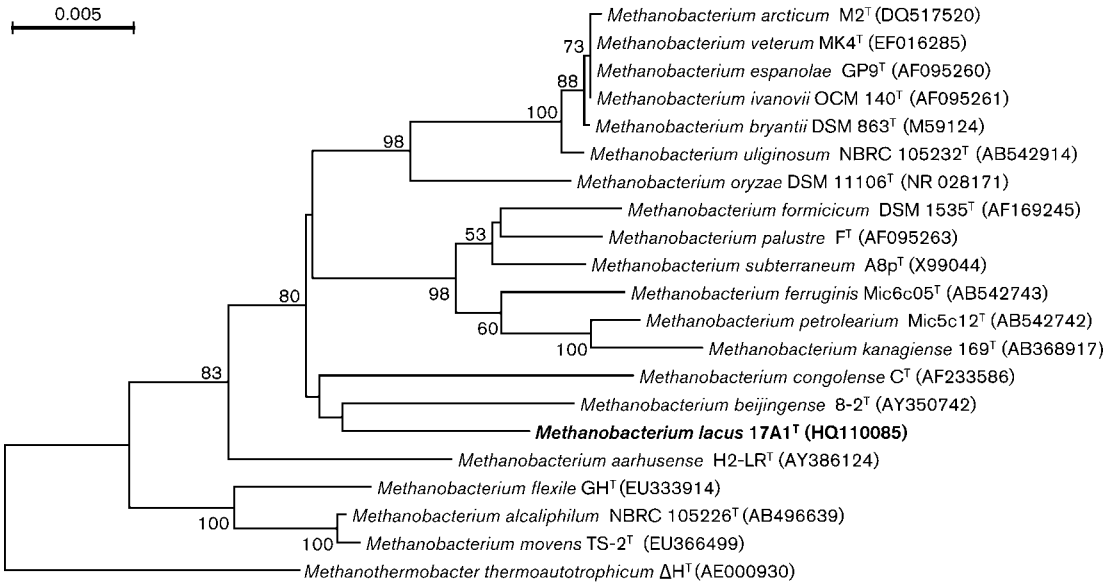

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships between strain 17A1^T and other methanogens. Bootstrap values (>50 %) shown on branches were calculated from 1000 resamplings. Bar, 0.005 substitution per nucleotide position.

Table 1. Phenotypic characteristics of strain 17A1^T and the type strains of five closely related species of the genus *Methanobacterium*

Strains: 1, 17A1^T; 2, *Methanobacterium beijingense* 8-2^T (Ma *et al.*, 2005); 3, *Methanobacterium veterum* MK4^T (Krivushin *et al.*, 2010); 4, *Methanobacterium formicicum* MF^T (Bryant & Boone, 1987); 5, *Methanobacterium palustre* F^T (Zellner *et al.*, 1988); 6, *Methanobacterium espanolae* GP9^T (Patel *et al.*, 1990). −, Negative; +, positive; ND, not determined; E, essential; NR, not required; S, stimulatory.

Characteristic	1	2	3	4	5	6
Cell morphology (μm)						
Length	2–15	3–5	2–8	2–15	2.5–5	3–22
Width	0.2–0.4	0.4–0.5	0.4	0.4–0.8	0.5	0.8
Gram staining	−	−	−	+	+	+
Catabolic substrates						
Formate	−	+	−	+	+	−
2-Propanol + CO_2	−	−	−	−	+	−
Methanol (50 mM) + H_2	+	ND	+	ND	ND	ND
Methylamine (20 mM) + H_2	−	ND	+	ND	ND	ND
Growth requirements						
Chemoautotrophic	+	−	+	−	+	+
Yeast extract	S	E	NR	ND	ND	S
Vitamin	S	NR	NR	ND	ND	S
Acetate	S	S	S	S	ND	NR
Temperature for growth (°C)						
Range	14–41	25–50	10–46	ND	ND	20–40
Optimum	30	37	28	37–45	37	35
pH for growth						
Range	5.0–8.5	6.5–8.6	5.2–9.4	ND	ND	4.7–7.0
Optimum	6.5	7.2–7.7	7.2–7.4	6.6–7.8	7	5.6–6.2
NaCl for growth (M)						
Range	0–0.4	0–0.5	0–0.3	ND	0–0.3	ND
Optimum	0.1	ND	0.03	ND	0.2	ND
DNA G+C content (mol%)*	37.0 (Lc)	38.9 (T_m)	33.8 (T_m)	41–42 (Bd)	34 (T_m)	34 (T_m)

*Determined by HPLC (Lc), melting point (T_m) or buoyant density (Bd) analysis.

