

Methanobacterium aarhusense sp. nov., a novel methanogen isolated from a marine sediment (Aarhus Bay, Denmark)

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Strain H2-LR^T, a 5–18 µm long and 0.7 µm wide filamentous, mesophilic, moderately halophilic, non-motile hydrogenotrophic methanogen, was isolated from marine sediment of Aarhus Bay, Denmark, 1.7 m below the sediment surface. On the basis of 16S rRNA gene comparison with sequences of known methanogens, strain H2-LR^T could be affiliated to the genus *Methanobacterium*. The strain forms a distinct line of descent within this genus, with *Methanobacterium oryzae* (95.9% sequence identity) and *Methanobacterium bryantii* (95.7% sequence identity) as its closest relatives. The 16S rRNA-based affiliation was supported by comparison of the *mcrA* gene, which encodes the α-subunit of methyl-coenzyme M reductase. Strain H2-LR^T grew only on H₂/CO₂. The DNA G + C content is 34.9 mol%. Optimum growth temperature was 45 °C. The strain grew equally well at pH 7.5 and 8. No growth or methane production was observed below pH 5 or above pH 9. Strain H2-LR^T grew well within an NaCl concentration range of 100 and 900 mM. No growth or methane production was observed at 1 M NaCl. At 50 mM NaCl, growth and methane production were reduced. Based on 16S rRNA gene sequence analysis, the isolate is proposed to represent a novel taxon within the genus *Methanobacterium*, namely *Methanobacterium aarhusense* sp. nov. The type strain is H2-LR^T (= DSM 15219^T = ATCC BAA-828^T).

INTRODUCTION

At the time of writing, the genus *Methanobacterium* comprised nine species with validly published names, *Methanobacterium formicicum* (Bryant & Boone, 1987), *Methanobacterium bryantii* (Bryant *et al.*, 1967), *Methanobacterium espanolae* (Patel *et al.*, 1990), *Methanobacterium ivanovii* (Jain *et al.*, 1987), *Methanobacterium palustre* (Zellner *et al.*, 1989), *Methanobacterium subterraneanum* (Kotelnikova *et al.*, 1998), *Methanobacterium uliginosum* (König, 1985), *Methanobacterium oryzae* (Joulian *et al.*, 2000) and *Methanobacterium congolense* (Cuzin *et al.*, 2001). All these species are hydrogenotrophic mesophiles (Boone, 2001). In this communication, we describe a novel mesophilic, hydrogenotrophic methanogenic taxon isolated

from marine sediment (1.7 m below sediment surface) in Aarhus Bay, Denmark.

METHODS

Source of inoculum. Sediment cores were collected in February in Aarhus Bay, a semi-enclosed embayment on the east coast of Jutland, Denmark (56° 09' 20" N 10° 19' 24" E), using a hydraulic piston corer. The sulphate–methane transition zone, defined by low sulphate and methane concentrations in combination with anaerobic methane oxidation and stimulated sulphate reduction, was determined by profiles of sulphate and methane concentrations and reduction and oxidation rates (Thomsen *et al.*, 2001). Sediment from the transition zone (from 170–190 cm) was mixed with a half volume of oxygen-free sea water, and the resulting slurry was used as inoculum.

Medium. Artificial marine medium for the cultivation of strain H2-LR^T was prepared according to Widdel & Bak (1992). It contained the following compounds. Solution A (g l⁻¹ demineralized water): NaCl, 20; MgCl₂·6H₂O, 3; KCl, 0.7; NH₄Cl, 0.2; KH₂PO₄, 0.2; CaCl₂·2H₂O, 0.2. Solution B: NaHCO₃, 2.5 g in 30 ml demineralized water. Solution C: 1.5 ml l⁻¹ of 0.04 g Na₂S·9H₂O ml⁻¹

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The GenBank accession numbers for the 16S rRNA gene and *mcrA* gene sequences of *Methanobacterium aarhusense* H2-LR^T are AY386124 and AY386125, respectively.

