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Ecology of *Methanonatronarchaeia*

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Abstract

Methanonatronarchaeia represents a deep-branching phylogenetic lineage of extremely halo(alkali)philic and moderately thermophilic methyl-reducing methanogens belonging to the phylum *Halobacteriota*. It includes two genera, the alkaliphilic *Methanonatronarchaeum* and the neutrophilic Ca. Methanohalarchaeum. The former is represented by multiple closely related pure culture isolates from hypersaline soda lakes, while the knowledge about the latter is limited to a few mixed cultures with anaerobic haloarchaea. To get more insight into the distribution and ecophysiology of this enigmatic group of extremophilic methanogens, potential activity tests and enrichment cultivation with different substrates and at different conditions were performed with anaerobic sediment slurries from various hypersaline lakes in Russia. *Methanonatronarchaeum* proliferated exclusively in hypersaline soda lake samples mostly at elevated temperature, while at mesophilic conditions it coexisted with the extremely salt-tolerant methylotroph *Methanosalsum natronophilum*. *Methanonatronarchaeum* was also able to serve as a methylotrophic or hydrogenotrophic partner in several thermophilic enrichment cultures with fermentative bacteria. Ca. Methanohalarchaeum did not proliferate at mesophilic conditions and at thermophilic conditions it competed with extremely halophilic and moderately thermophilic methylotroph *Methanohalobium*, which it outcompeted at a combination of elevated temperature and methyl-reducing conditions. Overall, the results demonstrated that *Methanonatronarchaeia* are specialized extremophiles specifically proliferating in conditions of elevated temperature coupled with extreme salinity and simultaneous availability of a wide range of C_1-methylated compounds and H_2/formate.

INTRODUCTION

Methyl-reducing pathway of methanogenesis is a 'hybrid' between the classical methylotrophic pathway, whereby C_1-methylated compounds, such as methanol, methylamines and methylated sulfides, are dismutated to methane and CO_2, and the hydrogenotrophic pathway forming methane from CO_2 with H_2, CO or formate as the electron donors. The methyl-reducing methanogens are only capable of utilizing C_1-methylated compounds as electron acceptors, while using external electron donors which known identity so far is limited to H_2 and formate. The reason for inability of the methyl-reducers to oxidize methyl group to CO_2 is explained either by a partial or complete inactivation of the reversible archaeal Wood–Ljungdahl (WL) pathway from CO_2 to methane consisting of six enzymes highly conserved in methanogens. While being discovered long time ago in two gut-inhabiting members of *Methanosphaera* (order *Methanobacteriales*) (Fricke et al., 2006; Miller & Wolin, 1985; Van de Wijngaard et al., 1991) and *Methanomicrococcus* (order *Methanosarcinales*) (Sprenger et al., 2000, 2005; Thomas et al., 2021), the methyl-reduction was considered rather as a curiosity

than an important alternative of the classical methylotrophy in methanogens. However, this view has been changed with a recent discovery of several deep-branching phylogenetic lineages of archaea utilizing this variation of methanogenesis for energy generation (Kurth et al., 2020; Söllinger & Urich, 2019). These include the following.

Members of the order *Methanomassiliicoccales* (phylum *Thermoplasmatota*), with *Methanomassiliicoccus luminyensis* as the only known pure culture representative, lack all the genes of the WL pathway except for the terminal McrABG-HdrABC complex responsible for the reduction of methyl-CoM to methane. They are using H_2 as the external electron donor and are found mostly in guts of mammals and termites and in freshwater habitats (Borrel et al., 2015; Dridi et al., 2012; Hervé et al., 2020; Iino et al., 2013; Lang et al., 2015; Paul et al., 2012). The energy-generating mechanism in *Methanomasiliicoccales* is different from that present in classical methanogens. It includes two different heterodisulfide reductase systems: one present normally in hydrogenotrophic methanogens consisting of a soluble bifurcating HdrABC-MvhADG hydrogenase complex oxidizing H_2 and reducing ferredoxin, while the second is a membrane-associated HdrD/Fpo-like complex apparently involved in reoxidation of ferredoxin with generation of a transmembrane proton gradient used by a membrane ATP synthetase (Kröninger et al., 2019; Lang et al., 2015).

