**Methanosarcina soligelidi** sp. nov., a desiccation- and freeze-thaw-resistant methanogenic archaeon from a Siberian permafrost-affected soil

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A methanogenic archaeon, strain SMA-21ᵀ, was isolated from a permafrost-affected soil by serial dilution in liquid medium. The cells were non-motile, stained Gram-negative and grew as irregular cocci with a diameter of 1.3–2.5 µm. Optimal growth was observed at 28 ± 0.6 °C, pH 7.8 and 0.02 M NaCl. The strain grew on H₂/CO₂, methanol and acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethylamine, dimethylamine, trimethylamine or dimethyl sulfide. Major membrane lipids of strain SMA-21ᵀ were archaeol phosphatidylglycerol, archaeol phosphatidylethanolamine and the corresponding hydroxyarchaeol compounds. The G+C content of the genomic DNA was 40.9 mol%. The 16S rRNA gene sequence was closely related to those of *Methanosarcina mazei* DSM 2053ᵀ (similarity 99.9 %) and *Methanosarcina horonobensis* HB-1ᵀ (similarity 98.7 %). On basis of the level of DNA–DNA hybridization (22.1 %) between strain SMA-21ᵀ and *Methanosarcina mazei* DSM 2053ᵀ as well as of phenotypic and genotypic differences, strain SMA-21ᵀ was assigned to a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina soligelidi* sp. nov. is proposed. The type strain is SMA-21ᵀ (= DSM 20065ᵀ = JCM 18468).

**Permafrost and the associated soils cover more than 25 % of the land surface (Zhang et al., 1999). These ecosystems play an essential role for the Earth’s climate due to the large amount of organic carbon preserved in the soils and sediments (Tarnocai et al., 2009) and because of the rapidly warming of permafrost in response to global climate change (Romanovsky et al., 2010). Methanogenic archaea are the object of particular interest in permafrost studies, because of their key role in the Arctic methane cycle and their significance for the global methane budget (Graham et al., 2012). Recent studies have shown that methanogens from permafrost environments are extremely stress tolerant and can still be metabolically active at sub-zero temperatures (Morozova & Wagner, 2007; Wagner et al., 2007).

Although the abundance and diversity of methanogens in permafrost environments is similar to that of communities of comparable temperate soil ecosystems (Wagner et al., 2005), only a few novel strains have been isolated and described from permafrost habitats. *Methanobacterium arcticum* was isolated from Holocene permafrost deposits (Shcherbakova et al., 2011) and *Methanobacterium veterum* was obtained from ancient permafrost, with an age of three million years, after long-term anaerobic cultivation (Krivushin et al., 2010). Another study described novel strains of methanogens from different low-temperature environments including strain MT, enriched from an Arctic tundra soil, which represents a new ecotype of *Methanosarcina mazi* (Simankova et al., 2003). Recently, three different methanogenic archaea closely related to *Methanosarcina mazi* and species of the genus...**

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Methanosarcina soligelidi* SMA-21ᵀ is JF812255.
Methanobacterium have been described from permafrost deposits of different ages (Rivkina et al., 2007).

In this study, we describe the characteristics of a novel methanogenic archaean, strain SMA-21T from a permafrost environment in north-east Siberia.

Strain SMA-21T was isolated from a permafrost-affected soil located on the northern tip of the island Samoylov (72° 22’ N 126° 28’ E), Lena Delta, Siberia. According to the USA soil taxonomy guidelines, the soil was characterized as Psammentic Aquorthel (Soil Survey Staff, 1999). The soil sample was collected in July 2002 from the soil surface (0–5 cm soil depth) and transported under frozen conditions to Potsdam, Germany.

Enrichment of methanogenic archaea was started by adding 2 g fresh soil material into a serum bottle (125 ml) containing 50 ml anaerobic medium of the following composition (l−1): NH4Cl, 1.0 g; MgCl2.6H2O, 1.0 g; CaCl2.2H2O, 0.4 g; KH2PO4, 0.5 g; NaHCO3, 12 g; Na2S.3H2O, 0.5 g; trace element solution (Balch et al., 1979), 10 ml; vitamin solution (Bryant et al., 1971) 10 ml; and 2 ml resazurin (7-hydroxy-3H-phenoxazin-3-on-10-oxide) indicator solution. The bottles were sealed with black rubber stoppers and pressurized with H2/CO2 (80:20, v/v, 150 kPa) as substrate. Cultures were incubated in the dark at 28 °C. After methane production was observed in the headspace, 5 ml of the culture was anaerobically transferred into a new bottle of sterile medium, which was supplemented with the antibiotics erythromycin and phosphomycin (each 50 μg/ml) to suppress growth of non-methanogenic microorganisms (Hilpert et al., 2001). This procedure was repeated until a pure culture was obtained. All further incubations including the purity check were done without any antibiotics. Purity of the strain was confirmed by light microscope examination, the absence of growth in rich medium containing (l−1) 4 g glucose, 2 g yeast extract and 2 g peptone, and denaturing gradient gel electrophoresis analyses of DNA extracts obtained from the culture. The strain was maintained by three-monthly transfer into liquid medium. After regrowth at 28 °C, the culture was stored at 5 °C. All preparation steps were done under strictly anaerobic conditions.

