Methanobacterium subterraneum sp. nov., a new alkaliphilic, eurythermic and halotolerant methanogen isolated from deep granitic groundwater

Svetlana Kotelnikova,1,3 Alberto J. L. Macario2 and Karsten Pedersen3

Deep subterranean granitic aquifers have not been explored regarding methanogens until now. Three autotrophic methane-producing Archaea were isolated from deep granitic groundwater at depths of 68, 409 and 420 m. These organisms were non-motile, small, thin rods, 0·1–0·15 µm in diameter, and they could use hydrogen and carbon dioxide or formate as substrates for growth and methanogenesis. One of the isolates, denoted A8p, was studied in detail. It grew with a doubling time of 2·5 h under optimal conditions (20–40 ºC, pH 7·8–8·8 and 0·2–1·2 M NaCl). Strain A8p is eurythermic as it grew between 3·6 and 45 ºC. It was resistant to up to 20 mg bacitracin l–1. The G+C content was 54·5 mol%, as determined by thermal denaturation. Phylogenetic studies based upon 16S rRNA gene sequence comparisons placed the isolate A8p in the genus Methanobacterium. Phenotypic and phylogenetic characters indicate that the alkaliphilic, halotolerant strain A8p represents a new species. We propose the name Methanobacterium subterraneum for this species, and strain A8p (= DSM 11074?) is the type strain.

Keywords: Methanobacterium subterraneum sp. nov., alkaliphile, eurythermy, halotolerance, methanogen

INTRODUCTION

Mesophilic, rod-shaped and hydrogen-consuming methanogenic Archaea are placed in the Methanobacterium and Methanobrevibacter genera. Typically, species belonging to the genus Methanobrevibacter are short rods with G+C contents lower than 32 mol%, while Methanobacterium species are long rods with G+C contents higher than 32 mol%. At present the genus Methanobacterium is composed of seven mesophilic and six thermophilic species. On the basis of phylogenetic comparative analysis of 16S rRNA, it was suggested to transfer Methanobacterium thermotolerificum, Methanobacterium thermophilum and Methanobacterium wolfeii to the genus Methanobacterium (7). The mesophilic species of the genus Methanobacterium were isolated from sewage sludge (10), peat bog (45), marshy soil (21), bleach kraft mill sludge (31), and an oilfield (5). Methanobacterium alcaliphilum, isolated from alkaline lake sediments, has a pH optimum between 8·1 and 9·1 (8). Four species, Methanobacterium formicicum, Methanobacterium ivanovii, Methanobacterium palustre and Methanobacterium bryantii are autotrophic. No members of Methanobacterium have been described before that can grow below 10 ºC.

In this paper we describe the phenotypic and phylogenetic characteristics of an autotrophic, halotolerant, eurythermic and alkaliphilic methanogen that is able to grow below 10 ºC. On the basis of these characteristics, we propose a new species, Methanobacterium subterraneum.

The organism described here was isolated from deep granitic groundwater and is the first example of a methanogen isolated from the deep subterranean biosphere (32). In this habitat, methanogens may represent chemoautolithotrophic organisms that initiate food chains in the oligotrophic deep sub-
surface environment at the expense of geologically produced hydrogen.

**METHODS**

**Sources of organisms.** The Åspö hard rock laboratory (HRL) tunnel is located on the Baltic coast under the island of Åspö, in the vicinity of the Simpevarp nuclear power plant north of Oskarshamn, South-East Sweden. The host rock is a ~ 1800 Ma old granodiorite belonging to the Fennoscandian shield. The tunnel has a total length of 3600 m, is approximately 5 x 5 m (height x width) and proceeds down with an inclination of about 14°. It starts at the coastline and continues about 1700 m under the sea floor where it spirals down to 460 m below sea level under the island of Åspö (34, 35). Microbiological data from boreholes in the tunnel to a depth of 192 m and a length of 1420 m and in surrounding surface boreholes have been published previously (35-37). Groundwater was sampled from core-drilled surface and tunnel boreholes at 10-440 m below ground. The methanogens described here were called A8p, 3067 and C2BIS and came from boreholes denoted KR0012A, KA3067A and HD0025A, respectively. These boreholes corresponded to tunnel lengths of 500, 3067 and 3200 m, and to depths below sea level of 68, 409 and 420 m, respectively. The samples were inoculated in the enrichment medium described below.