Candidate class Methanofastidiosa (solely metagenomic data) includes marine methyl-reducing methanogens most probably using methylated sulfides as electron acceptor and H_2 as electron donor to form methane. The CO_2-reducing archaeal WL pathway genes are also completely missing in the genome of this group (Nobu et al., 2016).

Candidate phylum Verstraetearchaeota was first discovered in highly active methanogenic enrichments and anoxic freshwater habitats by detecting an unusually deep-branching *mcrA* gene sequences in five metagenomes (Vanwonterghem et al., 2016) and, later on, a sixth MAG was reconstructed from a Yellowstone hot spring (Borrel et al., 2019). On the basis of genomic analysis these archaea were concluded to be methyl-reducing methanogens with a similar energy-generation machinery as found in *Methanomassiliicoccus*. Since all those habitats contained other conventional methanogens, it is still not clear whether this deep phylogenetic lineage, a member of the TACK superphylum, actually includes methanogens. For now, the only evidence in favour of this conclusion is a presence of deeply branched sequences of *mcr*-like genes. Same is true for another such deep-branching archaeal lineage *Candidate* phylum Bathyarchaeota proclaimed as methanogenic based exclusively on the presence of genes encoding an Mcr-like complex (Evans et al., 2015). However, later on it was discovered that those genes in Ca. Bathyarchaeota are more related to the *mcr* homologues of anaerobic butane oxidizer Ca. Syntropharchaeum butanivorans, a member of the order *Methanosarcinales* (Wang et al., 2021). The latter example is a fair warning for conclusions solely based on meta(genomic) data without activity and cultivation evidences.

The last deep phylogenetic lineage of methyl-reducing methanogens discovered so far was the class *Methanonatronarchaeia*, a member of the phylum *Halobacteriota* (Sorokin et al., 2017, 2018; Sorokin & Merkel, 2019). It includes two distantly related genera. The genus *Methanonatronarchaeum* includes multiple pure cultures isolated from various hypersaline soda lakes and a single metagenome from a Siberian soda lake. The *Candidate* genus Methanohalarchaeum is represented by a few highly enriched cultures obtained from hypersaline salt lakes with neutral pH in south Russia. Both are extreme halophiles and moderate thermophiles, and, in addition, *Methanonatronarchaeum* is also obligate alkaliphilic. Methanogenic system in these extremophilic archaea is different from all other methyl-reducers known so far. First, they have two membrane complexes encoding [NiFe] hydrogenase and [Mo] formate dehydrogenase, enabling them effective utilization of both H_2 and formate as the extracellular electron donors. Next, they have a membrane heterodisulfide reductase HdrDE and methanophenazine-like electron carriers forming a membrane cytochrome *b*-containing respiratory chain resembling those present in the members of *Methanosarcinales*. And lastly, in their chromosomes they have genes encoding four out of six enzymes of the archaeal C_1 WL pathway and, in that sense, they form an intermediate case between *Thermoplasmata*/Ca. Methanofatidiosa/Ca. Verstraetearchaeota methyl-reducers completely lacking all six WL complexes and *Methanosphaera* with the complete set of the CO_2-reducing enzymes (Kurth et al., 2020; Söllinger & Urich, 2019).

The main goal of this work was to investigate in more detail ecology of *Methanonatronarchaeia*, i.e. to understand more of its ecological niche and possible interactions (competition/syntrophy) with other extremely halo(alkali)philic methanogens and anaerobic bacteria inhabiting sulfidic sediments of hypersaline lakes.

