

Delft University of Technology

Methanonatronarchaeum thermophilum gen. Nov., sp. nov. and 'Candidatus methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprising a new euryarchaeal class Methanonatronarchaeia classis nov.

Sorokin, Dimitry Y.; Merkel, Alexander Y.; Abbas, Ben; Makarova, Kira S.; Rijpstra, W. Irene C.; Koenen, M.; Sinninghe Damsté, Jaap S.; Galinski, Erwin A.; Koonin, Eugene V.; Van Loosdrecht, Mark C.M. DOI

10.1099/ijsem.0.002810

Publication date 2018 **Document Version**

Accepted author manuscript

Published in

International Journal of Systematic and Evolutionary Microbiology

Citation (APA)

Sorokin, D. Y., Merkel, A. Y., Abbas, B., Makarova, K. S., Rijpstra, W. I. C., Koenen, M., Sinninghe Damsté, J. S., Galinski, E. A., Koonin, E. V., & Van Loosdrecht, M. C. M. (2018). Methanonatronarchaeum thermophilum gen. Nov., sp. nov. and 'Candidatus methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprising a new euryarchaeal class Methanonatronarchaeia classis nov. International Journal of Systematic and Evolutionary Microbiology, 68(7), 2199-2208. Article 002810. https://doi.org/10.1099/ijsem.0.002810

Important note

To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.

International Journal of Systematic and Evolutionary Microbiology

Methanonatronarchaeum thermophilum gen. nov., sp. nov, and 'Candidatus Methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprise a new euryarchaeal class Methanonatronarchaeia classis nov.

--Manuscript Draft--

Manuscript Number:						
Full Title:	Methanonatronarchaeum thermophilum gen. nov., sp. nov, and 'Candidatus Methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprise a new euryarchaeal class Methanonatronarchaeia classis nov.					
Article Type:	Taxonomic Description					
Section/Category:	New taxa - Archaea					
Keywords:	nethanogenesis nethyl-reducing nypersaline salt lakes soda lakes Methanonatronarchaeia					
Corresponding Author:	Dimitry Y Sorokin, Ph.D., Dr.Sci. Winogradsky Institute of Microbiology, Research Centre of Biotechnology RAS Moscow, NA RUSSIAN FEDERATION					
First Author:	Dimitry Y Sorokin, Ph.D., Dr.Sci.					
Order of Authors:	Dimitry Y Sorokin, Ph.D., Dr.Sci.					
	Alexander Y Merkel, PhD					
	Ben Abbas, MSc					
	Kira S Makarova, PhD					
	Irene W.C. Rijpstra					
	Michel Koenen					
	Jaap S. Sinninghe Damsté, PhD					
	Erwin A. Galinski, PhD					
	Eugene V. Koonin, PhD					
	Mark C.M. van Loosdrecht, Eng.,PhD					
Manuscript Region of Origin:	RUSSIAN FEDERATION					
Abstract:	Methanogenic enrichments from hypersaline lakes at moderate thermophilic conditions resulted in cultivation of an unknown deep lineage of euryarchaeota related to the class Halobacteria. Both soda and salt lake isolates belong to methyl-reducing methanogens that utilize C1 methylated compounds as electron acceptor and H2 or formate as electron donor. They are extreme halophiles, growing optimally at 4 M total Na+ and represent the first example of methanogens employing the "salt-in" osmoprotection mechanism. The salt lake subgroup is neutrophilic, whereas the soda lake isolates are obligate alkaliphiles, with an optimum around pH 9.5. Both grow optimally at 50oC. The genetic diversity inside the two subgroups is very low, indicating that the soda and salt lake clusters consist of a single genetic species each. The phylogenetic distance between the two subgroups is in the range of distant genera, whereas the distance to other euryarchaea is below 83% identity of the 16S rRNA. These isolates and closely related environmental clones from hypersaline habitats (SA1 group) form a novel class-level clade in the phylum Euryarchaeota that is strongly supported by bootstrap analysis. On the basis of distinct phenotypic and genetic properties, the soda lake isolates are classified into a new genus and species					

	Methanonatronarchaeum thermophilum with the type strain AMET1T (DSM 26684T=NBRC 110805T=UNIQEM U982), and the salt lake methanogens - as a candidate genus and species 'Ca. Methanohalarchaeum thermophilum'. Together with uncultured SA1 group clones, these organisms are proposed to form a new class Methanonatronarchaeia within the phylum Euryarchaeota.
--	--

