Methanosalsum natronophilum sp. nov., and Methanocalculus alkaliphilus sp. nov., haloalkaliphilic methanogens from hypersaline soda lakes

Dimitry Y. Sorokin,1,2 Ben Abbas,2 Alexander Y. Merkel,1 W. Irene C. Rijpstra,3 Jaap S. Sinninghe Damsté,4,5 Marina V. Sukhacheva5 and Mark C. M. van Loosdrecht2

Correspondence
D. Y. Sorokin
d.sorokin@tudelft.nl;
soroc@inmi.ru

1Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia
2Department of Biotechnology, TU Delft, The Netherlands
3Department of Marine Organic Biogeochemistry, NIOZ Netherlands Institute for Sea Research, The Netherlands
4Department of Earth Sciences – Geochemistry, Faculty of Geosciences, Utrecht University, Utrecht, The Netherlands
5Institute of Bioengineering, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia

Two groups of haloalkaliphilic methanogenic archaea were dominating in enrichments from hypersaline soda lake sediments at pH 10. At moderate salt concentrations with formate or H2 as electron donor, methanogens belonging to the genus Methanocalculus were enriched, while at high salt concentrations with methylated substrates, a group related to Methanosalsum zhilinae was dominating. For both groups, several pure cultures were obtained including the type strains AMF2T for the Methanocalculus group and AME2T for the Methanosalsum group. The Methanocalculus group is characterized by lithoheterotrophic growth with either formate (preferable substrate) or H2 at moderate salinity up to 1.5–2 M total Na+ and obligate alkaliphilic growth with an optimum at pH 9.5. According to phylogenetic analysis, the group also includes closely related strains isolated previously from the low-salt alkaline Lonar Lake. The novel Methanosalsum group is characterized by high salt tolerance (up to 3.5 M total Na+) and obligate alkaliphilic growth with an optimum at pH 9.5. It has a typical methylotrophic substrate profile, utilizing methanol, amino acids and dimethyl sulfide (at low concentrations) as methanogenic substrates. On the basis of physiological and phylogenetic data, it is proposed that the two groups of soda lake methanogenic isolates are assigned into two novel species, Methanocalculus alkaliphilus sp. nov. (type strain AMF2T=DSM 24457T=UNIQEM U859T) and Methanosalsum natronophilum sp. nov. (type strain AME2T=DSM 24634T=NBRC 110091T).

Methanogenic euryarchaea are an important functional group of secondary anaerobes participating in the global carbon cycle. They are active at a very broad range of environmental conditions including extreme temperatures, salt and pH (Hedderich & Whitman, 2013). Saline soda lakes represent a unique ‘double extreme’ habitat, with a salt content up to saturation and a high pH from 9.5 to 11. In saline habitats, methylotrophic methanogenesis is usually the dominant pathway since lithotrophic and acetoclastic methanogens are outcompeted by sulfate-reducing bacteria (Oremland & King, 1989; McGenity, 2010). Until now, little was known about the identity of methanogenic archaea in soda lakes. Early work in hypersaline alkaline lakes of Wadi al Natrun (Egypt) resulted in the isolation and characterization of two different methanogens capable of...
of growth at elevated pH: moderately halophilic, methylo-
trophic Methanosalum zhilinae (formerly Methanoalhalophilus
zhilinae) (Boone et al., 1986; Mathrani et al., 1988; Boone &
Baker 2001) and low salt-tolerant and alkali-tolerant, hydro-
genotrophic Methanobacterium alcaliphilum (Worakit et al., 1986).
A strain of Methanosalum zhilinae, very similar in properties to
the type strain, was later obtained from the hypersaline soda
lake Magadi (Kevbrin et al., 1997).
Recent intensive microbiological and molecular ecological
investigation of various saline alkaline lakes also dem-
strated a presence of two different subgroups of obligately
haloalkaliphilic, lithotrophic and methanogenic archaea
belonging to the genus Methanocalculus. The low salt-
tolerant subgroup has first been detected in the slightly
saline, alkaline Lonar Lake (Surakasi et al., 2007; Antony
et al., 2012), and an extremely salt-tolerant species, described
as Methanocalculus natronophilus, has recently
been obtained from a hypersaline soda lake in SW-Siberia
(Zhilina et al., 2013). The latter is remarkable, since in its
salt tolerance Methanocalculus natronophilus far exceeded
the highest limit of 2 M Na⁺ known so far for the hydro-
genotrophic methanogenesis in halophilic Methanocalculus
halotolerans (Ollivier et al., 1998).