EXPERIMENTAL PROCEDURES

Samples and potential methanogenic activity incubations

Top 10 cm sediment layer together with a near-bottom brines was taken into three corers with internal diameter of 3 cm and extruded into 500 ml glass Schott

bottles filling it completely without bubbles. These were used in the laboratory for potential activity incubations. Another 25–30 long cm core was used to determine methane and HS^-/FeS concentrations with 5 cm steps as described previously (Sorokin et al., 2015a). The sampled hypersaline lakes and the basic chemical parameters of their brines and sediments are described in Supplementary Table S1.

To probe for the presence and activity of *Methanonatronarchaeia*, one part of homogenized sediment sample from the 10 top cm layer was either mixed with one part of the near bottom brines directly (native incubations) or with one part of sodium carbonate–bicarbonate buffer containing 4 M total Na^+ (pH 10) and incubated anaerobically at variable temperatures (between 30°C and 60°C) in the presence of various C_1 methylated acceptors with or without addition of formate as an external electron donor. Gas samples were taken periodically for methane analysis, as described previously (Sorokin et al., 2015a; Sorokin et al., 2017).

Molecular analysis of the methanogenic populations in sediment incubations and enrichments

Three molecular methods were used to monitor development of methanogens in sediment potential activity incubations, which might be considered also as primary enrichment cultures. The methanogen-specific detection of the *mcrA* genes was done by using *mcrA*-DGGE with an optimized protocol including a two-step amplification as described in detail by Sorokin et al. (2015a). Briefly, the optimization included preincubation of sediment samples with 50 mM EDTA + 1 mg/ml skimmed milk overnight on ice to improve the yield and purity of DNA extracted further with the Soil DNA Extraction kit (MoBio Laboratories) and nested *mcrA* amplification with two sets of primers, one set based on *mlas_f*/*mcrA_r* according to Steinberg and Regan (2008) and the second—with the DGGE primers *mlas_f_inosine*/*mcrA_r_inosine* + *GC clamp*. The *mcrA* genes at used DGGE conditions resolved mostly in bundles of 2–6 closely associated bands with identical sequences which were verified with the reference pure cultures of *Methanocalculus natronophilus*, *Methanosalsum natronophilum*, *Methanonatronarchaeum thermophilum* AMET1 and *Ca*. Methanohalarchaeum thermophilum HMET1.

The other two methods were based on detection of 16S rRNA gene. In two cases a *q*PCR 16S rRNA gene counting with *Methanonatronarchaeum*-specific primers was used to detect a basic level of the population in two sediment samples from hypersaline soda lakes in Kulunda Steppe. The primer set was AMET-772F (5′-GGGGCACAAACCGGATTAGA-3′) and AMET-830R (5′-CAGACGTGCTACGGCTTACA-3′). Quantification was performed according to Kubista et al. (2006) on the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) using qPCRmix-HS SYBR Kit (Evrogen, Russia). Furthermore, classical cloning technique was applied to obtain nearly full-length 16S rRNA gene sequences from a sediment-free highly enriched thermophilic methyl-reducing culture from hypersaline salt lake Baskunchak. For this PCR amplification was conducted with primers A25F (5′-CTG.GTT.GAT.CCT.GCC.AG-3′) and U1517R (5′-ACG.GCT.ACC.TTG.TTA.CGA.CTT-3′) and the amplified fragments were purified using QIAquick PCR purification kit (QIAGEN, Germany) and cloned using TOPO TA Cloning (Thermo Fisher Scientific). In total 12 clones were used for direct PCR on the plasmid using the plasmid primers MF/MR yielding a near 1500 bp fragments. Those and the near full-length 16S rRNA genes amplified from pure AMET cultures obtained in this study were then identified by the Sanger sequencing. Finally, the NGS-based 16S rRNA gene profiling was applied to a number of methanogenic activity incubations and enrichments. Libraries of the V3–V4 region of the 16S rRNA gene for Illumina MiSeq high-throughput sequencing were prepared according to the protocol (Gohl et al., 2016). Data analysis was carried out using DADA2 software (Callahan et al., 2016) and SILVA 138.1 database (Quast et al., 2013). Raw reads are deposited in the NCBI SRA database under BioProject number PRJNA824466.