Methanonatronarchaeum thermophilum gen. nov., sp. nov, and 'Candidatus Methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprise a new eurvarchaeal class Methanonatronarchaeia classis nov. Dimitry Y. Sorokin^{a,b*}, Alexander Y. Merkel^a, Ben Abbas^b, Kira S. Makarova^c, W. Irene C. Rijpstra^d, M. Koenen^d, Jaap S. Sinninghe Damsté^{d,e}, Erwin A. Galinski^f, Eugene V. Koonin^c and Mark C.M. van Loosdrecht^b ^aWinogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia 15 16 17 18 ^bDepartment of Biotechnology, TU Delft, The Netherlands ^cNational Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA ^dDepartment of Marine Microbiology and Biogeochemistry, NIOZ Netherlands Institute for Sea Research, and Utrecht University, The Netherlands e Department of Earth Sciences – Geochemistry, Faculty of Geosciences, Utrecht University, Utrecht, The Netherlands ^fInstitute of Microbiology and Biotechnology, Rheinische Friedrich-Wilhelms University, Bonn, Germany *Author for correspondence: D.Y. Sorokin; Tel: (7-495)1350109, Fax: (7-495)1356530; e-mail: soroc@inmi.ru; d.sorokin@tudelft.nl Running title: Methanonatronarchaeum thermophilum gen. nov., sp. nov, and 'Candidatus Methanohalarchaeum thermophilum' The genome of the type strain $AMET1^{T}$ and the metagenome of $HMET1^{T}$ have been deposited in the GenBank under the numbers MRZU00000000 and MSDW00000000, respectively. The 16S-rRNA gene sequences of the AMET strains are deposited under the numbers KY449317-KY4493127.

41 Methanogenic enrichments from hypersaline lakes at moderate thermophilic conditions 42 resulted in cultivation of an unknown deep lineage of euryarchaeota related to the class 43 Halobacteria. Both soda and salt lake isolates belong to methyl-reducing methanogens 44 that utilize C_1 methylated compounds as electron acceptor and H_2 or formate as electron 45 donor. They are extreme halophiles, growing optimally at 4 M total Na⁺ and represent 46 the first example of methanogens employing the "salt-in" osmoprotection mechanism. 47 The salt lake subgroup is neutrophilic, whereas the soda lake isolates are obligate 48 alkaliphiles, with an optimum around pH 9.5. Both grow optimally at 50°C. The genetic 49 diversity inside the two subgroups is very low, indicating that the soda and salt lake clusters consist of a single genetic species each. The phylogenetic distance between the 50 51 two subgroups is in the range of distant genera, whereas the distance to other 52 euryarchaea is below 83% identity of the 16S rRNA. These isolates and closely related 53 environmental clones from hypersaline habitats (SA1 group) form a novel class-level 54 clade in the phylum Eurvarchaeota that is strongly supported by bootstrap analysis. On 55 the basis of distinct phenotypic and genetic properties, the soda lake isolates are 56 classified into a new genus and species Methanonatronarchaeum thermophilum with the type strain AMET1^T (DSM 26684^T=NBRC 110805^T=UNIQEM U982), and the salt lake 57 58 methanogens - as a candidate genus and species 'Ca. Methanohalarchaeum 59 thermophilum'. Together with uncultured SA1 group clones, these organisms are 60 proposed to form a new class *Methanonatronarchaeia* within the phylum Euryarchaeota. 61

62

63

64

65

66 In hypersaline habitats, methylotrophic methanogenesis is usually considered to be the 67 dominant pathway [1-2]. The organisms responsible for this process are members of the order Methanosarcinales. In neutral pH conditions, Methanosarcinales are represented by the high 68 salt-tolerant genera Methanohalophilus and Methanohalobium that can grow at up to 4 M 69 70 NaCl [2-4], and a single methylotrophic genus Methanonatronum has been identified that can 71 grow in hypersaline soda brines [5-8]. All these methanogens, although able to tolerate salt-72 saturating conditions, belong to moderate halophiles that grow optimally at salinity around 2 73 M total Na⁺ and utilize the bacterial type of osmoprotection based on organic compatible 74 solutes [9-10].

75

Our recent exploration of methanogenic archaea in sediments of hypersaline inland lakes has 76 shown that, at elevated temperatures, a previously unknown group of extremely 77 78 halo(natrono)philic methanogens started to outcompete the salt-tolerant Methanosarcinales members when formate was supplied on the top of C_1 methylated compounds as 79 80 methanogenic substrate. This suggested the methyl-reducing nature of the novel group [11]. 81 In this hybrid methanogenic pathway, the C_1 methylated compounds are used as electron 82 acceptors only, whereas external H₂ is required as the electron donor. This pathway, until 83 recently, had been considered rare, having been characterized in only two species of 84 methanogens, Methanosphaera stadtmanae (Methanobacteriales) and Methanomicrococcus 85 blatticola (Methanosarcinales) [12-14]. However, virtually all recent discoveries of novel 86 deep lineages of methanogens involve methyl-reducers, including the Thermoplasmata 87 methanogens [15-16], the Candidate class "Methanofastidiosa" [17] and the Candidate phyla 88 "Bathyarchaeota" [18] and "Verstraetearchaeota" [19]. These findings indicate that methyl-89 reduction has so far been overlooked as an important methanogenic pathway that might be 90 able to compete with both classical methylotrophic and lithotrophic pathways. Here, we

91 describe the phenotypic and genetic properties of the novel group of extremophilic methyl92 reducing methanogenic euryarchaea from hypersaline lakes which we propose as founding
93 members of a new class *Methanonatronarchaeia*.

94

95 The source of the isolates was surface layer (5-15 cm) of anaerobic sediments from 96 hypersaline salt and soda lakes from various geographical locations as shown in **Table 1**. 97 Overall, eleven pure cultures of haloalkaliphilic and three highly enriched cultures of 98 halophilic methyl-reducing methanogens were obtained at 4 M total Na⁺ and 37-60°C.

