Methanolobus zinderi sp. nov., a methylo-trophic methanogen isolated from a deep subsurface coal seam

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A methanogenic organism from the domain *Archaea* (SD1^T) was isolated from saline water released from a coal seam located 926 m below the surface via a methane-producing well near Monroe, Louisiana, USA. Growth and methanogenesis were supported with methanol, monomethylamine, dimethylamine or trimethylamine, but not with dimethylsulfide, formate, acetate or H₂/CO₂. Cells grew in high-salt minimal medium but growth was stimulated with yeast extract or tryptone. Cells were single, non-motile, irregular coccoids 0.5–1.0 µm in diameter and the cell wall contained protein. Conditions for the maximum rate of growth were 40–50 °C, 0.2–0.6 M NaCl, 100–≥200 mM MgCl₂, and pH 7.0–8.0. The G+C content of the genomic DNA was 42 ± 1 mol %. A comparison of 16S rRNA gene sequences indicated that strain SD1^T was most closely related to *Methanolobus oregonensis* DSM 5435^T with 96 % gene sequence similarity. It is proposed that strain SD1^T represents a novel species, *Methanolobus zinderi* sp. nov. The type strain is SD1^T (=ATCC BAA-1601^T=DSM 21339^T).

Deep subsurface coal deposits are habitats with potential for anaerobic methanogenic consortia that convert coal to methane within abundant water-filled natural fractures called cleats. Stable carbon isotope analyses from Permian coal beds in Australia (Smith & Pallasser, 1996) and Wilcox coal beds in northern Louisiana, USA (McIntosh *et al.*, 2007), indicate that a portion of the coal bed methane (CBM) is biological in origin. Sequences of 16S rRNA genes obtained from the water of a coal seam in northern Japan, located between 843 and 907 m below the surface, indicated the presence of methanogenic species belonging to the genera *Methanoculleus* and *Methanolobus* (Shimizu *et al.*, 2007). Further, unidentified microbial cells have been observed in water from a coal seam in northern Louisiana (Warwick & MacIntosh, 2007). However, methane-producing isolates from coal beds have yet to be reported. Here we describe the characteristics of strain SD1^T, a methane-producing species isolated from water from a northern Louisiana coal seam.

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Abbreviation: CBM, coal bed methane.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of *Methanolobus zinderi* sp. nov. SD1^T are EU711413^T and EU715818^T, respectively.

Graphs showing the effects of temperature, Na⁺, Mg²⁺ and pH on the growth of strain SD1^T are available with the online version of this paper.

Strain SD1^T was isolated from a sample of water obtained from a Wilcox coal seam via a CBM well located approximately 40 miles south of Monroe, LA, USA. The sample was taken at the well head in sterile glass jars filled to the top and then sealed to exclude air before transport to the laboratory. The coal seam is situated 926 m below the surface where the temperature is 51 °C and the associated water has a pH of 7.8 and contains 26 mM Mg²⁺ and 0.87 M Na⁺.

An anaerobic chamber (Coy Manufacturing) was used for isolation and the Hungate method (Hungate, 1969) with modifications (Miller & Wolin, 1974; Sowers *et al.*, 1984) was used for enrichment cultures and growth studies. The high-salt minimal medium was as described by Sowers *et al.* (1993) with the following substrates where indicated: 250 mM methanol, 100 mM trimethylamine, 100 mM dimethylamine, 100 mM monomethylamine, 100 mM sodium acetate or 160 kPa H₂/CO₂ (80:20) headspace gas mixture. The enrichment medium was high-salt minimal medium containing 250 mM methanol and a head space of N₂/CO₂/H₂ (75:20:5). The specific growth rate was determined by measuring the absorbance of cultures at 600 nm. Growth as a function of pH was determined with high-salt minimal media containing 250 mM methanol with the indicated pH range obtained by adjusting the ratio of CO₂ and N₂ contained in the head space.

Growth experiments were performed in anaerobic culture tubes (16 × 150 mm) that contained 10 ml high-salt minimal medium with the indicated substrate and a head space of N₂/CO₂ (80:20) unless indicated otherwise. Tubes were sealed with a butyl rubber stopper that was secured with an aluminium crimp collar. Growth was monitored spectrophotometrically at 600 nm with a Bausch and Lomb Spectronic 20. Headspace gases were quantified by GC as described by Ferry & Wolfe (1976).