demineralized water. Solution A was prepared and autoclaved in an inverted Erlenmeyer flask (Widdel & Bak, 1992). The hot solution was cooled under an atmosphere of oxygen-free N₂ in an ice-containing water bath while being constantly stirred with a magnetic stir-bar. Solutions B and C were prepared in a 100 ml glass bottle and a 20 ml Hungate tube, respectively, and autoclaved under an atmosphere of oxygen-free N₂. After cooling, solutions B and C were transferred to the glass vessel containing solution A and mixed with a magnetic stir-bar. The gas phase of the medium vessel was flushed with a gas mixture of N₂/CO₂ (9:1). The pH was adjusted to 7.2 by addition of a sterile solution of HCl (1 M). The following minerals and vitamins were then added to the medium (Widdel & Bak, 1992): trace element solution SL 10a, 2 ml l⁻¹; Na₂SeO₃ (0.01 mM), 2 ml l⁻¹; Na₂WO₄ (0.01 mM) 2 ml l⁻¹; vitamin mix, 1 ml l⁻¹; thiamine chloride (100 mg l⁻¹), 1 ml l⁻¹; vitamin B₁₂ (50 mg l⁻¹), 1 ml l⁻¹. The medium was distributed aseptically into 50 ml screw-capped glass bottles. A cocktail of antibiotics [vancomycin (100 µg ml⁻¹), kanamycin (100 mg ml⁻¹), streptomycin (200 µg ml⁻¹)] was added to the culture bottles prior to inoculation (0.2 ml in 50 ml).

Enrichment and isolation. Prior to inoculation, 17 ml medium was removed from the 50 ml culture bottle and the antibiotics were added. After inoculation (2 ml), the remaining headspace was flushed with a mixture of filtered H₂ and CO₂ (9:1, v/v). The bottle was closed with a butyl-rubber stopper. The inoculated bottles were incubated at 30 °C in the dark. After 10 transfers, isolates were obtained by repeated deep agar dilutions (Pfennig, 1978). Well-isolated colonies were withdrawn with Pasteur pipettes and transferred to Hungate tubes containing medium. The glass tubes were sealed with butyl-rubber stoppers and flushed with sterile-filtered H₂ and CO₂ (9:1, v/v). The purity of the isolated cultures was checked microscopically and by inoculation of growing cultures into complex medium containing pyruvate (5 mM), fumarate (5 mM), glucose (5 mM) and yeast extract (0.5%) but no antibiotics.

Microscopy. Cultures were routinely checked by phase-contrast microscopy. Samples for transmission electron microscopy were placed on a carbon-celloidin copper grid (200 mesh) and stained with a drop of 1% (w/v) uranyl acetate. Cells were observed with a JEOL 1200EX transmission electron microscope, 120 kV.

The Gram-type of the isolate was determined using Burke's staining method (Gerhardt *et al.*, 1994).

Physiological studies. The following substrates were tested: methanol (20 mM), formate (20 mM) + acetate (1 mM), acetate (1 mM), trimethylamine (gas) (2 ml in 50 ml), dimethyl sulphide (50 and 100 µl pure solution in 50 ml corresponding to ~13 and 27 mM), methane thiol (gas) (2 ml in 50 ml), ethanol (20 mM), 2-propanol (20 mM), CO (12 ml) and H₂ + 1 mM acetate. Growth was determined microscopically by comparison with unamended cultures and by methane measurement. Growth experiments were carried out in 16 ml glass tubes sealed with butyl-rubber stoppers. Experiments were started by inoculating 10 ml of a freshly grown culture into 50 ml fresh, amended medium. Aliquots (7 ml) of the inoculated cultures were then dispensed into 16 ml glass tubes and flushed with N₂/CO₂ (80/20) or H₂/CO₂. Salinity and pH tests were performed at 30 °C in the dark. Optimal growth conditions were scored by measuring methane concentrations 48 h after inoculation.