99

100 The extremely haloalkaliphilic methyl-reducing AMET isolates were enriched and further purified by serial dilution using mineral base medium containing 4 M total Na⁺ (2 M Na⁺ as 101 sodium carbonates + 2 M NaCl), 5 g l^{-1} KCl and and 1 g l^{-1} of K₂HPO₄ at pH 9.5 (4 mM 102 103 NH₄Cl was added after sterilization). The extremely halophilic, neutrophilic methyl-reducing HMET cultures were enriched in 4 M NaCl/5 g l⁻¹ KCl, buffered at pH 7 by K₂HPO₄-KH₂PO₄ 104 (total 3 g l^{-1}) and supplemented with 0.5 g l^{-1} of NH₄Cl. After sterilization, both types of the 105 106 mineral basic media were supplemented with two trace metal solutions, MgCl₂ and vitamins 107 as described previously [8]. Further additions included CoM (50 µM), yeast extract (100 mg/l) 108 and either 0.1 mM hydrotroillite (FeS x nH₂O) or heat-sterilized anaerobic sediment slurries (approx. 10 cm³ l⁻¹) from either soda lakes or salt lakes. 50 mM each of MeOH and sodium 109 110 formate were added as substrates and 0.5 mM sodium sulfide as a reductant. The media were 111 dispensed into serum bottles (from 30 to 100 ml) at 75% volume capacity and made anoxic by 112 sterile argon flushing-evacuation. Final reduction of the media was achieved by adding by syringe of a drop/10 ml of 10% dithionite solution in 1 M NaHCO₃. The colonial growth of 113 strain AMET1^T was achieved in soft agar by mixing the 4 M complete sterile liquid alkaline 114 115 medium and 4% agarose (0.8% final) at 50°C and pouring 20 ml portions onto plates

119

120 Phase contrast microphotographs were obtained using a Zeiss Axioplan Imaging 2 121 microscope (Göttingen, Germany). For the total cell electron microscopy, the cells were 122 centrifuged and resuspended in 3 M NaCl, fixed with paraformaldehyde (final concentration 123 3%, v/v) for 2 h at room temperature, then washed again with the same NaCl solutions. The 124 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 3.0 M NaCl for 1 week at 4°C, washed 125 126 and resuspended in 3 M NaCl, stained overnight with 1% (w/v) uranyl acetate, dehydrated in 127 ethanol series, and embedded in Epon resin. Thin sections were poststained with 1% (w/v) 128 lead citrate. The core membrane lipids were obtained by acid hydrolysis (5% HCl in methanol 129 by reflux for 3 h) of the freeze-dried cells and subsequent analysis by HPLC-MS for GDGTs 130 and archaeol derivatives according to [20]. Intact polar lipids were obtained by Bligh Dyer 131 extraction of freeze-dried cells and subsequent HPLC-MS analysis as described in [21]. The presence of intracellular organic compatible solutes in the lyophilized cells of strain AMET1^T 132 was analyzed by HPLC and ¹H-NMR after extraction with EtOH and the intracellular 133 134 potassium concentration was measured using ICP-MS. The cell protein was analyzed by the 135 Lowry method after removal of cell-bound FeS by several washing with acidic 4 M NaCl 136 solution.

137

In total, eleven pure and three highly enriched mixed methanogenic cultures were isolated from anaerobic sediments of hypersaline soda and salt lakes, respectively, at 4 M total Na⁺ and methyl-reducing conditions, i.e. with either methanol or trimethylamine (TMA) as the

electron acceptor and formate or H₂ as the electron donor (Table 1). In addition to the 141 142 extreme salinity, all but one AMET strain were enriched and isolated at elevated 143 temperatures, between 48 and 60°C. The soda lake AMET isolates were successfully purified from bacterial satellites using a combination of antibiotic treatment (strepromycin + 144 vancomycin, or rifampicine 100 mg l^{-1} each) and filtration (0.45 µm). For the salt lake HMET 145 146 cultures, although this procedure efficiently eliminated bacteria, a small fraction (approx. 5%) 147 of other, non-methanogenic haloarcheal cells persisted in the serial dilutions. Furthermore, the 148 growth rate and yield of the HMET cultures were extremely low compared to the AMET 149 isolates, which made their purification problematic. All cultures exhibited obligate 150 dependence on external CoM and FeS. Moreover, only three of the eleven AMET isolates 151 grew in the minimal medium with these additions, whereas the rest were dependent on the 152 presence of sterilized sediments either from soda (AMET) or salt (HMET) lakes. What 153 exactly these organisms needed from the sediments, remains unclear, although a test with 154 separated pore brines and the solid phase demonstrated that the latter was far more efficient as 155 a growth factor. To our knowledge, similar observations have been reported in only one other 156 case, for an unidentified methylotrophic methanogenic culture obtained from alkaline saline 157 Mono Lake, but that culture most probably belonged to a classical methylotroph, because it 158 grew in the presence of methanol as the only substrate [22].