**Media and culturing techniques.** The anaerobic technique described by Hungate (17) was used. A total of 19 boreholes were screened during 1995-1996 for the presence of methanogens with various carbon and energy sources. Enrichment cultures were obtained in a medium prepared with filter-sterilized groundwater which was collected from boreholes KR0012A or KA3067A. The groundwater was supplemented with (l-l) 10 ml trace element solution (44), 1.0 g yeast extract and 1 mg resazurine. The following carbon and energy sources were used (l-l): (i-2): 3.4 g sodium acetate, 2.0 g formate, hydrogen and carbon dioxide (80: 20%, 152 kPa), 2.0 g methanol or 1.0 g trimethylamine. Five milliliter portions of this medium were distributed under oxygen-free nitrogen gas in Hungate-type gas-tight, anaerobic culture tubes (Bellico Glass 2047, 17 ml) and sterilized at 121°C for 20 min. After cooling, the following sterile, anoxic solutions were added (l-3): 5 ml vitamin solution SL-6 (44), 1.0 g coenzyme M, 2.0 g NaHCO3, 0.25 g cysteine-HCl and 0.25 g NaaS, 9H2O. The pH of the enrichment medium was adjusted twice, before sterilization and after the final additions, with 0.1 M NaOH or 0.1 M HCl to pH values corresponding to those of the groundwaters used for inoculation (7.25-7.5). The enrichment tubes were inoculated with 0.5 ml groundwater from the boreholes within 2 h of sampling and incubated at room temperature for up to 5 months. The final headspace in the enrichment tubes was approximately 11 ml. The enrichment cultures which actively produced methane were subcultured by serial dilutions in the presence of 0.5 g vancomycin l-1. Pure cultures were obtained by mixing 1 ml of the last dilution of the culture which produced methane with 5 ml fresh enrichment medium with 20 g agar l-1 (45°C) plus 0.5 g vancomycin l-1, and then solidified in a thin layer by rolling in butyl-rubber-stoppered, aluminium crimp-sealed tubes (Bellico Glass 2048, 22 ml). For the cultivation of pure cultures, including all physiological experiments, an artificial Åspö medium (ASPM) was used that mimicked the chemical composition of the groundwater from the KR0012B borehole. It contained (l-l): 0.4 g NH4Cl, 0.03 g MgCl2, 0.45 g NaCl, 0.5 g KH2PO4, 0.003 g FeCl3.7H2O, 10 ml trace element solution (44), 0.001 g resazurin, 2.0 g NaHCO3, 0.25 g cysteine-HCl and 0.25 g Na2S.9H2O. The following carbon and energy sources were used: 2.0 g formate l-1, or hydrogen and carbon dioxide (80:20, 152 kPa). The medium did not contain buffer before sterilization, and the pH value of the medium, which became 7.0 after sterilization, was adjusted to 7.8-8.0 before inoculation with anoxic bicarbonate (10% stock solution) and NaOH (0.1 M stock solution). All experiments were repeated at least once and all tests were done at least in duplicate. Means of repeated tests are reported and used for the graphs presented.

**Microscopy.** Phase-contrast and fluorescence microscopy were performed with an Olympus BH-2 phase-contrast microscope and a Carl Zeiss Axioscope equipped with UV lamps. Phase-contrast images were obtained with an Olympus C35AD camera and an AD Exposure Control Unit. Autofluorescence of whole cells was observed with an LP 420 excitation filter (13). Acridine orange direct counts (AODCs) were done as described previously (14). Ultrathin sections of the cells were obtained by glutaraldehyde fixation (2.5%, 2 h) followed by osmium tetroxide fixation (1%, 2 h) in 0.1 M phosphate buffer (pH 7.4) at 4°C. The cells were embedded in Epon 812, then thin-sectioned and stained with uranyl acetate and lead citrate. The thin sections were studied with an EAL 1200EX electron microscope.