For DNA isolation, stationary phase cells were collected by centrifugation for 5 min at 6000 g at 4 °C. Cells were lysed by incubation for 1 h at 37 °C in buffer containing 10 mM Tris/HCl (pH 7.5), 1 mM EDTA (pH 8.0), 0.3 % (w/v) SDS and 1.5 µg ml⁻¹ RNase (Roche). Genomic DNA was extracted from the cell lysate using a Purgene Genomic DNA Extraction kit (Gentra Systems) following the manufacturer's guidelines. The DNA was further purified by two rounds of phenol/chloroform extraction/ethanol precipitation to remove all residual proteins.

The G + C content of the genomic DNA of strain CD1^T was determined by the thermal denaturation method, essentially as described by Mandel & Marmur (1968). Purified genomic DNA was diluted in standard SSC buffer to an absorbance of 0.4 at 260 nm and was dialysed overnight against 1000 volumes SSC. The DNA melting temperature was calculated by monitoring the change in absorbance at 260 nm over increasing temperatures on a spectrophotometer (model 50; Varian Cary) in combination with a Peltier thermostat-equipped accessory and an anaerobic fluorescence cuvette [2 mm × 1 cm (path length)] (Starna). Genomic DNA from *Escherichia coli* K-12 served as a standard.

For phylogenetic analyses, the 16S rRNA gene was amplified using the *Archaea*-specific primer 3f (5'-TCCGGTTGATCCTGCCGG-3') and the universal primer 1423r [5'-ACGGN(AT)ACCTTGTTACGAGTT-3'] (McInerney *et al.*, 1995) and an Advantage HF2 PCR kit (BD Biosciences). The amplified PCR product was sequenced directly using an ABI 3100 automated DNA sequencer. The above mentioned PCR primers were utilized for sequencing. The sequence between positions 64 and 1401 was obtained in this fashion. A modified inverse PCR approach was employed to sequence the 5' and 3' ends of the 16S rRNA gene. Genomic DNA was cleaved using the blunt cutter *Pst*I and the fragments were self-ligated to form circular molecules. PCR amplification using outward facing primers complementary to the ends of the sequenced portion of the 16S rRNA gene above produced two PCR products of 1.2 kb and 2.2 kb, respectively. The ends of these PCR fragments were sequenced as above using the PCR primers for sequencing. The 2.2 kb fragment yielded the 5' and 3' ends of the 16S rRNA gene. A 745 bp segment of the methyl coenzyme M reductase (*mcrA*) gene was amplified using the primer set ME1[5'-C(AC)ATGCA(AG)AT(ACT)GG(AT)ATGTC-3'] and ME2 [5'-TCAT(GT)GC(AG)TAGTT(AGT)GG(AG)

TAGT-3'] as described previously (Hales & Winstanley, 1996) and was sequenced directly as described above for the 16S rRNA gene.

The sequences of 1332 bases of the 16S rRNA gene and 490 bases of the *mcrA* gene from various species were aligned using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were deduced using the neighbour-joining algorithm (Saitou & Nei, 1987). Bootstrap values were calculated using the neighbour-joining method and 1000 replicate datasets and re-evaluated using the SEQBOOT, DNAPARS and CONSENSE programs implemented within the PHYLIP package.

Results and Discussion

Methanogens were enriched using high-salt minimal medium (Sowers *et al.*, 1993) containing 250 mM methanol as the growth substrate. A 5 ml water sample taken from a deep subsurface coal seam was added to 100 ml medium in a 160 ml stoppered serum vial (Miller & Wolin, 1974) containing a head space of N₂/CO₂/H₂ (75:20:5) and incubated at 37 °C. After methane production subsided, subcultures were established with a 5 ml inoculum from which isolates were obtained by plating on solid medium containing 2 % Nobel agar (Difco) in an anaerobic chamber (Coy Manufacturing). Plates were incubated in an atmosphere of N₂/CO₂ (80:20) at 37 °C. Cells from plates containing a single colony type were cultured in 10 ml liquid medium under a head space of N₂/CO₂ (80:20) at 37 °C. When methane production subsided, serial dilutions were replated and incubated as described above. An isolated colony, containing cells of uniform morphology, was cultured in the liquid medium as described above and designated strain SD1^T.

Colonies on agar plates appeared after 5 days and reached a diameter of 2–3 mm after 14 days. The opaque colonies were circular, raised with a smooth surface and entire margins, opaque, dark yellow to light brown in colour and dry in texture. Cells were non-motile, irregular coccoids of 0.5–1.0 µm in diameter (Fig. 1a, b) that stained Gram-negative. Aggregates were not observed. Thin-section transmission electron microscopy showed features resembling vacuoles (Fig. 1c) and a cell envelope similar to that of other methanogens containing a protein S-layer adjacent to the cell membrane (Fig. 1d). Cell lysis occurred immediately following the addition of SDS (final concentration 0.1 %) to the growth medium, consistent with the presence of a protein cell wall.