In a single case of strain $AMET1^{T}$, which can grow in presence of FeS alone, colonial growth was achieved. The colonies were disc-shaped, up to 1 mm in diameter and yellowcolored. The typical cell morphologies of the AMET and HMET type strains are shown on **Figs. 1** and **2**. The cells are irregular angular cocci of a characteristic small size (mean cell diameter is 0.4 µm). The cells of AMET strains were motile and possess multiple archaella, whereas no motility was observed for the cells in HMET cultures. Both groups have a thin, monolayer cell wall covered with a thick EPS layer. In addition, invaginations of cytoplasmic 166 membrane and large electron transparent inclusions (possibly, polyhydroxyalkanoates) were 167 visible in the cells of HMET1. The cells lyzed immediately upon downshift in salinity below 168 2 M Na⁺. The absence of blue autofluorescence indicated the absence of deazoflavine (F_{420}) 169 normally present in classical methylotrophic methanogens.

The analysis of organic compatible solutes in cells of strain AMET1^T (grown at 4 M Na⁺, pH 9.5) gave negative results. However, intracellular cation analysis demonstrated molar concentrations of K^+ . These observations indicate that the novel methanogens employ the haloarchaeal type ("salt-in") osmoprotection mechanism which has not yet been demonstrated for any other halophilic methanogens.

The core membrane lipids of AMET1^T and HMET1^T are primarily composed of a 175 mixture of GDGT-0 and archaeol (C20-C20 diphytanylglycerol diether) (Supplementary 176 177 Table S1). AMET1 also contained small quantities of GDGT-1, which was not detected in HMET1^T. In addition to archaeol, minor amounts of two monophytanyl glycerol ethers (2-178 C20 MGE and 1-C20 MGE) in AMET1^T and only 1-C20 MGE in HMET1^T were detected. 179 180 The complete absence of extended archaeols (C20-C25 and C25-C20 DGE) in membrane 181 lipids differentiated the extremely halophilic methanogens from haloarchaea [23]. The intact 182 polar lipid compositions of the two organisms were clearly different. In the alkaliphilic AMET1^T, the dominant polar lipids were phosphatidylglycerol (PG) and PG-PG with GDGT-183 0 as the core lipid. In the halophilic strain HMET1^T, the dominant polar lipids were identified 184 185 as dihexose derivatives of both archaeol and GDGT-0 (Supplementary table S1).

Both AMET and HMET strains use the methyl-reducing pathway of methanogenesis, whereby the C₁ methylated compounds, such as methanol, methylamines or methylated sulfides are used only as electron acceptors, whereas H₂ serves as the external electron donor. For the AMET strains the best electron acceptor was methanol. Methylamines, including mono-, di- and trimethyamine and tetramethyammonium, can also be utilized in ammonia-

191 free media but were highly toxic at alkaline conditions and the growth was much less active. 192 The growth with dimethylsulfide demanded gradual adaptation starting from 2 mM, but after 193 several steps, the best adapted strain, AMET6-2, was able to grow in presence of up to 20 mM 194 DMS. On the other hand, although possible in principle, the utilization of methanethiol was 195 irregular and no adaptation was observed to this toxic methylated compound. The neutrophilic 196 HMET strains preferred trimethylamine as the acceptor over methanol and growth with the 197 other C₁ methyl compounds was not observed. The two groups also differed in their preferred 198 *e*-donor: while the AMET strains clearly preferred formate, the HMET strains used H₂ more 199 actively. Utilization of formate as the e-donor, as well as DMS as the acceptor, have not been 200 demonstrated previously for any cultured methyl-reducing methanogens.

201 A unique property of the novel methyl-reducing methanogens is their extreme halophily. Both groups grew within the range of Na⁺ concentrations that, among the cultured 202 203 archaea, are typical only for haloarchaea, i.e. from 3 to 5 M, with an optimum at 204 approximately 4 M. This preferred range of salt concentration is compatible with the evidence 205 indicating that these organisms employ the "salt-in" strategy for osmoprotection. The AMET 206 group from soda lakes belongs to obligate alkaliphiles growing within the pH range (at 4 M 207 Na⁺ and 48°C) from 8.2 to 10.2 (optimum at 9.5-9.8). In contrast to most of the extremely 208 natronophilic bacteria isolated from hypersaline soda lakes, the new archaea depend on molar 209 concentrations of NaCl and grow optimally in a medium containing 2 M NaCl and 2 M (Na) 210 carbonates. The HMET strains were typical neutraphiles with a pH range for growth from 6.5 211 to 8. Furthermore, both groups preferred elevated temperatures for growth despite being isolated from moderate habitats. They grew optimally at 50°C and some of the strains 212 213 tolerated up to 60°C.

The Maximum Likelihood phylogenetic tree of 16S rRNA was constructed using PhyML 3.0 with the Smart Model Selection [24], the SPR (Subtree Pruning and Regrafting) 216 type of tree improvement [25] and the aLRT (Approximate likelihood-ratio test) for branch 217 support [26]. Only nearly complete sequences of 16S rRNA genes from the SILVA database 218 [27] were included in the calculation. The results show that the AMET and HMET groups 219 form two compact clades, with a maximum distance inside the groups of 1.5%. The distance 220 between the two groups was about 10%, indicating that they represent two distinct genera. 221 However, no close relatives of these organisms were identified among the cultivated members 222 of Euryarchaeota, whereas among uncultured archaeal clones, the novel methanogens were 223 clearly related to the SA1 group detected in various hypersaline habitats [28-30]. Further 224 phylogenetic reconstruction [11] showed that the closest relatives of the AMET-HMET group 225 in Euryarchaeota were haloarchaea of the class Halobacteria (Fig. 3) which, again, is compatible with the extreme halophily and the likely "salt-in" osmotic strategy of the novel 226 227 methanogens.