**Gas chromatography.** Methane was determined with a Varian GC-3700 gas chromatograph equipped with a 2 m x 3.175 mm steel column packed with Porapak Q mesh 80/100 (Varian), with nitrogen as carrier gas at a flow rate of 30 ml min-1, and a flame-ionization detector. The response of the detector to methane was linear. The injector, column and detector temperatures were isothermal at 100, 100 and 200 °C, respectively. Calibration, registration and integration of methane peaks was done with a Star Chromatography Workstation, version 4.5 (Varian).

**Growth determination.** The studied isolate A8p grew in aggregates and it was, therefore, not possible to use measurements of optical density in the cultures for biomass determinations. Instead, the linear relation between methane production and biomass formation during the exponential growth phase (38) was used to calculate specific growth rates for the experiments described below. This relation was confirmed in experiments where methane production during growth of the isolates was found to be linearly correlated with the increase in cell number as determined by AODC. Growth at low methane production rates (< 0.05 h-1) was confirmed by AODC. Inoculations which did not show a cell count increase and a corresponding methane production after 20 d incubation were regarded as negative.

**Susceptibility tests.** The susceptibility of A8p to antibiotics was determined by adding them at 1-2000 mg l-1. The minimum concentration (MIC) at which the antibiotic causing 15% reduction in growth rate was considered to be inhibitory.

**Determination of growth requirements.** Cultures of A8p were transferred three times in a modified ASPM (pH 7.8), without vitamins and organic compounds, but with 40 mM formate. Subsequently, an array of possible growth factors was added to the modified ASPM and inoculated in duplicate with the vitamin-depleted A8p culture. The control did not contain any additions. Growth of the cultures was monitored during incubation at 35°C for 120 h. The experiments were repeated twice. Growth curves were obtained for each repetition of the studied compounds and growth rates were calculated. The differences between the
mean growth rate for an added compound compared to the control were evaluated with the Student’s t-distribution test (1) at a significance level of P = 0.05.

Determination of growth parameters. Methane production was monitored at 1–3 d intervals in ASPM. The mean specific growth rate for each incubation temperature was calculated from the exponential methane-production phase. The effect of pH on growth of A8p was determined using different pH buffers in ASPM. They were: a mixture of 26 mM sodium acetate and 18 mM acetic acid (pH 4.8–5.5), 20 mM MES (pH 5.7–6.5), 20 mM HEPES (pH 5.5–7.2), 20 mM PIPES (pH 6.2–7.6), 20 mM sodium bicarbonate (pH 7.0–8.0), 30 mM Tricine (pH 7.5–8.0) and 20 mM Tris (pH 8.2–8.8). The culture was inoculated into five repetitions of each buffer. The pH was measured before and after inoculation, as well as during growth and never varied more than 0.15 units. The mean specific growth rate for each incubation pH was calculated from the exponential methane production phase.

The salt tolerance of A8p was determined in ASPM at 35 °C and pH 8.5, supplemented with NaCl. The experiment was performed in duplicate and repeated twice. The specific growth rate at each NaCl concentration was calculated from the methane production between 20 and 177 h incubation.

Antigenic fingerprinting. Partial antigenic fingerprinting of the new isolates was performed using calibrated antibody probes as described previously (24, 25).

Determination of DNA composition. Standard procedures (26, 43) were used for DNA extraction from the culture with the following modifications. Cells were lysed by repeated freezing and thawing followed by 2 h incubation in 50 mM ammonium bicine, 50 mM EDTA buffer (pH 8.0) 10 mg SDS ml⁻¹ and 20 mg DTT ml⁻¹ at 60 °C. Extracted DNA was dissolved in 0.1 M Tris pH 8.0 SSC buffer and dialysed against the same buffer. The G+C content was determined by a thermal denaturation method (27) with a Cary Varian Thermal Spectrophotometer, using the DNA of Escherichia coli Brosius numbering) 5'-GTC

Methanobacterium subterraneum sp. nov.