Phase-contrast microscopy (Axioskop 2; Zeiss) was performed in the exponential growth phase. Cells were irregular cocci, around 1.3–2.5 μm in diameter (Fig. 1) and non-motile. Cells stained Gram-negative, but the reaction was sometimes variable. Lysis of the cells was observed in a 0.01% (w/v) SDS solution.

Growth and substrate utilization were determined by culturing strain SMA-21T in the medium described above and growth rate was estimated by measuring the concentration of methane in the gas phase (Powell, 1983). The methane concentration was measured by gas chromatography as described previously (Koch et al., 2009). All growth tests were performed in triplicate at 28 °C. The effect of temperature on growth was tested with H2/CO2 (80:20, v/v) as substrate at 0, 5, 10, 16, 22, 28, 33, 38, 44, 54 and 64 °C. Growth of strain SMA-21T was observed at 0–54 °C, with optimum growth at 28 °C (Fig. 2a). The pH of the growth medium was adjusted to pH 4.1–9.9 with 1 M HCl and 1 M NaOH, respectively. Growth was observed between pH 4.8 and 9.9, with optimum growth at pH 7.8 (Fig. 2b). The salinity range was determined in medium with 0.02–0.6 M NaCl. Optimum growth was measured at 0.02 M, and growth was observed at salt concentrations of up to 0.6 M (Fig. 2c). All preparation steps were done under strictly anaerobic conditions.

Intact membrane lipids were examined for strain SMA-21T and its closest relative Methanosarcina mazei (similarity 99.9%) using a method described by Zink & Mangelsdorf (2004). The intact lipids were detected with a HPLC electrospray interface MS (HPLC-ESI-MS) system. Furthermore, ether cleavage experiments were conducted.

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**Fig. 1.** Phase-contrast micrograph of cells of strain SMA-21T. Bar, 10 μm.
to determine the lipid side chain inventory following a method described by Gattinger et al. (2003). Compounds were detected using a GC-MS system. Both archaea possessed the same set of membrane diether lipids, detected in the HPLC-ESI-MS negative ion mode. The major lipids were archaeol phosphatidylglycerol (ArPG; [M-H]− at m/z = 805), hydroxyarchaeol phosphatidylglycerol (Hydroxy-ArPG; [M-H]− at m/z = 821), archaeol phosphatidylethanolamine (ArPE; [M-H]− at m/z = 774) and hydroxyarchaeol phosphatidylethanolamine (Hydroxy-ArPE; [M-H]− at m/z = 790; Table 1). GC-MS measurement on the side chain inventory supported the results obtained from the intact lipid analysis. The major compound in the GC-MS run was phytane, being the ether cleavage product of archaeol. Tetraether lipids or biphytanes, respectively, were not detected. Archaeol phospholipids were also the main phospholipids of Methanococoides burtonii isolated from the Ace Lake, Antarctica (Nichols et al., 2004).

Environmental stress tolerance was tested as previously described (Morozova & Wagner, 2007). Briefly, oxygen sensitivity and desiccation was tested by adding cell suspensions of 10^8 cells ml^-1 onto microscope coverslips, which were exposed to aerobic conditions or completely dried. After certain time periods, cells were anaerobically resuspended and methane production was checked. Freeze–thaw resistance was tested by freezing cultures of 10^8 cells ml^-1 immediately in medium at −78.5 °C. After 24 h the frozen cells were thawed at room temperature and either frozen again at −78.5 °C or methane production was measured. In addition, cells were slowly frozen (0.2 °C min^-1) to −20 °C. Initial methane production rates were measured before freezing and compared with those obtained after thawing for samples held at −20 °C for a period of 1–2 years. The results indicated a high survival potential of strain SMA-21T against air exposure (up to 72 h), desiccation (up to 25 days), freeze–thaw cycles down to −78.5 °C and long-term freezing (up to 2 years at −20 °C).