The methane production rate was determined from consecutive methane concentration measurements. Growth was also followed microscopically. The growth response of the strains to temperature was determined in a temperature gradient block. Duplicate cultures were incubated at a temperature range from 5 to 50 °C at 3–5 degree intervals. Samples for methane measurement were taken every 48–76 h. The salinity range of the isolate was tested at NaCl concentrations

from 50 to 1000 mM NaCl at 100 mM intervals. The pH range was tested in intervals from 5 to 9. The requirement for vitamins was tested by consecutive transfers into vitamin-depleted substrate-amended culture medium and comparison with vitamin-containing controls.

The effect of the antibiotics chloramphenicol (5 µg ml⁻¹), bacitracin (10 µg ml⁻¹), and penicillin G (2000 µg ml⁻¹) was tested. Five ml aliquots of the cultures were transferred into fresh medium containing one of the three antibiotics. Duplicate cultures of each strain plus one control culture were incubated for 1 week at 30 °C. The impact of antibiotics was determined by comparing the growth of cultures containing these antibiotics with controls.

The tolerance of the isolate to demineralized water was tested by resuspending the pellet of a centrifuged culture in demineralized water. The effect on the cells was followed by microscopic inspection. The effects of SDS and Triton X-100 were tested by adding increasing amounts of each compound to 5 ml culture to give final concentrations of 0.05, 0.1, 1 and 4%. The samples were shaken and the cultures were checked regularly by phase-contrast microscopy and compared to unamended controls.

The G+C content of genomic DNA was determined at the Identification Service of the DSMZ (Braunschweig, Germany). The DNA was isolated according to Visuvanathan *et al.* (1989) and purified as described by Cashion *et al.* (1977) and the G+C content was determined by HPLC analysis (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984).

Phylogenetic analysis. DNA was extracted and purified as described previously (Henckel *et al.*, 1999). DNA extracts were analysed by agarose (1%) gel electrophoresis and visualized after staining with ethidium bromide.

16S rRNA gene amplicons (~1350 bp) were obtained using the following primer pair: Ar109f (5'-ACKGCTCAGTAAACACGT-3') (Großkopf *et al.*, 1998) and Ar1383r (5'-CGGTGTGTGCAAGGA-GCA-3'). The PCR contained, in a total volume of 50 µl, 1 × PCR buffer II, 50 µM each dNTP, 1.5 µM MgCl₂, 0.5 µM each primer, 1.25 U AmpliTaq DNA polymerase and 1 µl of a 1:10 dilution of the DNA template. The PCRs were started by immediately placing the reaction tubes into the preheated (94 °C) thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The thermal program was as follows: an initial denaturation step (94 °C, 4 min) was followed by 28 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 90s). After a final extension step (72 °C, 6 min) samples were kept at 4 °C until further analysis.

mcrA gene fragments were amplified using the following primer combination: ME1 (5'-GCMATGCARATHGGWATGTC-3') and ME2 (5'-TCATKGCRTAGTTDGGRTAGT-3') (Hales *et al.*, 1996), yielding ~740 bp amplicons. In addition, a new forward primer, MR1 (5'-GACCTCCACTWCGTVAACAACGC-3') (Simankova *et al.*, 2003), was used in combination with ME2, yielding amplicons of ~1100 bp. The PCR mixtures and thermal cycling conditions were as previously described (Lueders *et al.*, 2001).

PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequencing of PCR products was performed using the BigDye terminator cycle sequencing kit on an ABI 377A DNA sequencer (Applied Biosystems). In addition to primers Ar109f and Ar1383r, the primer Ar915r (5'-GTGCTCCCCGCCAATTCT-3') (Großkopf *et al.*, 1998) was used for sequencing of 16S rRNA gene amplicons, and primers ME1, ME2 and MR1 were used to sequence *mcrA* amplicons. Alignment and reconstruction of phylogenetic trees were performed using the ARB software package (Strunk & Ludwig, 2000) as previously described (Lueders *et al.*, 2001).