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**RESULTS**

**Enrichment and isolation**

Groundwater from deep granitic rock aquifers (10–440 m below sea level) was used as the inoculant. These groundwaters were anoxic and oligotrophic (2.0–11.0 mg organic carbon l⁻¹), with different salinities (537–13 300 mg Cl⁻¹) and bicarbonate concentrations (53–326 mg HCO₃⁻ l⁻¹) (29).
Active methanogenesis was observed in enrichment media containing 2.0 g l\(^{-1}\) formate and 1.0 g trimethylamine l\(^{-1}\) inoculated with groundwater from the boreholes KR0012A and HD0025A, corresponding to sample dates 2 February 1995 and 25 November 1995 and to 68 and 420 m below sea level, respectively. Abundant methane production was also observed in an enrichment culture supplied with hydrogen and carbon dioxide (80:20, 152 kPa) inoculated 25 November 1995 from borehole KA3067A, 409 m depth. The pH increased from 7.2–7.5 to 9.3–9.6 in these cultures concurrent with growth and methane production in the medium with formate. All the enrichment cultures could grow and produced copious amounts of methane when inoculated into the medium with formate or with hydrogen and carbon dioxide and they grew best at alkaline pH values (7.8–8.8). Small, non-motile and autofluorescing rod-shaped cells were observed by light microscopy in all these enrichments. The cultures were purified by serial dilution in the....
groundwater-based media containing 2.0 g formate l⁻¹ and 0.5 g vancomycin l⁻¹ and subsequent inoculation in roll tubes. Colonies were observed after 2 weeks. Single colonies from tubes with methane production were selected and transferred into liquid ASPM with 2.0 g formate l⁻¹. The resulting cultures were morphologically homogeneous. Only autofluorescing cells were observed. Inoculation into ASPM without carbon and energy sources but instead containing 20 mM sulfate and 20 mM lactate or 2 g glucose l⁻¹ and 1 g peptone l⁻¹, showed no growth. Thus, the cultures were axenic. The cells of the studied isolates had differing autofluorescence intensities and varied slightly in length. The isolates were designated as strains A8p (68 m depth), 3067 (409 m depth) and C2BIS (420 m depth).

**Morphology and ultrastructure**

Surface colonies of strain A8p were circular, granular, colourless and 0.5-1.5 mm in diameter. Cells of isolate A8p were non-motile, slightly curved, small and short rods, 0.6-1.2 μm in length and 0.1-0.15 μm in diameter, often growing in aggregates (Fig. 1a, d). These aggregates floated with gas bubbles when cultivated in ASPM with formate. Ultrathin sections showed a single-layered electron-dense cell wall of about 0.5 nm in thickness (Fig. 1b), typical of Gram-positive bacteria. The cell wall was tightly fitted to the cytoplasmic membrane. The cells divided after septum formation that was initiated peripherally (Fig. 1b, c).

Colonies of strain C2BIS were circular, granular, yellow, 1.0-2.0 mm in diameter. The cells were straight rods with a cell wall ultrastructure typical of Gram-positive bacteria and with round ends, i.e. a morphology similar to A8p. Under 420 nm light, bright blue-green autofluorescence was observed. Colonies and cells of strain 3067 had a morphology similar to that of C2BIS. Because the 16S rRNA sequence of strain A8p (68 m depth), 3067 (409 m depth) and strains A8p (68 m depth), 3067 (409 m depth) and C2BIS (420 m depth).

**Antibiotic susceptibility**

Growth of strain A8p was not inhibited by (l⁻¹): 1 g vancomycin, 500 mg benzylpenicillin, 1 g ampicillin, 1 g methicillin, 2 g streptomycin, 800 mg nalidixic acid, 14 mg neomycin or 20 mg bacitracin, while its growth rate was reduced one order of magnitude by (l⁻¹): 40-100 mg chloramphenicol, 20 mg neomycin and 40 mg bacitracin.