Our methanogenesis activity measurements combined
with the mcrA gene molecular detection have demon-
strated that, in contrast to saline habitats with neutral
pH, lithotrophic methanogenesis can be active up to
extremely high salt concentrations in soda lakes (Nolla-
Ardèvol et al., 2012; Sorokin et al., 2015). This work
also resulted in isolation of multiple pure cultures of
haloalkaliphilic methanogens from Siberian soda lakes,
among which two groups were substantially different in
properties and phylogeny from the so far described
methanogens found in haloalkaline lakes. In this paper,
these groups are proposed as two novel species of methy-
lotrophic and lithotrophic haloalkaliphilic methanogens,
respectively.

The source of the isolates was the surface layer (2–10 cm)
of anoxic sediments from hypersaline soda lakes in Kulunda
Steppe obtained in 2009–2012. The lake properties and
the strain origin are described previously (Sorokin et al.,
and lithotrophic methanogens belonging to the genus
Methanocalculus were enriched and purified, either by
serial dilutions or by a combination of serial dilutions and
plating using sodium carbonate mineral medium con-
taining 0.6 M total Na⁺ and buffered at pH 10, sup-
plemented with 2 mM acetate as carbon source and either
50 mM formate or H₂ (1 atm) as the electron donor.
Three extremely salt-tolerant methylotrophic isolates were
enriched and further purified by serial dilution (since
colony formation was not achieved) using sodium carbon-
ate mineral base containing 2–3 M total Na⁺ at pH 10 and
supplemented either with 50 mM MeOH or 5 mM of
trimethylamine or dimethyl sulfide. In the case of trimethy-
lamine, ammonium was omitted from the medium. Details
of media composition, isolation procedure, as well as
analytical methods and phylogenetic analysis, are described
previously (Sorokin et al., 2015).

Phase-contrast micrographs were obtained using a Zeiss
Axioplan Imaging 2 microscope. For whole-cell electron
microscopy, cells were centrifuged and resuspended in
0.5 (strain AMF2T) or 1.0 (strains AME2T) M NaCl sol-
sion, fixed with glutaraldehyde (final concentration 3 %,
v/v) for 2 h at 4 °C, then washed again with the same
NaCl solutions. The fixed cells were positively contrasted with
1 % (w/v) uranyl acetate. For thin sectioning, the
cell pellets were fixed in 1 % (w/v) OsO₄ containing 0.5–
1.0 M NaCl for 48 h at room temperature, washed, stained
overnight with 1 % (w/v) uranyl acetate, dehydrated in an
increasing ethanol series, and embedded in Epon resin.
Thin sections were stained with 1 % (w/v) lead citrate.
Membrane lipid analyses were performed by methods
described in Sinninghe Damsté et al. (2011) and Weijers
et al. (2009). The isolation of DNA and subsequent deter-
mination of the G+C content was performed according to
Marmur (1961) and Marmur & Doty (1962), respectively.
DNA–DNA hybridization between low and high salt-toler-
ant alkali-tolerant Methanocalculus strains was performed by
the thermal denaturation method (De Ley et al., 1970).