DNA was isolated using a Microbial DNA Isolation kit (MoBio Laboratories) according to the manufacturer’s protocol. For 16S rRNA gene amplification, general archaean primers were used: ArUn4F (TCYGGTTGAT-CCTGCCR) and Arc1492R (GGCTACCTTGTTACGA-CIT). Sequencing by GATC Biotech (Konstanz, Germany) resulted in a 1337 bp gene product. Alignments were performed with all known isolates of the genus Methanosarcina. Sequences were obtained from GenBank and aligned using the integrated SINA alignment tool from the ARB-SILVA website (Pruesse et al., 2007). Sequences were checked manually using the ARB program (Ludwig et al., 2004) and evolutionary distances were calculated based on the neighbour-joining algorithm. Reconstruction of a phylogenetic tree was done by using the neighbour-joining method (Saitou & Nei, 1987; Fig. 3) and a termini filter that is implemented in the ARB program. To evaluate the tree topologies, a bootstrap analysis with 1000 replications was performed. For strain SMA-21T, highest 16S rRNA
gene sequence similarities were found to the type strains of Methanosarcina mazei DSM 2053\textsuperscript{T} (99.9\%) , Methanosarcina horonobensis HB-1\textsuperscript{T} (98.7\%) and Methanosarcina siciliae T4/MT (97.9\%). Determination of G+C content of DNA was done by HPLC according to the method of Mesbah et al. (1989). The DNA G+C content of strain SMA-21\textsuperscript{T} was 40.9 mol\%. DNA–DNA hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with \textit{in situ} temperature probe (Varian). Although high 16S rRNA gene sequence similarity was observed between strain SMA-21\textsuperscript{T} and related members of the genus Methanosarcina, the DNA G+C content differences and the DNA–DNA hybridization values indicated that strain SMA-21\textsuperscript{T} represents a novel species of the genus Methanosarcina. The DNA–DNA hybridization values are shown in Table 1.

### Table 1. Characteristics of strain SMA-21\textsuperscript{T} and related members of the genus Methanosarcina

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Irregular cocci</td>
<td>Irregular cocci</td>
<td>Irregular cocci</td>
<td>Irregular cocci</td>
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<tr>
<td>Cell dimension ((\mu)m)</td>
<td>1.3–2.5</td>
<td>1.0–3.0</td>
<td>1.4–2.9</td>
<td>1.5–3.0</td>
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<tr>
<td>Gram stain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Temperature range for growth ((^\circ)C)</td>
<td>0–54</td>
<td>23–50</td>
<td>20–42</td>
<td>25–45</td>
</tr>
<tr>
<td>Optimum temperature ((^\circ)C)</td>
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<td>30–40</td>
<td>37</td>
<td>40</td>
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<tr>
<td>pH range</td>
<td>4.8–9.9</td>
<td>6.1–8.0</td>
<td>6.0–7.8</td>
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<tr>
<td>Optimum pH</td>
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<td>7.0</td>
<td>7.0–7.3</td>
<td>6.5–6.8</td>
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<tr>
<td>Tolerance of NaCl (M)</td>
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<td>0–1.7</td>
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<tr>
<td>Optimum NaCl for growth</td>
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<td>0.1</td>
<td>0.4–0.6</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>(\text{H}_2/\text{CO}_2)</td>
<td>+</td>
<td>+ *</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
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<td>– *</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monomethylamine</td>
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<td>– *</td>
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<tr>
<td>Dimethylamine</td>
<td>–</td>
<td>+ *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>40.9</td>
<td>42.0</td>
<td>41.4</td>
<td>42–43</td>
</tr>
<tr>
<td>ArPG</td>
<td>+</td>
<td>+ *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hydroxy-ArPG</td>
<td>+</td>
<td>+ *</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>ArPE</td>
<td>+</td>
<td>+ *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hydroxy-ArPE</td>
<td>+</td>
<td>+ *</td>
<td>ND</td>
<td>ND</td>
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</table>

*Data obtained in this study.*

![Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences of the novel strain, SMA-21\textsuperscript{T}, within the genus Methanosarcina (with Methanopyrus kandleri AV19\textsuperscript{T} as the outgroup). The tree was reconstructed by the neighbour-joining algorithm, but all branches were also found in maximum-likelihood (Fitch, 1971) and maximum-parsimony trees (Felsenstein, 1981). Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values \(\geq 50\%\) are shown. Bar, 0.01 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
and Methanosarcina mazei, the results of DNA-DNA hybridization indicated only 22% genomic relatedness (two replications). According to the recommendation of Wayne et al. (1987) and based on phylogenetic and physiological characteristics according to the minimal standards for the description of new taxa of prokaryotic strains (Tindall et al., 2010), a novel species of the genus Methanosarcina is proposed, named Methanosarcina soligelidi sp. nov.

Description of Methanosarcina soligelidi sp. nov.

Methanosarcina soligelidi (so.li.ge’li.di. L. n. solum soil; L. adj. gelidus –a –um icy cold, very cold; N.L. gen. n. soligelidi of a icy cold soil, referring to the isolation of the type strain from permafrost-affected soil).

Cells are strictly anaerobic, Gram-negative staining, irregular cocci that are 1.3–2.5 μm in diameter. Cells grow on H2/CO2, methanol and acetate, but not on formate, ethanol, 2-butanol, 2-propanol, monomethylamine, dimethylamine, trimethylamine or dimethyl sulfide. Optimal conditions for growth (range) are 28 °C (0–54 °C), pH 7.8 (pH 4.8–9.9) and 0.02 M NaCl (0.02–0.6 M). Major membrane lipids are archaeol phosphatidylglycerol, archaeol phoshatidylethanolamine and the corresponding hydroxy-archaeol compounds.

The type strain, SMA-21T (= DSM 20065T = JCM 18468), was isolated from a permafrost-affected soil on Samoylov Island, Lena Delta, Siberia. The DNA G+C content of the type strain is 40.9 mol %.

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References