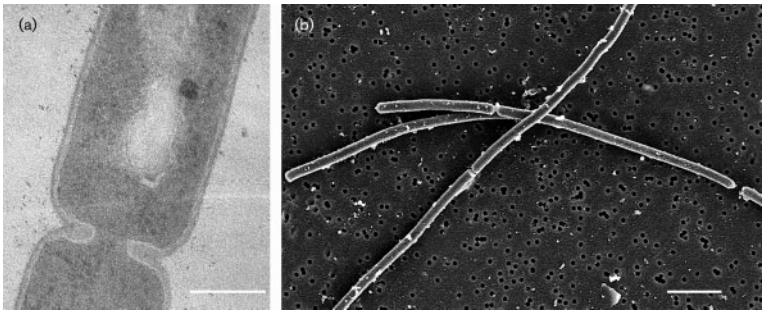

Fig. 2. Transmission (a) and scanning electron micrographs (b) of cells of strain 17A1^T. Bars, 200 nm (a) and 2 μm (b).

$+H_2$ for growth (Table 1) and methane production but did not grow on methanol (30 mM), methylamine (20 mM) $+H_2$, formate (30 mM), acetate (30 mM), 2-propanol (20 mM) or isobutanol (20 mM). This catabolite substrate profile is similar to that of *Methanobacterium veterum* (Krivushin *et al.*, 2010) with the exception that *Methanobacterium veterum* is able to grow on methylamine $+H_2$ (Table 1). The isolate grew on H_2/CO_2 at temperatures between 14 and 40 °C, with optimal growth at 30 °C. This is the second-lowest temperature optimum known for species of *Methanobacterium*, the lowest being 28 °C for *Methanobacterium veterum* (Krivushin *et al.*, 2010). Strain 17A1^T grew in medium Mc at pH values between 5.0 and 8.6, with optimal growth at pH 6.5. The latter value is significantly lower than the corresponding pH optima for the growth of *Methanobacterium beijingense* (Ma *et al.*, 2005) and *Methanobacterium veterum* (Krivushin *et al.*, 2010). Although the novel strain grew in medium Mc without NaCl, growth was optimal when the medium contained 0.1 M NaCl. There was no growth when the NaCl concentration exceeded 0.4 M. The doubling time of strain 17A1^T in medium Mc with 0.1 M NaCl, at pH 6.8 and 25 °C, was 22 h. Penicillin (0.01 %, w/v), vancomycin (0.01 %, w/v) and kanamycin (0.001 %, w/v) decreased growth of the novel strain by 49 %, 27 % and 17 %, respectively. At a concentration of 0.1 % (w/v), penicillin and vancomycin decreased growth by 87 % and 63 %, respectively.

Based on the morphological, physiological and phylogenetic evidence, strain 17A1^T represents a novel species in the genus *Methanobacterium*, for which the name *Methanobacterium lacus* sp. nov. is proposed.

Description of *Methanobacterium lacus* sp. nov.

Methanobacterium lacus (la′cus. L. gen. n. *lacus* of a lake, indicating the site of isolation).

Cells are non-motile, long (2–15 μm) rods that usually grow as filaments. Cells stain Gram-negative, autofluoresce under ultraviolet light (λ_{420}) and are resistant to lysis in 0.1 % (w/v) SDS or distilled water. Surface colonies on agar are small, round and greyish white, with entire edges. Cells are obligately anaerobic and grow as chemoautotrophs. Grows at 14–40 °C (optimum 30 °C) and at pH 5.0–8.6 (optimum pH 6.5). Optimal growth occurs in the presence of 0.1 M NaCl. Growth is reduced by penicillin, vancomycin and kanamycin. Catabolic substrates for growth include H_2/CO_2 and methanol $+H_2$ but not formate, acetate, methylamine $+H_2$, 2-propanol or iso-butanol. Produces methane. Vitamins, yeast extract, acetate and rumen fluids are not required but stimulate growth.

The type strain, 17A1^T (=DSM 24406^T=JCM 17760^T), was isolated from the deep sediment of Lake Pavin, France. The genomic DNA G+C content of the type strain, as determined by HPLC, is 37.0 mol%.

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