**Determination of growth requirements**

Strain A8p was transferred four times in ASPM with hydrogen as the energy source and bicarbonate as the electron acceptor and carbon source. The strain was autotrophic; it could use hydrogen and carbon dioxide (80:20; 152 kPa) with a growth rate of 0.2-0.3 h⁻¹ and did not require any growth factors or vitamins for growth. Strain A8p grew in ASPM (specific growth rate, 0.2-0.4 h⁻¹) with formate (2 g l⁻¹) as the substrate. In the absence of exogenous bicarbonate or carbon dioxide, methane formation from hydrogen diminished. The addition of methanol, trimethylamine or pyruvate did not stimulate methanogenesis from hydrogen. In the bicarbonate-buffered medium without hydrogen, only small amounts of methane were formed (< 0.5%). The presence of n-propanol (5 mM), isobutanol (5 mM), n-butanol (5 mM), or ethanol (5 mM) in the bicarbonate-buffered medium did not increase methane production in comparison with the control. Thus, these alcohols were not hydrogen donors for methanogenesis. The addition of acetate (30 mM), methanol (20 mM), pyruvate (20 mM), trimethylamine (20 mM), fructose (10 mM), glucose (10 mM), or dimethyl sulfide (4-5 mM) did not lead to an increase in methane production. Therefore, these compounds were also not substrates for methanogenesis by strain A8p.

Tryptone (1 g l⁻¹), sodium acetate (1 g l⁻¹), paraninobenzoic acid (100 mg l⁻¹), vitamin B₁₂ (4 mg l⁻¹), biotin (0.4 mg l⁻¹), coenzyme M (0.1 mg l⁻¹), CaCl₂ (0.5 g l⁻¹), MgCl₂ (0.5 g l⁻¹), Na₂MoO₄ (2-20 mg l⁻¹), Na₂WO₄ (2 mg l⁻¹), FeCl₃ (2 mg l⁻¹), CuCl₂ (2 mg l⁻¹) and NiCl₂ (0.2 mg l⁻¹) were tested as growth factors for strain A8p. Statistical evaluation showed that in ASPM with hydrogen and carbon dioxide as energy and carbon sources and supplemented with the different growth factors listed above, strain A8p showed growth rates close to those of the controls without additions, i.e. these compounds did not influence methanogenesis by strain A8p. However, growth was reduced by yeast extract, Casamino acids, isobutyric acid, n-butyric acid, Na₂SeO₃, ZnCl₂, CoCl₂, NiCl₂ and MnCl₂ (Table 1). Growth in medium with vitamin solution SL-6 (5 ml l⁻¹) (44) was slower than that in a

**Table 1. Effect of different growth factors on the growth of strain A8p**

<table>
<thead>
<tr>
<th>Growth factor added</th>
<th>Conc (mg l⁻¹)</th>
<th>Growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM (control)</td>
<td>-</td>
<td>0.178</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>2</td>
<td>0.082</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>2</td>
<td>0.085</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>2</td>
<td>0.062</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>2</td>
<td>0.095</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>20</td>
<td>0.107</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2000</td>
<td>0.101</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>1000</td>
<td>0.109</td>
</tr>
<tr>
<td>Isobutyric acid</td>
<td>5</td>
<td>0.121</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>5</td>
<td>0.113</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>5</td>
<td>-*</td>
</tr>
</tbody>
</table>

* Growth not observed.
function of the incubation temperatures and varied from 5 to 144 h. Minimal lag phases and maximal specific growth rates were observed at 20–40 °C. The strain showed growth and methanogenesis at 3.6–13 °C. Growth at 3.6, 6, 13.6 and 20 °C was confirmed by AODCs. At 3.6 °C, the number of cells had increased by more than two orders of magnitude, indicating active growth. In a separate experiment we verified that growth (increase in cell numbers) and methane production rates were linearly correlated during exponential growth at 6 °C (not shown). Growth and methane production were not detected at 2 or 50 °C. The organism is, therefore, mesophilic and eurythermic.