Genome sequencing, assembly and genome-based phylogenetic analysis

The HMW DNA from bacteria-free methanogenic cultures was extracted and purified using the Microbial DNA isolation kit (MoBio Laboratories). Whole-genome sequencing of strain AMET6-2 was carried out on a NextSeq system (Illumina, CA, USA) using the reagent kit providing for 2 × 100 bp reading. Assembly of reads by Unicycler v0.4.8 (Wick et al., 2017) resulted in circular chromosome with a total size of 1 398 990 bp for pure culture of *Methanonatronarchaeum* AMET6-2. It was deposited in GenBank under number CP095400 (assembly GCA_023016325). For genome-based phylogenetic reconstructions, 122 archaeal single-copy conservative marker genes were used as described previously (Parks et al., 2020). The trees were built using the IQ-TREE 2 program (Nguyen et al., 2015) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast approximation for phylogenetic bootstrap (Hoang et al., 2018) as well as approximate likelihood-ratio test for branches (Anisimova & Gascuel, 2006). The genomic index (ANI/AAI/DDH) distances between the type strain of *Methanonatronarchaeum thermophilum* AMET1 and *Methanonatronarchaeum* AMET6-2 were calculated according to Pritchard et al. (2016), Rodriguez-R and

Konstantinidis (2016) and Buchfink et al. (2015), respectively.

Cultivation conditions

Cultivation and purification of novel AMET and HMET cultures from hypersaline lakes were done in accordance with previously developed protocols (Sorokin et al., 2017). In short, the cultivation was performed in media containing 4 M total Na^+, either as 1:1 mixture of sodium carbonates and NaCl at pH 9.5 (for soda lakes) or pure NaCl at pH 6.6–7.0 (for salt lakes). The media were supplemented with 1% (vol./vol.) of autoclaved anoxic sediment mix from corresponding lakes (as a 1:1 sediment: brine slurries), 50 μm of CoM (Sigma-Aldrich), 0.5 mM Na_2S, vitamin and trace metal mixtures (Pfennig & Lippert, 1966) and various C_1-methylated compounds as electron acceptors at concentrations of 10–50 mM. Formate (50 mM) was used as an electron donor (but in some cases was omitted) and acetate (2 mM)/yeast extract (0.2 g L^{-1}) served as the C-source. Sterile argon flushing-evacuation was applied to achieve anoxic conditions and the final medium reduction was done by adding 0.2 ml L^{-1} of 10% filter sterilized dithionite solution in 1 M $NaHCO_3$.

RESULTS AND DISCUSSION

Potential activity of methylotrophic methanogenesis at elevated temperatures

It has already been shown (Sorokin et al., 2017) that the methyl-reducing *Methanonatronarchaeia* are moderate thermophiles. Therefore, in this work extended incubation experiments were aimed to investigate how they compete with classical halo(alkali)philic methylotrophic methanogens at moderate to high temperatures in sediments of various hypersaline lakes. In hypersaline alkaline soda lakes of Kulunda Steppe (Altai, Russia) only methylotrophic methanogenesis was active and it was markedly stimulated by the formate addition, suggesting that the methyl-reducing methanogens dominated the process at a combination of salt-saturating moderately thermophilic conditions (Figure 1). A similar situation was observed in sediments from hypersaline salt lakes with neutral pH in Kulunda Steppe, south Russia and Crimea (Figure 2). The overall trend in ratio of potential methanogenic rates with methyl substrates in the absence and in the presence of formate as an external electron donor was increasing in favour of the methyl-reducing methanogens with increasing temperature (Figure 3).