In total, eleven lithotrophic methanogenic isolates were
obtained at moderate salt concentrations with either for-
mat or H₂ as substrates and three high salt-tolerant
methylotrophic methanogens from sediments of hypersa-
line soda lakes in Kulunda Steppe. The isolates within
their corresponding groups were very similar genetically
and phenotypically. Therefore, only representative type
strains for the groups, AMF2T for the Methanocalculus
group and AME2T for the Methanosalum group, have
been chosen for in-depth characterization. The typical
cell morphology and ultrastructure of the type strains are
shown in Figs 1 and 2. The Methanocalculus isolates have
angular coccoid flattened cells that are highly motile by
multiple archaella, while cells of the Methanosalum isolates
are also coccoid and angular, but somewhat smaller, non-
motile and not flattened. Both strains have thin, apparently
proteinaceous cell walls (cells are easily lysed in hypotonic
solutions and in the presence of SDS), and both have bright
blue autofluorescence indicating the presence of deazoflu-
vine (F420).

All Methanocalculus strains in this study were able to grow
with formate (preferable) or H₂ as electron donor, and pro-
duce methane in the presence of acetate as carbon source
(yeast extract did not stimulate growth). Optimal growth
occurred in carbonate-based media at pH 9.5 and moderate
salinity of 0.3–0.6 M total Na⁺. The Methanosalum strains
grew best with methanol (50 mM) as the substrate, but
were also able to use methylamines and dimethyl sulfide
at low concentrations (<5 mM). Optimal growth occurred
in carbonate-based media at pH 9.5 and moderate salinity
of 1–2 M total Na⁺, but all three strains also grew in
at least up to 3 M total Na⁺, in which they were clearly
different from the moderately salt-tolerant Methanosalum...
Fig. 1. Cell morphology of *Methanocalculus* strain AMF2<sup>T</sup> grown with formate at pH 10 and 0.6 M Na<sup>+</sup>. (a), phase-contrast microscopy; (b and c), electron microscopy of total cells and thin sections, respectively. (N), nucleoid; CS - cross sections showing that the cells are flattened.

Fig. 2. Cell morphology of *Methanosalsum* strain AME2<sup>T</sup> grown with methanol at pH 10 and 2 M Na<sup>+</sup>. (a), phase-contrast microscopy; (b and c), electron microscopy of total cells and thin sections.
zhilinae. More detailed phenotypic properties of the novel isolates in comparison with the nearest relatives are given in Table 1.

The membrane lipid analysis demonstrated that both examined strains AMF2\textsuperscript{T} and AME2\textsuperscript{T} produce diether and tetraether lipids in approximately equal abundance. LC/MS analyses identified the glycerol dibiphytanyl glycerol tetraether with no cyclopentane moieties (GDGT-0) as the most abundant tetraether for both species. The diether core lipids in strain AMF2\textsuperscript{T} consist of three components: archaeol (86\%) and 1- and 2-phytanyl glycerol ether (PGE; 12 and 2\%, respectively). These latter components are likely artefacts formed during acid hydrolysis from diether lipids containing unsaturated phytanyl moieties. The presence of such components was confirmed by LC-ESI-MS\textsuperscript{n} of the Bligh/Dyer extract of strain AMF2\textsuperscript{T}. This also indicated that the head groups of the intact polar lipids (IPLs) are unknown and require further study. Strain AME2\textsuperscript{T} had a more complicated diether lipid composition. It also contained archaeol (55\%), and 1- and 2-phytanyl glycerol ether (15 and 5\%, respectively). In addition, hydroxy archaeols (18\%) and the macrocyclic dibiphytanyl glycerol ether (7\%) were present. This complex composition of diether lipids is in agreement with that reported for their close relatives, *Methanosalsum zhilinae* DSM 4017\textsuperscript{T} and *Methanohalobium estigatatum* Z-7303\textsuperscript{T} (Koga et al., 1998). LC-ESI-MS\textsuperscript{n} of the Bligh/Dyer extract of strain AME2\textsuperscript{T} indicated again a complicated pattern of IPLs in which archaeol containing a phosphatidylglycerol head group was identified as a minor constituent.