The optimum pH for growth and methane production at 35 °C in ASPM was 7.8–8.8 (Fig. 2b). This optimum was confirmed by AODCs at the end of incubation. Growth and methane production were observed at pH 9.2 and 6.5, but not above pH 9.2 or below 6.5. The duration of the adaptation phase was the same at pH 7.7–9.1 and increased at pH values below 7.7 and above 9.1. The reproducibility of cell growth at pH 9.0 was confirmed by subculturing strain A8p in ASPM at pH 9.0 twice. The level of growth and methane production observed during subculturing was reproducible. Thus, strain A8p is alkaliphilic.

Although the growth rate of A8p was highest at 0.2 M NaCl, this strain could grow up to a salinity of 1.40 M (Fig. 2c). In ASPM with added NaCl (0.25–1.40 M) A8p cells formed huge aggregates (Fig. 1a, d). Strain A8p is able to both grow and produce methane in a wide range of salinities, consequently, it is halotolerant.

**Antigenic fingerprinting**

The partial antigenic fingerprints of the new isolates demonstrated that they were antigenically unrelated to reference methanogens from the genera *Methanobacterium* and *Methanobrevibacter*.

**G + C content of the DNA**

The G + C content of strain A8p was 54.5 ± 0.5 mol%. The thermal melting point of DNA was 77.3 ± 0.2 °C. These results are means of four independent measurements.

**Phylogenetic analysis**

The 16S rRNA genes of the isolates were amplified using archaea-specific primers and then sequenced. The 16S rRNA genes of the isolates A8p and C2BIS were sequenced at positions 38–1534 and 22–1512, respectively (*E. coli* Brosius numbering). The 16S rRNA gene sequences of isolates A8p and C2BIS were obtained without any unambiguous or unknown bases and were identified for 1474 positions. Isolate 3067
could not be sequenced completely (the sequenced fragment was between positions 570 and 1178) due to what seemed to be blockage of the sequencing process at position 571 by a tertiary structure. The 16S rRNA gene of isolate 3067 differed by 0.7% (13 bases) from A8p and C2BIS (610 bases compared). Only unambiguous bases were included in the comparison. The sequenced fragment of isolate 3067 included both variable and conservative regions of the 16S rRNA gene, and the differences were situated in the variable regions. The partial 16S rRNA gene sequence of the isolate 3067 was not included in the phylogenetic analysis. A phylogenetic tree was constructed based on the 16S rRNA gene sequences of A8p and C2BIS in comparison with organisms in the EMBL and GenBank databases (Fig. 3). The phylogenetic tree shows that the isolates A8p and C2BIS were most closely related to the mesophilic species of the genus Methanobacterium. The highest similarity, 97.2%, was observed with Methanobacterium formicicum. The 16S rRNA genes of this organism differed in 38 positions over 1450 bases from A8p and C2BIS. The sequence similarities of the 16S rRNA genes of A8p and C2BIS to the other organisms were as follows: Methanobacterium wolfei, 94.2%; Methanobacterium bryantii, 92.5%; Methanobacterium thermoautotrophicum CB-12, 92.2%; Methanobacterium thermoautotrophicum TFT and Methanobacterium thermoautotrophicum THF, 92.1%; Methanobacterium thermoautotrophicum FTF, 92.0%; Methanobacterium thermoautotrophicum Z-245 and Methanobacterium thermoautotrophicum ΔH, 91.8%.

DISCUSSION

The level of similarity among the 16S rRNA genes (99.3–100%) of three new strains A8p, C2BIS and 3067, isolated from different granitic groundwater, implies that they can be regarded as closely related organisms unless evidence of phenotypic differences is revealed. The rod-like morphology and similar substrate specificity of the new isolates attest that they are phenotypically similar. Therefore, one organism, strain A8p, was chosen for detailed studies.

The resolution of 16S rRNA sequencing analysis between closely related organisms is generally low, but it is reliable for generic identification. The sequence similarities of the 16S rRNA gene of A8p showed that it was related to sequenced Methanobacterium species. The dissimilarity levels were 2.8–8.2%. The levels of dissimilarity were within the range found between other species of this genus. Thus, the results of 16S rRNA gene comparison indicated that our isolate belongs to the genus Methanobacterium. Phylogenetically, A8p was most closely related to Methanobacterium formicicum (97.2% similarity). However, this evolutionary distance is sufficiently large that the two strains most probably belong in separate species (7, 12, 19, 41). The phenotypic characteristics of A8p confirmed that the isolate belongs to the genus Methanobacterium and that it can be distinguished from phylogenetically related taxa as shown in Table 2.