The range of C_1-methylated compounds utilized at thermophilic, methyl-reducing conditions in sediments of hypersaline lakes reported previously was limited to methanol (MeOH) and trimethylamine (TMA) (Sorokin et al., 2017) and only recently it has been shown for the type strain of *Methanonatronarchaeum thermophilum* that it can also grow on methylamine (MA) and dimethylamine (DMA) (Steiniger et al., 2022). In this work we also tested MA and DMA and methylated sulfides [methanethiol (MT) and dimethylsulfide (DMS)] in methanogenic sediment incubations. In hypersaline soda lakes all of these compounds were used as the electron acceptors to form methane in presence of formate as the electron donor in the following activity order: MeOH > TMA > DMA > MA > DMS > MT (Figure 4). A much slower methane formation was also

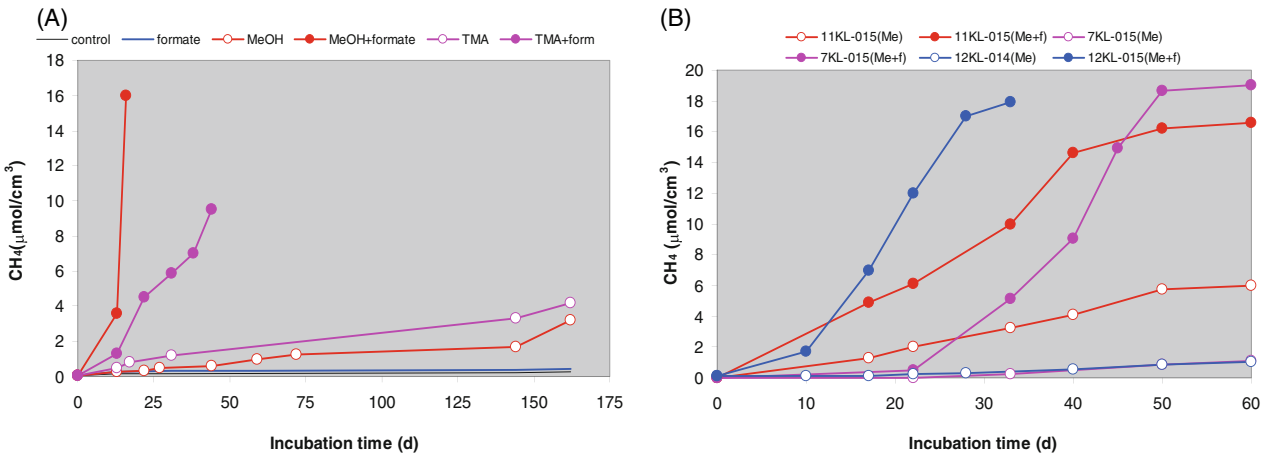

FIGURE 1 Potential methanogenic activity in sediment slurry from hypersaline soda lakes in Kulunda Steppe (Altai, Russia) incubated at moderately thermophilic conditions. (A) Tanatar-1 (2014) at 48°C; (B) Stamp Lake (11KL-015) at 54°C, soda crystallizer (7KL-015) and Crooked Lake (12KL-014) at 48°C. Substrates were added at 5 mM. The endogenous rates (without substrates) were subtracted. Mean values from duplicate experiments. MeOH (or Me), methanol; TMA, trimethylamine; (+f), with formate addition as an external electron donor