Phylogenetic analyses of sequences of the 16S rRNA gene (Fig. 3a) and the functional marker McrA (Fig. 3b) showed that the low-salt-tolerant and high-salt-tolerant *Methanocalculus* isolates from soda lakes formed two separate clusters; the former includes 11 isolates from the Siberian soda lakes and also, probably the Lonar lake isolates (Surakasi et al., 2007), while the high-salt-tolerant *Methanocalculus* strains from hypersaline Siberian lakes formed another cluster around the already described *Methanocalculus natronophilus* (Zhilina et al., 2013). The phylogenetic distance in 16S rRNA gene sequences between the representatives of two soda lake *Methanocalculus* clusters was around 1.2–1.5\% and for the partial McrA sequences was 4–5\%. These levels of divergence indicated that the two clusters might represent two different species. Indeed, DNA–DNA hybridization between the two representative strains (AMF2\textsuperscript{T} for the moderate salt-tolerant cluster and AMF5 for the *Methanocalculus natronophilus* cluster) was 32\%, which is far below the inter-species level of 70\%. Likewise, despite general phenotypic similarity to the type and the only species of the genus *Methanosalsum* - *Methanosalum zhilinae*, the phylogeny of three new *Methanosalum* isolates from Siberian soda lakes was clearly above the intraspecies level (3.2–4.5\% and 4\% difference for the ribosomal gene and McrA partial protein sequences, respectively).

### Table 1. Comparison of the properties of novel methanogenic isolates from soda lakes with their immediate haloalkaliphilic relatives

Data for strains AMF2\textsuperscript{T} and AME2\textsuperscript{T} from this study, for *Methanocalculus natronophilus* from Zhilina et al. (2013), and for *Methanosalum zhilinae* from Boone & Baker (2001), Kevbrin et al. (1997) and Koga et al. (1998). ND, Not determined; MeOH, methanol; DMS, dimethyl sulphide.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Methanocalculus</th>
<th>Methanosalum</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF2\textsuperscript{T}</td>
<td>Methanocalculus natronophilus</td>
<td>AME2\textsuperscript{T}</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Flattened angular coccoids</td>
<td>Irregular angular coccoids</td>
</tr>
<tr>
<td>Motility</td>
<td>+ Multiple archaella plus</td>
<td>+ Multiple archaella plus</td>
</tr>
<tr>
<td>Methanogenic substrates</td>
<td>H\textsubscript{2}, formate</td>
<td>H\textsubscript{2}, formate</td>
</tr>
<tr>
<td>Salt range for growth (optimum) (M Na\textsuperscript{+})</td>
<td>0.2–1.5 (0.6)</td>
<td>1.0–3.3 (1.4–1.9)</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>8–10.2 (9.5)</td>
<td>8–10.2 (9–9.5)</td>
</tr>
<tr>
<td>Maximum growth temperature (°C)</td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td>Membrane core lipids</td>
<td>Archaeol, GDGT-0</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>51.1</td>
<td>50.2</td>
</tr>
<tr>
<td>Isolation source</td>
<td>Siberian soda lakes</td>
<td></td>
</tr>
</tbody>
</table>
Novel haloalkaliphilic methanogens from soda lakes

Fig. 3. Phylogeny of novel haloalkaliphilic methanogens from Siberian soda lakes based on 16S rRNA gene (a) and McrA marker (b) sequence analysis. The 16S rRNA gene-based tree was reconstructed by using maximum-likelihood method RAxML (algorithm: rapid bootstrap analysis; consensus length 1287 nt positions). The bootstrap values (250 rounds) were all >70 % and therefore are not shown on the tree. For the McrA-based tree, the neighbour-joining method was applied on the basis of 132–137 aa positions (numbers at nodes represent mean bootstrap values from 1000 rounds). Bars, 0.10 (a) and 0.12 (b) substitutions per position.