228

Overall, on the basis of phylogenetic analysis and unique phenotypic properties, the novel moderately thermophilic and extremely halo(alkali)philic methyl-reducing methanogens from hypersaline lakes are proposed to form a new euryarchaeal class *Methanonatronarchaeia* including the alkaliphilic AMET isolates from soda lakes as a new genus and species *Methanonatronarchaeum thermophilum*, and a candidate genus and species '*Ca*. Methanohalarchaeum thermophilum' from salt lakes.

235

236 DESCRIPTION OF *METHANONATRONARCHAEUM* GEN. NOV.

Metha.no.na.tron.ar.chae'um. N.L. n. *methanum* [from French n. *méth(yle)* and chemical
suffix *-ane*], methane; N.L. pref. *methano-*, pertaining to methane; N. Gr. n. *natron*,
arbitrarily derived from the Arabic n. *natrun* or *natron*, soda; N. L. neut. n. *archaeum* [from
Gr. adj. *archaios*, *-e*, *-on* ancient] archaeon; N. L. neut. n. *Methanonatronarchaeum* a sodaloving archaeon forming methane

Extremely halo(alkali)philic and moderately thermophilic methanogens that use the methylreducing pathway of methanogenesis. Utilize the "salt in" osmoprotection strategy. Found in
hypersaline alkaline lakes. Member of the phylum Euryarchaeota.

ther.mo.phi'lum Gr. adj. *thermos*, hot; N. L. adj. philum (from Gr. adj. philos -ê -on), friend,
loving; N. L. adj. thermophilum, thermophilic).

249

250 The species description is based on eleven isolates. Cells are small irregular cocci, 0.4-0.5 µm 251 in size, motile by 1-5 archaella. The cell wall is a thin monolayer covered with EPS. The cells 252 lyze at salinity below 2 M Na⁺. Accumulate potassium as compatible solute. The F_{420} -253 dependent cell autofluorescence is absent. The colonies are yellowish, lens-shaped, up to 1 254 mm. The core lipids are dominated by archaeol (C20-C20 DGE). Strictly anaerobic 255 methanogens utilizing MeOH, methylamines and dimethylsulfide as electron acceptor and 256 formate or H₂ as electron donor. Heterotrophic, can utilize yeast extract or acetate as C-257 source. Growth depends on external CoM, FeS/or sterilized anaerobic sediments from soda 258 lakes. Obligately alkaliphilic with a pH range for growth from 8.2 to 10.2 (optimum at pH 259 9.5-9.7) and extremely halo(natrono)philic, growing optimally at 4 M total Na⁺. Moderately thermophilic, with an optimum at 50°C and the upper limit for growth at 60°C. The G + C260 content of the genomic DNA in the type strain is 38 mol% (genome). The type strain, 261 AMET1^T (DSM 26684=NBRC 110805=UNIQEM 982), was isolated from sediments of 262 263 hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The accession number of 16S 264 rRNA gene sequence of the type strain in GenBank is KY449317.

265

266DESCRIPTIONOF'CANDIDATUSMETHANOHALARCHAEUM267THERMOPHILUM'

Metha.no.hal.ar.chae'um. N. L. n. *methanum* [from French n. *méth(yle)* and chemical suffix -*ane*], methane; N. L. pref. *methano-*, pertaining to methane; Gr. n. *hals halos*, sea salt, NaCl;
N. L. neut. n. *archaeum* [from Gr. adj. *archaios*, *-e*, *-on* ancient] archaeon; N. L. neut.
n. *Methanohalarchaeum* a salt-loving archaeon forming methane;

ther.mo.phi'lum Gr. adj. *thermos*, hot; N. L. adj. philum (from Gr. adj. philos -ê -on), friend,
loving; N. L. adj. thermophilum, thermophilic).

274

275 The description is based on three highly enriched monomethanogenic cultures. Cells are small, irregular, non-motile cocci, 0.4-0.5 µm. The cell wall is a thin monolayer covered with 276 277 EPS. The cells lyze at salinity below 2 M NaCl. The F₄₂₀-dependent cell autofluorescence is absent. The core lipids are dominated by archaeol (C20-C20 DGE). The colony formation 278 279 was not observed. Strictly anaerobic methanogens utilizing MeOH and trimethylamine as 280 electron acceptor and H₂ or formate as electron donor. Heterotrophic, utilize yeast extract as 281 C-source. The growth depends on external CoM and sterilized anaerobic sediments from salt 282 lakes. Extremely halophilic, grow optimally at 4-5 M NaCl. The pH optimum for growth is 7-

- 283 7.5. Moderately thermophilic with an optimum at 50°C and the upper limit for growth at
- 284 60° C. The G + C content of the genomic DNA in the type strain is 35.4 mol% (genome). The
- type strain, HMET1^T, was enriched from sediments of hypersaline lakes in Kulunda Steppe.
- 286 The accession number of 16S rRNA sequence of the type strain in GenBank is KY449328.
- 287

291

288 DESCRIPTION OF METHANONATRONARCHAEACEAE FAM. NOV.

- 289 The description is the same as for the genus *Methanonatronarchaeum*.
- 290 Type genus: *Methanonatronarchaeum* gen. nov.