Growth studies were conducted in high-salt minimal medium (Sowers *et al.*, 1993) containing the indicated substrate and a head space of N₂/CO₂ (80:20). Growth was monitored at 600 nm and methane was detected by GC. Strain SD1^T grew and produced methane with 250 mM methanol, 100 mM monomethylamine, 100 mM dimethylamine and 100 mM trimethylamine as carbon and energy sources in the absence of H₂. Dimethylsulfide (100 mM), acetate (100 mM), formate (100 mM) and H₂/CO₂ (80:20) were not utilized by strain SD1^T. Although not

Fig. 1. Morphology of cells of strain SD1^T. (a) Phase-contrast micrograph of whole cells. Bar, 5 μ m. (b) Scanning electron micrograph of whole cells. Bar, 0.5 μ m. (c) Thin-section transmission electron micrograph showing vacuole-like structures. Bar, 2 μ m. (d) Thin-section electron micrograph depicting the cell wall (CW) and cytoplasmic membrane (CM). Bar, 0.1 μ m.

required, growth was stimulated by tryptone or acetate. The following growth experiments were conducted in high-salt medium (Sowers *et al.*, 1993) containing 250 mM methanol as the substrate and a head space of N₂/CO₂ (80:20). The temperature range for growth was 25–50 °C, with the maximum rate between 40 and 50 °C (see Supplementary Fig. S1a in IJSEM Online). Growth was not observed at 55 °C. Sodium chloride was required for growth, was supported at concentrations of 0.05–1.8 M NaCl and the maximum growth rate occurred at concentrations between 0.2 and 0.6 M NaCl (Supplementary Fig. S1b in IJSEM Online). Growth was poor with no added MgCl₂ (Supplementary Fig. S1c). The optimal conditions for growth were between 10 and ≥ 200 mM MgCl₂, with the maximum rate between 100 and ≥ 200 mM (Supplementary Fig. S1c). Growth was supported between pH 6.0 and 9.0, with the maximum rate between pH 7.0 and 8.0 (Supplementary Fig. S1d).

The G + C content of the genomic DNA of strain was SD1^T determined to be 42 ± 1 mol%. Comparison of the 16S rRNA gene sequence against GenBank using the BLAST search tool revealed that strain SD1^T was most closely related to the methylotrophic species *Methanobolus oregonensis* (96%), *Methanobolus taylorii* (96%), *Methanobolus vulcani* (95%), *Methanobolus bombayensis* (94%) and several uncultured clones from a biodegraded oil well (97%). Other methylotrophic methanogenic species related to strain SD1^T included *Methanomethylovorans thermophila* (95%), *Methanomethylovorans hollandica* strain ZB (94%), *Methanococcoides burtonii* (93%) and *Methanohalophilus mahii* (93%). A phylogenetic tree constructed using 16S rRNA gene sequences showed that strain SD1^T clustered with members of the genus *Methanobolus*, although bootstrap analysis indicated a

clear branching of strain SD1^T from the recognized species of the genus *Methanobolus* (Fig. 2). This branching occurred in 100% of trees generated by the neighbour-joining or maximum-parsimony methods. The partial nucleotide sequence of the *mcrA* gene of strain SD1^T was most closely related to those of *Methanobolus bombayensis* (94%), *Methanobolus vulcani* (93%), *Methanobolus taylorii* (92%) and *Methanobolus taylorii* (91%). The deduced partial amino acid sequence of the methyl-coenzyme M methylreductase from strain SD1^T was most similar to that from *Methanococcoides burtonii* (90%). Neighbour-joining trees of partial *mcrA* sequences indicated that the gene sequence of strain SD1^T was closest to that of *Methanobolus taylorii* and clustered with other sequences from species of the genus *Methanobolus* (Fig. 2). However, it was clearly separated from other species of the genus *Methanobolus* with the branch occurring in 87% of all trees.