Cells of strain A8p were small, thin rods. The cell
Table 2. Comparison of phenotypic characteristics of mesophilic Methanobacterium species

<table>
<thead>
<tr>
<th>Property</th>
<th>M. formicicum</th>
<th>M. bryantii</th>
<th>M. ivanovii</th>
<th>M. alcaliphilum</th>
<th>M. espanolae</th>
<th>M. palustre</th>
<th>M. alienigenus</th>
<th>‘M. subterraneum’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Rod, 0-4 x 0-8</td>
<td>Rod, 0-5 x 0-1</td>
<td>Rod, 0-5 x 0-8</td>
<td>Rod, 0-5 x 0-8</td>
<td>Rod, 0-5 x 1</td>
<td>Rod, 0-5 x 3-22</td>
<td>Rod, 0-5 x 3-5</td>
<td>Rod, 0-2 x 3-24</td>
</tr>
<tr>
<td>Growth substrates</td>
<td>Filaments, clumps H₂ + CO₂, formate</td>
<td>Filaments, clumps H₂ + CO₂, 2-propanol + CO₂</td>
<td>Filaments, clumps H₂ + CO₂</td>
<td>Filaments H₂ + CO₂</td>
<td>Filaments H₂ + CO₂, formate</td>
<td>Filaments H₂ + CO₂, formate</td>
<td>Filaments H₂ + CO₂</td>
<td>Filaments H₂ + CO₂</td>
</tr>
<tr>
<td>Autotrophy Stimulatory factors</td>
<td>Acetate, cysteine</td>
<td>Acetate, cysteine</td>
<td>Acetate, cysteine</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Growth factors</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Vitamins</td>
<td>Vitamins</td>
<td>Vitamins</td>
<td>Vitamins</td>
<td>Vitamins</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>ND (6-6-8)</td>
<td>ND (6-9-7)</td>
<td>6-5-8-5 (70-76)</td>
<td>7-9-9 (80-91)</td>
<td>4-6-7 (5-6-6)</td>
<td>7</td>
<td>6-8-5 (6-8-10)</td>
<td>6-5-9-2 (78-88)</td>
</tr>
<tr>
<td>Salinity, M (optimum)</td>
<td>0.25</td>
<td>0.26</td>
<td>0.19</td>
<td>0.012</td>
<td>ND</td>
<td>0-0.3 (0.2)</td>
<td>ND</td>
<td>0-1.4 (0.2-1.25)</td>
</tr>
<tr>
<td>G + C, mol% Reference</td>
<td>38.4-48.0</td>
<td>31.0-38.0</td>
<td>36.6</td>
<td>57.0</td>
<td>34.0</td>
<td>34.0</td>
<td>29.0</td>
<td>54.5</td>
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ND, Not done.

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Methanobacterium that is able to grow at low temperatures. A8p is also capable of growth within a wide range of salinity values and at high pH values, which further suggests that it is well-adapted to the deep granitic groundwater environment. The salinity and pH of the studied groundwater at Åspö HRL increase from 0 to 0-5 M and from 7-5 to 8-5, respectively, down to a depth of 950 m (40). These figures coincide with the optimum salinity and pH values obtained for strain A8p. The temperature, salinity and pH ranges obtained for A8p are indicative of its habitual adaptation to an active life in deep granitic aquifers and support the suggestion of A8p as a new species.