Overall, on the basis of phenotypic and genetic differences, the novel moderately salt-tolerant alkaliphilic Methanocalculus isolates and extremely salt-tolerant alkaliphilic Methanosalum isolates from Siberian hypersaline soda lakes are proposed to be accommodated into two novel species – Methanocalculus alkaliphilus sp. nov. and Methanosalum natronophilum sp. nov.
Description of Methanocalculus alkaliphilus sp. nov.

Methanocalculus alkaliphilus [al.ka.li.phi’lus. N. L. n. alkali (from Arabic article al, the, and Arabic n. galii) ashes of saltwort; N.L. adj. philus (from Gr. adj. philos) loving; N.L. adj. alkaliphilus loving alkaline conditions].

Cells are angled flattened coccoids, 1.5–2.5 μm and motile by multiple peritrichous archaella. The cell wall consists of a thin proteinaceous layer. The cells from actively growing cultures show strong blue autofluorescence (F420). Colonies formed inside agar are 1 mm maximum, soft, disc-like and yellowish. Core membrane lipids included archaeol and GDGT-0. Obligately anaerobic, growing with either formate or H$_2$ as electron donor and acetate as carbon source with methane as the end-product. Ammonium is utilized as a nitrogen source. Optimum growth temperature is 35 °C (maximum at 41 °C). Obligately alkaliphilic with a pH for growth with formate from 8 to 10.2 (optimum at pH 9.5) and moderately salt-tolerant with the range from 0.2 to 1.5 M total Na$^+$ in sodium carbonate buffer at pH 9.5. The species encompasses 11 closely related isolates from south Siberian soda lakes and several isolates from alkaline Lake Crater Lake in India.

The type strain (AMF2$^T$ = DSM 24457$^T$ = UNIQEM U859$^T$) was isolated from mixed sediments of soda lakes in Kulunda Steppe (Altai, Russia). The G+C content of the DNA of the type strain is 51.1 mol% ($T_m$).

Description of Methanosalsum natronophilum sp. nov.

Methanosalsum natronophilum [na.tro.no.phi’lum. N. L. n. natron (arbitrarily derived from the Arabic n. natrun or natron), soda; N.L. pref. natrono-, pertaining to soda; N.L. adj. philum (from Gr. fem. adj. phile), friend, loving; N.L. adj. natronophilum (soda-loving)].

Cells are angled, non-motile coccoids, 0.7–2 μm, with strong blue autofluorescence (F420). The cell wall consists of a thin proteinaceous layer. Colony formation was not achieved at the cultivation conditions used. Core membrane lipids included the diether lipids archaeol, hydroxy archaeol, cyclic archaeol and the membrane-spanning lipid GDGT-0. Obligately anaerobic, growing best with methanol at high concentrations (up to 100 mM) or (much slower and at low concentrations) with methanamines and dimethyl sulfide. Yeast extract (100 mg l$^{-1}$) moderately stimulated growth with methanol. Ammonium is utilized as a nitrogen source, either free or produced from the methanamines. Optimum growth temperature is 37 °C (maximum at 43 °C). Obligately alkaliphilic with a pH range for growth with methanol from 8.2 to 10.2 (optimum at pH 9.5) and highly salt-tolerant with a range from 0.5 to 3.5 M total Na$^+$ in sodium carbonate buffer at pH 9.5.

The type strain (AME2$^T$ = DSM 24634$^T$ = NBRC 110091$^T$) was isolated from mixed sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The G+C content of the DNA of the type strain is 44.8 mol% ($T_m$).

Acknowledgements

This work was supported by the Netherlands Applied Science Foundation (STW, project 12226) and by the Russian Foundation for Basic Research (RFBR 13-04-00049). I.S.S.D. and M.C.M. v. L. were supported by gravitation grant SIAM (24002002).

References


