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

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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 °C, pH 7.8-8.8 and 0.2-1.2 M NaCl). Strain A8p is eurythermic as it grew between 3-65 °C. 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 110747) is the type strain.

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

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**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 thermoautotrophicum*, *Methanobacterium thermophilum* and *Methanobacterium wofei* to the genus *Methanothermobacterium* (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 °C.

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 °C. 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 CB21B and came from boreholes denoted KRO012A, KA3067A and HD0025A, respectively. These boreholes correspond 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 KRO012A or KA3067A. The groundwater was supplemented with (l-'): 0.4 g K,HPO,, 0.003 g FeCl,, 7H,O, 10 ml trace element solution (44), 0.001 g resazurin, 2.0 g NaHCO,, 0.25 g cysteine. HCl and 0.25 g Na,H,O. The following carbon and energy sources were used (l-'): 3-4 g sodium acetate, 2.0 g formate, hydrogen and carbon dioxide (80-20 %), 2.0 g methanol or 1-0 g trimethylamine. Five millilitre portions of this medium were distributed under oxygen-free nitrogen gas in Hungate-type gas-tight, anaerobic culture tubes (Bellco Glass 2048, 17 ml) and sterilized at 121 °C for 20 min. After cooling, the following sterile, anoxic solutions were added (l-'): 5 ml vitamin solution SL-6 (44), 1 mg coenzyme M, 2.0 g NaHCO,, 0.25 g cysteine. HCl and 0.25 g Na,S,H,O. 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 (Bellco 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 KRO012B borehole. It contained (l-'): 0.4 g NH,Cl, 0.03 g MgCl,, 0.45 g NaCl, 0.5 g K,HPO,, 0.003 g FeCl,, 7H,O, 10 ml trace element solution (44), 0.001 g resazurin, 2.0 g NaHCO,, 0.25 g cysteine. HCl and 0.25 g Na,H,O. 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 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 (1-0, 2 h) in 0.1 M phosphate solution of pH 7.6 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 were 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) of 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 \( P = 0.95 \).

**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 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 x 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* (Sigma) for 15 min at 37 °C and incubated at 95 °C for 1 min. The sequencing product was separated from the residual oil by tip dropping. Four microlitres of loading gel electrophoresis was performed on an ALF DNA Sequencer (Pharmacia Biotech). Ready-mixed Long Ranger Gel solution for DNA sequencing (FMS Bioproducts-Europe) and urea (ALF grade) were used in the gel solution. Sequenced fragments were obtained with 11 different primers and were aligned with the Genetics Computer Group (GCG) GELASSEMBLE procedure and verified manually.

**Sequence analysis.** The 16S rRNA gene sequences of the isolates A8p, 3067 and 2C2BIS were compared to sequences available in the EMBL database using the FASTA and BESTFIT procedures in the GCG program package. The similarity percentages between the 16S rRNA gene sequences of the isolates and the most closely related organisms in the database were calculated with BESTFIT, without considering uncertain and unknown positions.

A phylogenetic analysis was performed on A8p and 2C2BIS strains using the programs contained in PHYLIP version 3.5c package (15). Nucleotide positions that could be unambiguously aligned for all 16S rRNA genes compared were included in the analysis. The final data set comprised 1400 nucleotide positions, positions 38-1438 of 16 organisms. The sequences used for the tree construction were aligned using PILEUP (GCG). The distances were calculated using the DNADIST (18) and a tree was built running KITCH with contemporary tips. KITCH was run with a randomized input order of data with 20 jumbles, and 11759 trees were examined during execution. The organisms used for the tree construction are listed in the legend to Fig. 3.

**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 HCO\(_3\)\(^{-1}\)) (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

![Fig. 1. Morphology of strain A8p. (a–c) Electron microphotographs of thin sections of cells. (d) Phase-contrast micrograph of cells and cell aggregate. The bar indicates 50 nm in (a) and (b), 100 nm in (c) and 1 \(\mu\)m in (d). Abbreviations: M, cytoplasmic membrane; CW, cell wall; CP, cytoplasm; IN, invagination of cytoplasmic membrane before cell division; S, septum.](image-url)
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 6.5 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 strains A8p (68 m depth), 3067 (409 m depth) and C2BIS (420 m depth) 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).

**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), para-aminobenzoic 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 |
|-----------------|-----------------|-----------------|
| Growth factor added | Conc (mg l⁻¹) | Growth rate (h⁻¹) |
| ASPM (control) | - | 0.178 |
| ZnCl₂ | 2 | 0.082 |
| CoCl₂ | 2 | 0.085 |
| Na₂SeO₃ | 2 | 0.062 |
| NiCl₂ | 2 | 0.095 |
| MnCl₂ | 20 | 0.107 |
| Yeast extract | 2000 | 0.101 |
| Casamino acids | 1000 | 0.109 |
| Isobutyric acid | 5 | 0.121 |
| n-Butyric acid | 5 | 0.113 |
| Propionic acid | 5 | -* |

* Growth not observed.
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Determination of growth parameters

Methanogenesis by strain A8p was observed at 3-6-45 °C (Fig. 2a), with a broad optimum of 20-40 °C. The length of the lag phase differed greatly as a 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 38-1 534 and 22-1 512, 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
Methanobacterium subterraneum sp. nov.