292 DESCRIPTION OF ORDER METHANONATRONARCHAEALES ORD. NOV.

- 293 The description is the same as for the genus *Methanonatronarchaeum*.
- 294 Type genus: *Methanonatronarchaeum* gen. nov.

295 DESCRIPTION OF METHANONATRONARCHAEIA CLASSIS NOV.

- 296 The class *Methanonatronarchaeia* is defined on the basis of comparative sequence analysis of
- the 16S rRNA obtained from 11 pure cultures of the genus *Methanonatronarchaeum*, 3 highly
- enriched cultures of 'Candidatus Methanohalarchaeum' and the cloned sequences from
- uncultured SA1 group found in various hypersaline habitats of terrestrial and marine origin.
- 300 Type order: *Methanonatronarchaeales* ord. nov.
- 301

302 Fundings

This work was supported by the Netherlands Applied Science Foundation (STW, project 12226),
Gravitation (SIAM) grant 24002002 and by the Russian Foundation for Basic Research (RFBR 16-04-00035).

306307 Conflicts of interest

- 308 The authors declared no conflicts of interest
- 309
- 310 311

312 References313

- Oremland RS, King GM. Methanogenesis in hypersaline environments. In: *Microbial mats. Physiological ecology of benthic microbial communities* 1989; pp 180-190. Cohen Y, Rosenberg E (eds). American Society for Microbiology: Washington, DC.
- McGenity TJ. Methanogens and methanogenesis in hypersaline environments. In: Timmis
 KN (ed) *Handbook of Hydrocarbon and Lipid Microbiology* 2010; pp 665-679. Springer Verlag: Berlin, Heidelberg.
- 320 3. Paterek JR, Smith PH. *Methanophilus mahii* gen. nov., sp. nov., a methylotrophic
 321 halophilic methanogen. *Int J Syst Bacteriol* 1988; 38: 122–123.
- 322 4. Zhilina TN, Zavarzin GA. *Methanohalobium evestigatus*, gen. nov. sp. nov., the extremely
 323 halophilic methanogenic archaebacterium. *Dokl Akad Nauk USSR* 1987; 293: 464–468.

- 324 5. Boone DR, Baker CC. Genus VI. *Methanosalsum* gen. nov. In: *Bergey's Manual of*325 *Systematic Bacteriology*, second edition 2001; vol 1: 287-289. Boone DR et al. (eds).
 326 Springer-Verlag, New York.
- Mathrani JM, Boone DR, Mah RA, Fox GE, Lau PP. *Methanohalophilus zhilinae* sp.
 nov., an alkaliphilic, halophilic, methylotrophic methanogen. *Int J Syst Bacteriol* 1988; 38:
 139–142.
- 330 7. Kevbrin VV, Lysenko AM, Zhilina TN. Physiology of alkaliphilic methanogen Z-7936, a
 331 new strain of *Methanosalsus zhilinae* isolated from Lake Magadi. *Microbiology* (Moscow,
 332 English Translation) 1997; 66: 261–266.
- Sorokin DY, Abbas BA, Sinninghe Damsté JS, Sukhacheva MV, van Loosdrecht MCM.
 Methanocalculus alkaliphilus sp. nov., and *Methanosalsum natronophilus* sp. nov., novel
 haloalkaliphilic methanogens from hypersaline soda lakes. *Int J Syst Evol Microbiol* 2015;
 65: 3739-3745.
- 337 9. Menaia JAGF. Osmotics of halophilic methanogenic archaeobacteria". *Scholar Archive* 338 paper 1992; 133 (http://digitalcommons.ohsu.edu/etd).
- Roberts MF, Lai MC, Gunsalus RP. Biosynthetic pathways of the osmolytes Nε-acetyl-β lysine, β-glutamine, and betaine in *Methanohalophilus* strain FDF1 suggested by nuclear
 magnetic resonance analyses. *J Bacteriol* 1992; 174: 6688–6693.
- 342 11. Sorokin DY, Makarova K, Abbas B, Ferrer M, Golyshin PN. Discovery of extremely
 343 halophilic methyl-reducing methyl-reducing euryarchaea provides insights into the
 avoid evolutionary origin of methanogenesis. *Nature Microbiol* 2017; 2: article 17081.
- Fricke WF, Seedorf H, Henne A, Krüer M, Liesegang H. The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to
 methanol and H₂ for methane formation and ATP synthesis. J Bacteriol 2006; 188: 642–
 658.
- 349 13. Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT.
 350 *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing
 351 methanogen from the hindgut of the cockroach *Periplaneta americana*. *Int J Syst Evol*352 *Microbiol* 2000; 50: 1989-1999.
- Hedderich R, Whitman WB. Physiology and biochemistry of the methane-producing
 Archaea. In: *The Prokaryotes Prokaryotic Physiology and Biochemistry* 2013; pp 635Rosenberg E *et al.* (eds). Springer-Verlag: Berlin, Heidelberg.
- Borrel G, Parisot N, Harris HMB, Peyretaillade E, Gaci N et al. Comparative genomics
 highlights the unique biology of *Methanomassiliicoccales*, a *Thermoplasmatales*-related
 seventh order of methanogenic archaea that encodes pyrrolysine. *BMC Genom* 2015; 15:
 679.
- Beaul K, Nonoh JO, Mikulski L, Brune A. "Methanoplasmatales", *Thermoplasmatales*related archaea in termite guts and other environments, are the seventh order of
 methanogens. *Appl Environ Microbiol* 2012; 78: 8245-8253.