Analytical procedures. Methane was determined on a gas chromatograph (Packard model A438A) equipped with an FID detector (Chrompack) and a 1.5 m × 3.2 mm Carbopack BHT 100 column. Oven, injection port and detector temperatures were 100, 150 and 275 °C, respectively. N₂ was used as a carrier gas at a flow rate of 25 ml min⁻¹. The amount of methane produced was measured by injecting 200 µl gas from the headspaces of the cultures into a gas chromatograph. The amount of methane produced was calculated by comparison with standards.

RESULTS AND DISCUSSION

A methanogenic enrichment culture dominated by filamentous rods was obtained after 14 days. Visible colonies in agar shake tubes appeared after 3 weeks of incubation at 30 °C. The colonies were circular and greyish surrounded by a whitish zone. Phase-contrast microscopy of slides prepared from the colonies revealed that the cells resembled the cells of liquid culture in shape and size. Several morphologically identical cultures were obtained. Only one strain, designated H2-LR^T, was characterized in detail. Cells of H2-LR^T were straight or crooked rods, non-motile, 5–18 µm long and 0.7 µm wide. They stained Gram-positive. The cells occurred singly, in pairs or as long filaments. In liquid cultures, cells of strain H2-LR^T often attached to particles, forming clumps consisting of hundreds of cells. Cells did not possess pili or flagella (Fig. 1).

Cells of H2-LR^T did not lyse in milli-Q water or Triton X-100 (4% final concentration). Higher concentrations of Triton X-100 were not tested. The cells tolerated SDS (0.1% final concentration) for at least 75 min. They lysed at 1% (final concentration) SDS after a few minutes. Growth of H2-LR^T was suppressed by the addition of 5 µg chloramphenicol ml⁻¹. The addition of 2000 µg penicillin ml⁻¹ and 10 µg bacitracin ml⁻¹ slowed the growth of strain H2-LR^T. Chloramphenicol inhibits protein synthesis in *Bacteria*, but it does not affect protein synthesis in

methanogens. According to Whitman *et al.* (1992), members of the genus *Methanobacterium* may possess a particulate dehydrogenase enzyme that is sensitive to chloramphenicol. Strain H2-LR^T was also sensitive to bacitracin and penicillin G. Bacitracin inhibits the formation of lipid-bound precursors of murein in *Bacteria* (Whitman *et al.*, 1992); it may have the same effect on methanogens of the family *Methanobacteriaceae*, which have cell walls composed of pseudomurein (König, 1988).

Strain H2-LR^T grew with H₂ and bicarbonate/CO₂ as the sole carbon source. Growth was not stimulated by the addition of acetate (0.1 mM final concentration) to the growth medium. The strain can thus be considered chemoautotrophic, a growth mode that is common among members of the genus *Methanobacterium* (Boone, 2001). The strain did not grow on methanol, formate, acetate, ethanol, 2-propanol, CO, trimethylamine, dimethyl sulphide or methane thiol.

Strain H2-LR^T utilized hydrogen sulphide but not sulphate as a sulphur source, which is in agreement with what has been reported from other species within the genus *Methanobacterium* (Boone, 2001). Vitamins and yeast extract did not stimulate growth.

The optimum growth temperature was 45 °C. Methane production and growth were also observed at 15 °C, while, at 5 °C, only methane production was observed. Under optimal conditions, methane production was observed about 15 h after transfer into new growth medium. The temperature interval and, in particular, the temperature optimum have been reported from members of the mesophilic genus *Methanobacterium* (Boone, 2001; Joulain *et al.*, 2000) and was used to distinguish this genus from the related thermophilic genus, *Methanothermobacter* (Wasserfallen *et al.*, 2000). Strain H2-LR^T grew optimally within a pH range of 7.5–8. No growth was observed below pH 6 or above pH 9. Thus, the strain is slightly alkaliphilic, but falls well within the range that has been reported for the genus *Methanobacterium* (Boone, 2001). Strain H2-LR^T grew equally well within a broad NaCl concentrations range, from 50 to 900 mM, but not at 1000 mM. The lowest salt concentration was not determined. The range is typical for a halotolerant organism.