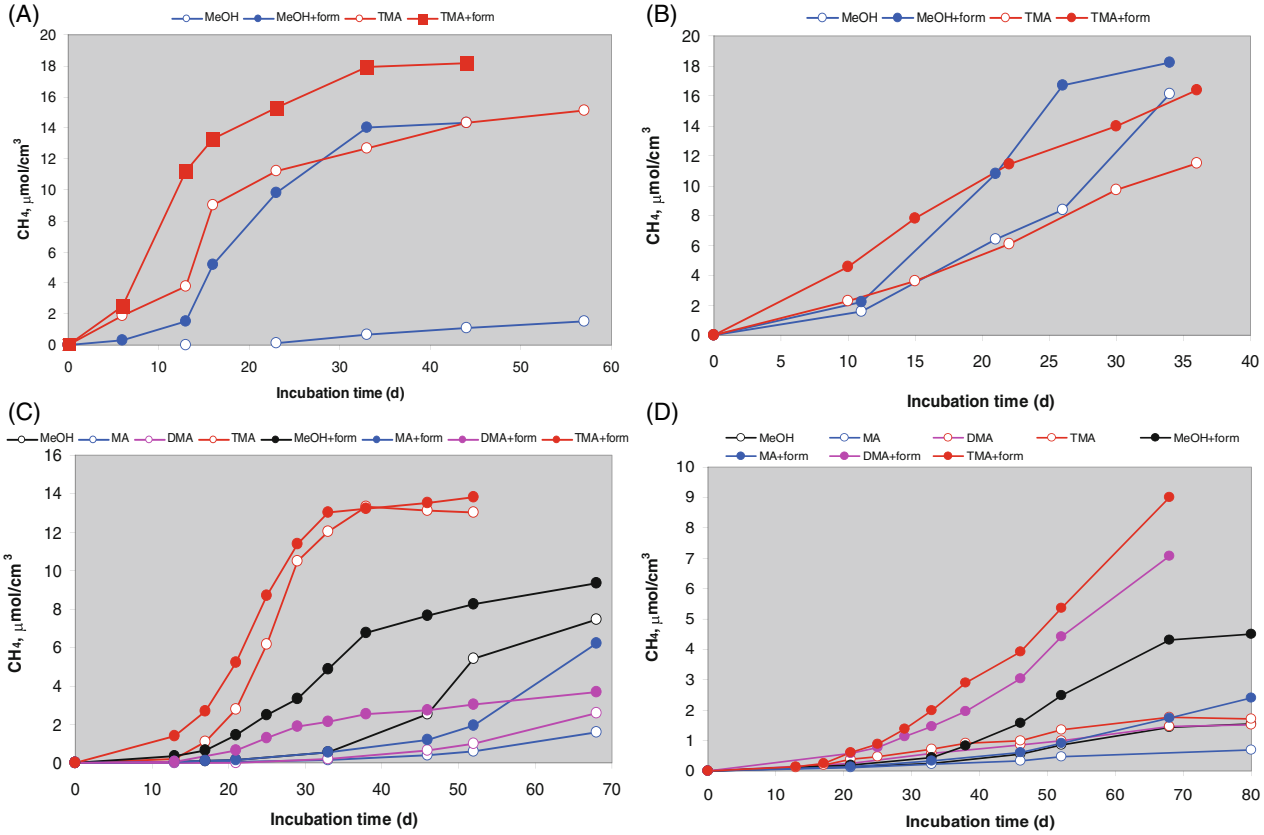

FIGURE 2 Potential methanogenic activity of methylotrophic methanogenesis at moderately thermophilic conditions in anaerobic sediment slurries from hypersaline salt lakes with neutral pH (Russia). (A) Mixed sediments from four hypersaline lakes in Kulunda Steppe, Altai (2014); (B) salt concentrator in Eupatoria (Crimea, 2015); (C) lake Baskunchak and (D) lake Elton (south Russia, 2016). Incubation temperatures: (A, B) = 48°C, (C, D) = 50°C. The endogenous rates were subtracted. The methanogenesis with formate alone was not observed. Mean values from duplicate experiments.

observed with methionine, which is known to act as a precursor for anaerobic MT formation via action of methionine lyase (Higgins et al., 2006). Interestingly, when the material from these incubations was transferred into sediment-free medium (1:100, vol./vol.), the DMS-dependent process became much more active than the others. In salt lakes with neutral pH this test identified MeOH and TMA as the most favourable substrates for methyl-reducing methanogenesis (probably because of lower TMA toxicity at neutral pH), while no activity was observed with methylated sulfides (Figures 2 and 3C).