- 363 17. Nobu MK, Narihiro T, Kuroda K, Mei R, Liu W-T. Chasing the elusive *Euryarchaeota*364 class WSA2: genomes reveal a uniquely fastidious methylreducing methanogen. *ISME J*365 2016; 10: 2478-24-87.
- Evans PN, Parks DH, Chadwick GL, Robbins SJ, Orphan VJ. Methane metabolism in
 the archaeal phylum *Bathyarchaeota* revealed by genome-centric metagenomics. *Science* 2015; 350: 434-438.
- 369 19. Vanwonterghem I, Evans PN, Parks DH, Jensen, PD, Woodcroft BJ. Methylotrophic
 370 methanogenesis discovered in the archaeal phylum *Verstraetearchaeota*. *Nat Microbiol*371 2016; 1: article 16170.
- Weijers JWH, Panoto E, van Bleijswijk J, Schouten S, Balk M *et al.* Constraints on the
 biological source(s) of the orphan branched tetraether membrane lipids. *Geomicrobiol J*2009; 26: 402-414.
- 375 21. Sinninghe Damsté JS, Rijpstra WIC, Hopmans EC, Jung MY, Kim JG. Intact polar and
 376 core glycerol dibiphytanyl glycerol tetraether lipids of group I. 1a and I. 1b *Thaumarchaeota*377 in soil. *Appl Environ Microbiol* 2012; 78: 6866-6874.
- 378 22. Oremland RS, Marsh L, Desmarais DJ. Methanogenesis in Big Soda Lake, Nevada: an
 379 alkaline, moderately hypersaline desert lake. *Appl Environ Microbiol* 1982; 43, 462-468.
- 380 23. Dawson KS, Freeman KH, Macalady JL. Molecular characterization of core lipids from
 381 halophilic archaea grown under different salinity conditions. Org Chem 2012; 48: 1-8.
- 382 24. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W *et al.* New algorithms and
 383 methods to estimate maximum-likelihood phylogenies: assessing the performance of
 384 PhyML 3.0. *Syst Biol* 2010; 59: 307-21.
- 385 25. Hordijk W, Gascuel O. Improving the efficiency of SPR moves in phylogenetic tree search
 386 methods based on maximum likelihood. *Bioinformatics* 2005; 21: 4338-47.
- 387 26. Anisimova M, Gascuel O. Approximate likelihood-ratio test for branches: A fast, accurate,
 388 and powerful alternative. *Syst Biol* 2006; 55: 539-52.
- 389 27. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T *et al.* The SILVA ribosomal RNA
 390 gene database project: improved data processing and web-based tools. *Nucl Acids Res* 2013;
 391 41: D590-D596.
- 392 28. Eder W, Schmidt M, Koch M, Garbe-Schonberg D, Huber R. Prokaryotic phylogenetic
 393 diversity and corresponding geochemical data of the brine-seawater interface of the Shaban
 394 Deep, Red Sea. *Environ microbiol* 2002; 4: 758-763.
- 395 29. Ferrer M, Werner J, Chernikova TN, Bargiela R, Fernández L. et al. Unveiling
 396 microbial life in the new deep-sea hypersaline Lake *Thetis*. Part II: a metagenomic study.
 397 Environ Microbiol 2012; 14: 268–281.
- 398 30. Jiang H, Dong H, Yu B, Liu X, Li Y. Microbial response to salinity change in Lake Chaka,
 a hypersaline lake on Tibetan plateau. *Environ Microbiol* 2007; 9: 2603-2621.

	Lake	Area	Brine para	meters	Enrichment conditions			
Strain			pН	Total salt g/l	Soluble carbonate alkalinity, M	Substrate	pН	T, ⁰C
AMET1	Mix 5 soda lakes	Kulunda Steppe	9.6-10.1	120-400	0.6-3.0	MeOH+formate		48
AMET3	Tanatar-1	(Altai, Russia)	10.1	350	2.8	Meon normate		48
AMET4	Picturesque Lake	2013-2015	9.8	250				48
AMET5	Mix 6 soda lakes		9.6-10.2	50-380	0.5-3.4	TMA +formate		48
AMET6-2	Tanatar-1		10.25	380	3.4	MeOH+formate		60
AMET7	Soda crystallizer		9.6	350	3.8		0.6	55
AMET8	Mix 6 soda lakes		9.6-10.2	50-380			9.0	30
AMET9	Soda crystallizer		10.1	340	3.9			43
AMET10	Stamp Lake		9.1	325	0.2			54
AMET2	Mix from 8 lakes	Wadi al Natrun (Egypt, 2000)	9.1-9.9	200-360	0.1-0.9	MeOH+formate		60
AMET-SI	Searles Lake	California	9.8	350	0.2	MeOH+formate	9.2	48
HMET1 (mixed culture)	Mix from 4 salt lakers	Kulunda Steppe 2014	7.5-8.1	280-340	-	TMA+H ₂		48
HMET-El (mixed culture)	Lake Elton	Southa Russia 2015	6.7	320	-	MaOH+formata	7.0	54
HMET-Eu (mixed culture)	Salt crystallizer	Crimea (Russia) 2015	7.2	220	-	weOn+ioiiilate		55