The adaptations to the specific environment might determine the unique phenotypic properties of the isolated methanogen when compared to established methanogens. The levels of salinity and pH tolerated by strain A8p were higher than those tolerated by other Methanobacterium strains (Table 2). Although its pH optimum is similar to that of Methanobacterium alcalophilum (8), strain A8p is salt-tolerant and consumes formate. A8p is autotrophic like four other organisms of the genus Methanobacterium: Methanobacterium formicicum, Methanobacterium bryantii, Methanobacterium ivanovii and Methanobacterium palustre (Table 2), but all these organisms except Methanobacterium ivanovii are able to use alcohols as electron donors, which strain A8p could not do. Strain A8p can grow on hydrogen and carbon dioxide or formate like Methanobacterium formicicum and Methanobacterium palustre, but strain A8p differed from these species by its temperature and salinity ranges and pH optimum. The difference in the G + C contents of strain A8p and Methanobacterium formicicum was 16.5 mol% (Table 2). In contrast to Methanobacterium ivanovii (4) and Methanobacterium
**Methanobacterium subterraneum** sp. nov.

*bryantii*, strain A8p uses formate, is alkaliphilic and halotolerant. Thus, strain A8p differs from known species of the *Methanobacterium* genus in its morphology, physiology, sensitivity to antibiotics, temperature and salinity ranges, pH optimum, substrate spectrum, and in 16S rRNA gene base composition (Table 2). These differences support placing A8p as a new species.

The antigenic fingerprinting data demonstrated that the three isolates described here are antigenically unrelated to reference methanogens and represent novel immunotypes within the genus *Methanobacterium*. Whether A8p, 3067 and C2BIS are of the same or different immunotypes, remains to be established. The antigenic fingerprinting indicated no cross-reaction of the isolates with other members of the family *Methanobacteriaceae*. The immunotypes of the isolates could not be affiliated to any of the reference strains.

The requirement for growth factors is considered to be a distinguishing phenotypic property for methanogens. The growth of strain A8p was inhibited by yeast extract, Casamino acids, isobutyric acid and n-butyrlic acid. Low concentrations of Se⁴⁺, Zn²⁺, Co⁴⁺ and high concentrations of Ni²⁺ and Mn²⁺ ions inhibited methanogenesis (Table 1). Growth of strain A8p was not stimulated by any growth factors and did not require vitamins for growth and methanogenesis, unlike many previously studied mesophilic species of the genus *Methanobacterium* (Table 2). The nutritional requirements of strain A8p are in accordance with the chemistry of the environment from which this organism was isolated. The deep groundwater contains trace amounts of metals and it is oligotrophic with concentrations of Ni²⁺ and Mn²⁺ ions inhibited methanogenesis (Table 1).

Growth is inhibited by yeast extract (2 g l⁻¹), Casamino acids (1 g l⁻¹), isobutyric acid (5 mg l⁻¹), n-butyrlic acid (5 mg l⁻¹), Na₂SeO₃ (2 mg l⁻¹), ZnCl₂ (2 mg l⁻¹), CoCl₂ (2 mg l⁻¹), NiCl₂ (20 mg l⁻¹) and MnCl₂ (20 mg l⁻¹). Vitamins are not essential for growth. Growth conditions: temperature 36–45 °C, pH 6.5–9.2, salinity 0.2–1.4 M NaCl. The G+C content of the DNA is 53.5 ± 0.5 mol% (as determined by thermal melting point). Isolated from granitic rock groundwater from the Åspö hard rock laboratory tunnel, South-Eastern Sweden. The type strain is A8pT (= DSM 11074⁴), and the reference strains are 3067 and C2BIS (= DSM 11075), isolated from granitic groundwater at the depths 68, 409 and 420 m below sea level, respectively. The 16S rRNA gene sequences of A8p⁷, 3067 and C2BIS have EMBL/GenBank accession numbers X99044, Y12592 and X99045, respectively.

**ACKNOWLEDGEMENTS**

The authors wish to thank Ann-Charlotte Erlandson for thorough sequencing of the 16S rRNA genes; Lotta Hallbeck for help with the phylogenetic tree construction and Demetra Xythalis for help with antigenic fingerprinting. S. K. thanks Karsten Pedersen and his group for support during Åspö expeditions and for having provided hospitality for 2 years. The research was supported by grants from The Swedish Nuclear Fuel and Waste Management CO (SKB) and from the Swedish Natural Science Research Council, grant number 8466/315/319/321.

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