Identity of methyl-reducing methanogens potentially active in hypersaline lake sediments

The *mcrA*-DGGE analysis of the methanogenic populations developed in hypersaline lake sediment incubations aimed at the comparative effect of incubation temperature/a presence of external reductant (formate) in addition to the methyl substrates (MeOH or TMA) indicated that methyl-reducing methanogens in hypersaline lakes were taking advantage mostly at elevated temperature (Figure 5). In hypersaline soda lakes (Figure 5A,B) classical salt-tolerant methylotrophic methanogens from *Methanosarcinales* (genus *Methanosalsum*) were only detectable at the lowest incubation temperatures between 30°C and 38°C, while at higher temperatures the only *mcrA* genes detected belonged to *Methanonatronarchaeum*-like methyl-reducers. In most of the cases, those *mcrA* sequences formed two distinct groups: group 1 (framed in red) was identical to the *mcrA1* of the type strain AMET1 of *Methanonatronarchaeum thermophilum*, while group 2 (framed in purple) was identical to the *mcrA* of the most phylogenetically distant strain AMET6-2 of *Methanonatronarchaeum* (Sorokin et al., 2017, 2018), representing a putative second species in the genus (see below) (Supplementary Figure S1). Thus, the *mcrA*-DGGE data of the soda lake methanogenic populations active at thermophilic conditions demonstrated the coexistence/competition of two different genotypes of *Methanonatronarchaeum* already in the primary enrichments and this was further confirmed on the level of highly enriched sediment-free cultures (see below). Only one out of the multiple pure culture strains of

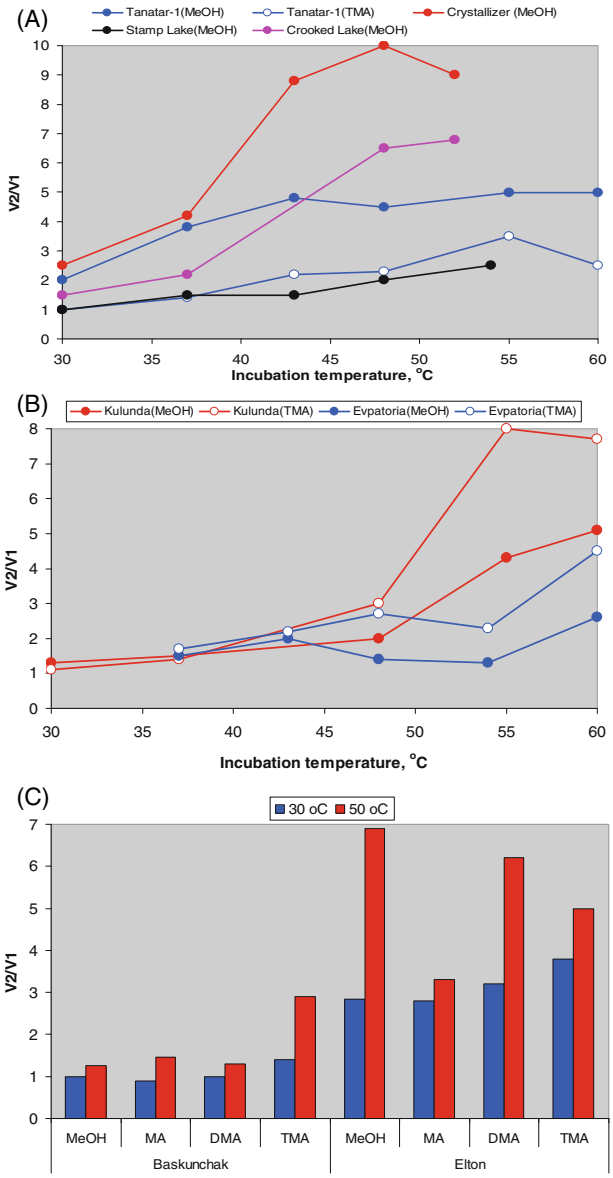

FIGURE 3 Differential influence of incubation temperature on the potential rates of classical methylotrophic (V1) and methyl-reducing (V2, with formate as *e*-donor) methanogenesis in sediment slurries from hypersaline lakes. MA, methylamine, DMA, dimethylamine, TMA, trimethylamine. (A) Hypersaline soda lakes in Kulunda Steppe (Altai, Russia); (B, C), hypersaline salt lakes with neutral pH in Kulunda Steppe (mixed sample) and in south Russia. The endogenous rates were subtracted. The methanogenesis with formate alone was not observed. Mean values from duplicate experiments