400
 401
 401 Table 1. Extremely halophilic and moderately thermophilic mixotrophic methanogens isolated from hypersaline lakes at 4 M total Na⁺ TMA - trimethylamine

- 402 Figure legends
- 403
- 404 **Fig. 1** Cell morphology of *Methanonatronarchaeum thermophilum* strain $AMET1^T$ grown 405 with MeOH+formate at pH 9.5, 4 M total Na⁺ and 48°C. (a), phase contrast microscopy; (b 406 and c), electron microscopy of total cells and thin sections, respectively. N, nucleoide; CM -407 cytoplasmic membrane; CW - cell wall.
- 408

409 Fig. 2. Cell morphology of '*Ca*. Methanohalarchaeum thermophilum' strain HMET1 grown
410 with TMA+H₂ at pH 7, 4 M NaCl and 50°C. (a), phase contrast microscopy; (b and c),
411 electron microscopy of total cells and thin section, respectively. N, nucleoide; ICPM 412 intracytoplasmic membranes; PHA - possible polyhydroxyalkanoate storage granule; CW 413 cell wall.

414

Fig. 3. Phylogeny of novel halo(alkali)philic methanogens from hypersaline lakes based on
the 16S rRNA gene sequence analysis. The bootstrap values above 70% are shown at the
nods. Bar, 0.10 changes per position.

418







Fig.2

Fig. 3



Methanonatronarchaeum thermophilum gen. nov., sp. nov, and '*Candidatus* Methanohalarchaeum thermophilum', extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes comprise a new euryarchaeal class *Methanonatronarchaeia* classis nov.

Dimitry Y. Sorokin^{a,b*}, Alexander Y. Merkel^a, Ben Abbas^b, Kira S. Makarova^c, W. Irene C. Rijpstra^d, M. Koenen^d, Jaap S. Sinninghe Damsté^{d,e}, Erwin A. Galinski^f, Eugene V. Koonin^c and Mark C.M. van Loosdrecht^b

Supplementary Table S1.

Membrane lipid composition of extremely halophilic methyl-reducing methanogens

A: core lipids

Strain	Di- and mone	ophytanyl gly	cerol ethers (%)	Glycerol dibiphytanyl glycerol tetraethers		
	Archaeol	2-C20 MGE	1-C20 MGE	GDGT-0/archaeol	GDGT-1	
AMET1	96.0	2.4	1.6	1:1	+	
HMET1	93.4	_	6.6	3:1	_	



B: Intact polar lipids composed of a core lipid with attached polar head group(s)

Strain	Archaeol							GDGT-0	
	PG	PGP	PGP-Me	PS	НРН	DH	Cardiolipin Ar-P-G-P- Ar*	PG PG	DH
AMET1	++		+	+				++	
HMET1	+	+			+	+++	+		+++

PG=Phosphatidylglycerol

PGP=Phosphatidylglycerolphosphate

PGP-Me=Phosphatidylglycerophosphate methyl ester

PS=Phosphatidylserine

HPH=hexosephosphatidylhexose

DH=dihexose

Ar-P-G-P-Ar = archaeol-phosphatidyl-glycerol-phosphatidyl-archaeol

(*small part of the cardiolipin with extended archaeol (C25/C20) was also present)

±



RUSSIAN ACADEMY OF SCIENCES WINOGRADSKY INSTITUTE OF MICROBIOLOGY RESEARCH CENTRE OF BIOTECHNOLOGY

117312 Russia, Moscow, Prospekt 60-let Oktyabrya, 7/2 Tel. (095) 135-21-39; Fax (095) 135-65-30; e-mail: inmi@inmi.ru

Moscow, Russia

12 September 2014

Confirmation of the availability of a strain for the purpose of valid publication of a new name according to the Bacteriological Code

The following information is confidential and serves only to allow the Microbiology journal to confirm that a strain has been deposited and will be available from the UNIQEM (Unique and Extremophilic Microorganisms Collection of Winogradsky Institute of Microbiology RAS).

Methanonatronaerchaeium thermophilum" strain AMET1(T) has been deposited in the UNIQEM under the number U982.

The strains are available in the open section of the UNIQEM and restrictions have not been placed on access to information concerning the presence of the strain in the UNIQEM. It will be included in published and online catalogues after publication of this number by the authors.

The strain has been checked for viability and is stored using 20% glycerol stock culture in liquid nitrogen.

Prof. Dr. Valery F. Galchenko Director of UNIQEM

BIL