The results of DNA distance matrix analyses of the 16S rRNA and partial *mcrA* gene sequences suggested that strain SD1^T was most closely related to organisms in the genus *Methanobolus*. Springer *et al.* (1995) compared 16S rRNA and *mcrA* gene sequence distances between members of the same genus or different genera within the family *Methanosarcinaceae* and found 16S rRNA gene distances between members of the same genus to be 0.0214 ± 0.0065 , whereas distances between members of different genera were 0.0807 ± 0.0096 . Although these standards did not immediately suggest an unequivocal genus assignment for strain SD1^T, the novel strain was closely related to members of the genus *Methanobolus* with a 16S rRNA gene sequence distance of 0.0351 ± 0.0037 . Similarly, Springer *et al.* (1995) found that *mcrA* gene distances within a given genus were 0.0937 ± 0.0313 , while distances

Fig. 2. Phylogenetic trees of 16S rRNA (a) and *mcrA* (b) gene sequences showing the relationship between strain SD1^T and members of the genera *Methanosarcina*, *Methanomethylovorans*, *Methanohalophilus*, *Methanococcoides* and *Methanolobus*, constructed using the neighbour-joining algorithm. The sequences were obtained from GenBank and accession numbers are shown in parentheses. Bars, 0.1 substitutions per nucleotide position.

between genera were 0.245 ± 0.038 . Applying these standards, it can be seen that strain SD1^T clearly represents a novel species.

Analyses of 16S rRNA genes suggest that a diversity of novel methanogens are present in a variety of deep subsurface environments which include faults (Moser *et al.*, 2005), thermal aquifers (Kimura *et al.*, 2005) rock aquifers (Chapelle *et al.*, 2002, Kotelnikova *et al.*, 1998), shales (Takai *et al.*, 2003), petroleum deposits (Cheng *et al.*, 2007, Grabowski *et al.*, 2005, Orphan *et al.*, 2000), methane gas fields (Mochimaru *et al.*, 2007a, b) and coal beds (Shimizu *et al.*, 2007). However, relatively few methanogens have been isolated from these environments and none have been isolated from coal beds. A better understanding of the microbiology of deep subsurface environments is essential to grasp the extent of the Earth's prokaryotic diversity and share of Earth's living protoplasm. The microbiology of coal beds contributes to this understanding and has the added benefit of enabling on-going efforts for the development of processes for microbial enhancement of CBM. Indeed, it is estimated that an increase of 16 % in CBM reserves could be generated by the microbial conversion of only 0.01 % of US coal (Scott, 1999). Furthermore, it is anticipated that the estimated 10^{14} m³ CBM present in deep coal seams worldwide will contribute substantially to future energy sources (Shimada, 1995).

Although several species of methanogens have been isolated from oilfields (Belyaev *et al.*, 1983; Cheng *et al.*, 2007; Ni & Boone, 1991; Nilsen & Torsvik, 1996; Ollivier *et al.*, 1997), isolates from methane gas fields or methane-producing coal beds have not been reported. The temperature, pH and salinity of the sampled coal water coincided with optimal conditions for growth of strain SD1^T, indicating that this strain is well adapted to its environment. The results provide direct microbiological evidence supporting the 16S rRNA gene sequence, isotopic and chemical analyses (McIntosh *et al.*, 2007; Scott, 1999; Shimizu *et al.*, 2007) that suggest that biogenic methane is produced in deep subsurface coal beds by anaerobic microbial consortia.

The results presented here are an important first step in defining the *in situ* microbial populations and processes necessary to develop methods for the microbial enhancement of CBM. The methylotrophic substrates used by strain SD1^T are characteristic of the genus *Methanolobus* and indicate that one-carbon compounds derived from coal, by either biogenic or thermogenic processes, are potentially important precursors to biogenic CBM. This proposal is supported by 16S rRNA gene sequences obtained from deep coal bed water in Japan that indicated a major presence of uncultured species of the genus *Methanolobus* (Shimizu *et al.*, 2007).

Description of *Methanolobus zinderi* sp. nov.

Methanolobus zinderi (zin' der.i. N.L. gen. masc. n. *zinderi* of Zinder, named in honour of Stephen H. Zinder for his outstanding contributions to our understanding of the microbiology and ecology of methanogenesis).

Cells are irregular coccoids, 1.0–2.0 µm in diameter. Cells are lysed by detergents. Gram-negative. Non-motile. Cells grow by forming methane from methanol and methylamines. Cells are unable to grow with H₂/CO₂ or acetate. The maximum rate of growth is between 40–50 °C, 0.2–0.6 M NaCl, 100–≥200 mM MgCl₂ and pH 7.0–8.0. No organic compounds are required for growth.

The type strain, SD1^T (=ATCC BAA-1601^T=DSM 21339^T), was isolated from a coal seam near Monroe, LA, USA. The G+C content of the genomic DNA of the type strain is 42 ± 1 mol%.

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