The DNA base comparison of H2-LR^T was 34.9 mol% G+C. This value falls into the range that has been reported for a strain of the species *Methanobacterium bryantii*, a close relative of strain H2-LR^T (34–38 mol%), but higher than its closest relative, *Methanobacterium oryzae* (31 mol%) (Boone, 2001; Joulain *et al.*, 2000).

A large fragment of the 16S rRNA gene (~1333 bp) was obtained from the isolate and sequenced. Phylogenetic analysis of 16S rRNA gene sequences revealed that strain H2-LR^T was related to known methanogenic taxa within *Methanobacteriaceae* (Fig. 2). Strain H2-LR^T was closely related to *Methanobacterium oryzae* strain Fpi^T (95.9%

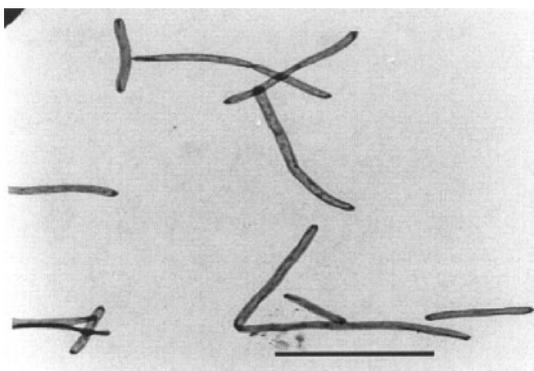


Fig. 1. Electron micrograph of strain H2-LR^T showing rod-shaped cells. The rods are straight or crooked, single, double or in chains. Original magnification, × 2750. Bar, 10 µm.

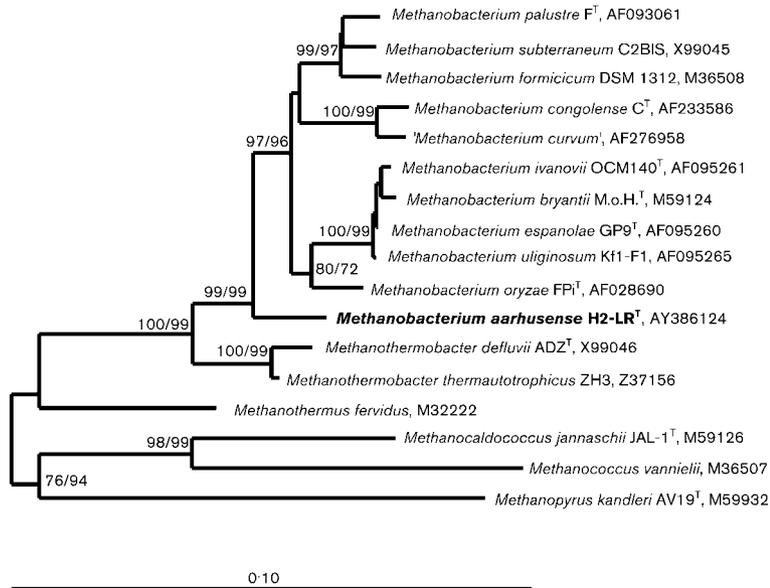


Fig. 2. Phylogenetic dendrogram of 16S rRNA gene sequences showing the position of *Methanobacterium aarhusense* strain H2-LR^T relative to other species of the genus *Methanobacterium* as well as selected reference sequences of methanogens. *Methanococcus jannaschii*, *Methanococcus vannielii* and *Methanopyrus kandleri* were used as outgroup references. The tree was reconstructed using distance matrix- (1208 nucleic acid positions; Jukes-Cantor distance correction) based neighbour-joining analysis. Bootstrap support (>70% indicated only) was obtained from neighbour-joining (first value) and maximum-parsimony analysis (second value) based on 1000 replications each. GenBank accession numbers are indicated. The scale bar represents a 10% estimated difference in nucleotide sequences.

sequence identity; Joulain *et al.*, 1998) and *Methanobacterium bryantii* M.o.H.^T (95.7% sequence identity). In addition to the 16S rRNA analysis, part of the *mcrA* gene, which encodes the α -subunit of the methyl-coenzyme M reductase, was amplified and sequenced (1124 bp). Phylogenetic analysis of the deduced McrA amino acid sequences confirmed the relationship of the novel isolate established by 16S rRNA gene analysis (Fig. 3); the closest relative as based on *mcrA* gene sequence analysis was *Methanobacterium bryantii* M.o.H.^T (93% amino acid sequence identity, 83% nucleic acid sequence identity).