Fig. 3. Most probable phylogenetic tree constructed using Jukes-Cantor distance matrix calculated by comparing the 16S rRNA genes (1400 bases) with a random input order of sequences. Distances were calculated by the number of substitutions per 100 bases by the Fitch-Margoliash method with contemporary tips. The resulting tree was the most probable from 11759 trees. The branch length for the given topology is a least-squares fit and is proportional to evolutionary distances. Bar, 1% difference in nucleotide sequences, as determined by measuring the length of the horizontal lines connecting two species. For the tree construction the 16S rRNA gene sequences from the following organisms with EMBL accession numbers were used: Methanobacterium formicicum MF, M36508; Methanobacterium bryantii M.o.H., M59124; Methanobacterium thermoautotrophicum FTF, X68711; Methanobacterium thermoautotrophicum ΔH, X68720; Methanobacterium Marburg, X15364, Methanobacterium thermoformicicum FTF, X68713; Methanobacterium thermoformicicum Z245, X68712; Methanocorpusculum bavaricum, X71838; Methanothermus fervidus, M59145; Methanosphaera stadmanae MCB-3, M59139, Methanoseta concilii, X16932, Methanosarcina barkeri 227, M59144; Methanogenium cariaci JR1, M59130; Pyrococcus furiosus, Z54172. The figure shows the part of the tree with strains most closely related to strain A8p.

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 wofeii, 94.2%; Methanobacterium bryantii 92.5%; Methanobacterium thermoautotrophicum CB12, 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. alginosus</th>
<th>M. subterraneum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Rod, 0-4 x 0-8 - 2 x 15</td>
<td>Rod, 0-5 x 0-1 x 1-5</td>
<td>Rod, 0-5 x 0-8 x 1-2</td>
<td>Rod, 0-5 x 0-6 x 2-5</td>
<td>Rod, 0-8 x 3-2</td>
<td>Rod, 0-8 x 3-5</td>
<td>Rod, 0-2 x 0-6 x 2-4</td>
<td>Short rod, 0-1-0-15 x 0-06 x 1-2</td>
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<td>Growth substrates</td>
<td>Filaments, clumps</td>
<td>Filaments, clumps</td>
<td>Filaments</td>
<td>Filaments</td>
<td>Filaments</td>
<td>Filaments</td>
<td>Aggregates</td>
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<tr>
<td>H₂ + CO₂, formate</td>
<td>2-propenyl + CO₂, isobutanol + CO₂</td>
<td>H₂ + CO₂</td>
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<td>H₂ + CO₂</td>
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<td>ND (6-9-7-2)</td>
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<td>7-0-9-9 (8-1-9-1)</td>
<td>4-6-7-0 (5-6-6-2)</td>
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<td>Salinity, M (optimum)</td>
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<td>0-19</td>
<td>0-612</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%</td>
<td>38-48-0</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>
</tr>
<tr>
<td>Reference</td>
<td>(10)</td>
<td>(11-6)</td>
<td>(4)</td>
<td>(8)</td>
<td>(31)</td>
<td>(45)</td>
<td>(21)</td>
<td>This study</td>
</tr>
</tbody>
</table>

ND, Not done.

Strain A8p had a wider temperature range than other mesophilic Methanobacterium species. It was capable of growth at very low temperatures (3-6-20 °C) but also at mesophilic temperatures. This property probably allows A8p to survive at different depths in granitic rock aquifers, in which the temperature increases with depth from below 10 °C at ground level by 1-2 °C for every 100 m. The increase in temperature over the sampled depth interval, 68-420 m, was from 8-5 to 16 °C (20). The temperature in various parts of this environment is stable but the groundwater in-}

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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-butyric acid. Low concentrations of Se4+, Zn2+, Co2+ 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 inhibitory amounts of fatty acids. Thus, the nutritional requirements of A8p reflect its environmental adaptation.

The isolates described here are the first examples of methanogens from deep granitic groundwater, which can be 10000 years old at depths of 400–500 m (34). They are representatives of life in the recently discovered deep biosphere (36) and may represent its inhabitants. The deep biosphere is then consequently postulated to be independent of the Sun-driven ecosystems on the Earth's surface (39, 42, 20, 28, 34). As our findings and other studies of the last years show, methanogenic Archaea probably play a significant role in subsurface biogeochemistry (20, 28, 39).

Because of the phylogenetic and phenotypic characteristics reported above, we propose that A8p is a new species. The proposed name is Methanobacterium subterraneum, with strain A8p as the type strain. The description follows.

Description of Methanobacterium subterraneum sp. nov.

Methanobacterium subterraneum (sub.ter.ra’ne.um. L. adj. neut. subterraneum underground, below the earth/sOIl surface).

Cells are non-motile, small and thin rods, 0.6–1.2 µm in length and 0.1–0.15 µm in diameter, often in aggregates but not in chains. The substrates used for growth and methane production include hydrogen and carbon dioxide and formate, but not methylanines, acetate, pyruvate, dimethyl sulfide, methanol or other alcohols plus carbon dioxide. It grows autotrophically in mineral medium without any organic additions. Growth is inhibited by yeast extract (2 g l⁻¹), Casamino acids (1 g l⁻¹), isobutyric acid (5 mg l⁻¹), n-butyric 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 54.5 ± 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 11074T), 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 A8pT, 3067 and C2BIS have EMBL/GenBank accession numbers X99044, Y12592 and X99045, respectively.

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