Based on morphological, physiological and phylogenetic data, we propose the newly isolated strain H2-LR^T to represent a novel taxon within the genus *Methanobacterium*, *Methanobacterium aarhusense* sp. nov.

Description of *Methanobacterium aarhusense* sp. nov.

Methanobacterium aarhusense (aar.hus.en'se. N.L. neut. adj. *aarhusense* from Aarhus, a city on the east coast of Jutland, Denmark, where the type strain was isolated).

Colonies are circular, greyish, surrounded by a white zone. After 6 weeks, up to 1 mm in diameter. Cells are straight or crooked rods, 5–18 μ m long and 0.7 μ m wide. Occur often in filaments and tend to attach to particles. Gram-positive. Non-motile. Methanogenic. Do not lyse after transfer into demineralized water or in 4% Triton X-100 medium. Lyses in 1% SDS medium. Member of the *Euryarchaeota* of the domain *Archaea*. H₂/CO₂ is the only substrate that supports growth. Chemoautotrophic. Does not grow on methanol, formate, acetate, ethanol, 2-propanol, CO, trimethylamine,

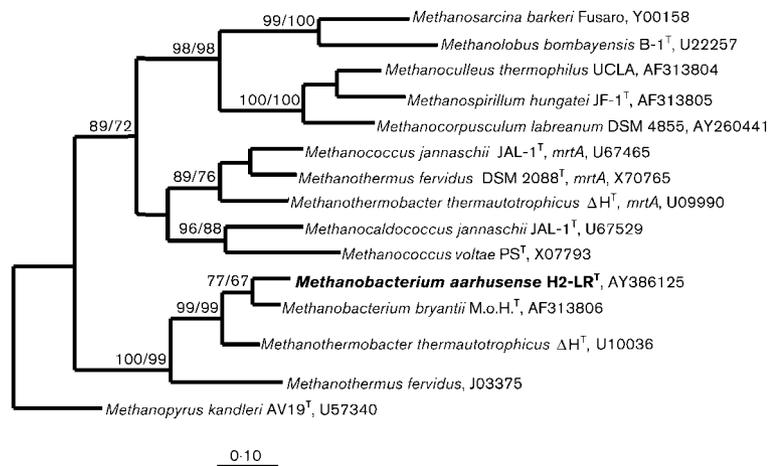


Fig. 3. Phylogenetic tree of deduced McrA and MrtA (methyl-coenzyme M reductase isoenzyme) amino acid sequences indicating the relationship of *Methanobacterium aarhusense* strain H2-LR^T to members of the genus *Methanobacterium* and other methanogenic archaea. The tree was reconstructed based on distance matrices (219 amino acid positions; PAM distance correction) by neighbour-joining analysis. The sequence of *Methanopyrus kandleri* was used as outgroup reference. Bootstrap support (>70% indicated only) was obtained from neighbour-joining (first value) and maximum-parsimony analysis (second value) based on 1000 replications each. GenBank accession numbers are indicated. The scale bar represents 10% estimated difference in amino acid sequences.

dimethyl sulphide or methane thiol. Growth is not stimulated by acetate, yeast extract or vitamins. Optimum growth temperature is 45 °C. Neither growth nor methane production is observed at 48 °C. Methane production, but no growth occurs at 5 °C. Optimum pH is between 7.5 and 8. No growth occurs at pH 5 or 9. DNA base composition is 34.9 mol% G + C (as determined by HPLC).

The type strain is H2-LR^T (=DSM 15219^T=ATCC BAA-828^T). Isolated from the methane–sulphate transition zone at 1.7 m below the sediment surface in Aarhus Bay, Denmark.

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