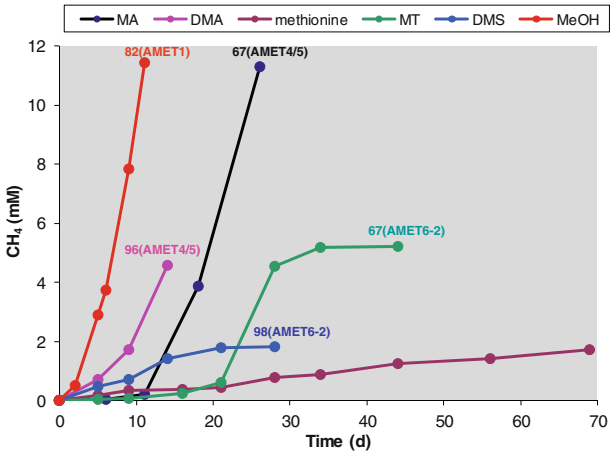

FIGURE 4 Methyl-reducing methanogenesis in sediment slurries from the mix Kulunda Steppe hypersaline soda lake sediments at 50°C, 4 M Na^+/pH 9.5 amended with formate as *e*-donor and different methyl acceptors (5 mM each). The numbers at the curve end-points indicate % of the AMET-like 16S rRNA amplicon sequences from the total archaeal population and the closest related AMET strains known in pure culture are shown in parentheses. AMET1 and AMET4/5 represent the type species *Methanonatronarchaeum thermophilum*, while AMET6-2 belongs to a putative new species of *Methanonatronarchaeum*. At the start of incubation the abundance of *Methanonatronarchaeia* determined by qPCR and 16S rRNA gene amplicon sequencing was ~1% from total archaeal population

Methanonatronarchaeum grew at temperatures below 37°C (Sorokin et al., 2017), while in several sediment samples its *mcrA* was still detectable at lower intensity after 30°C incubation (Figure 5A,B). This might have an explanation in the fact that low methanogenic activity was also seen in resting cells (pregrown at 48°C) of most AMET isolates at non-growing conditions (Sorokin et al., 2017).

It is also worth to notice that *mcrA* genes related to the ones from hydrogenotrophic genus *Methanobacterium* and methyl-reducing member of the *Methanomassiliicoccales* (Ca. Methanomethylophilus) were detected in native sediments of soda lakes (Figure 5A, B). But the former disappeared upon potential methanogenic activity incubations and the latter either dropped in intensity upon incubations at 30°C–38°C or disappeared at higher temperatures. This example shows that conclusions based on a mere presence of functional (or any other) markers in open natural systems/habitats without a proof of activity or ability to proliferate at close to in situ conditions may result in false conclusions on the functional importance of a certain taxon in such habitats. At least for soda lakes those two methanogenic genera have never been seen coming into the enrichments, even at moderate salinity of 0.5 M Na^+.

In the salt lake sediments, classical extremely halophilic methylotrophic methanogens from the order *Methanosarcinales* were detectable at the start of incubation (*Methanohalophilus* and *Methanohalobium*) and in the activity incubations at 48°C (*Methanohalobium*). The methyl-reducing *Ca*. Methanohalarchaeum thermophilum coexisted with *Methanohalobium* at 48°C, while at higher temperatures the methyl-reducer was the only methanogen detectable (Figure 5C) despite the fact that *Methanohalobium evestigatum* can grow up to 60°C with MeOH or TMA (Zhilina & Zavarzin, 1987). Apparently, the methyl-reducing

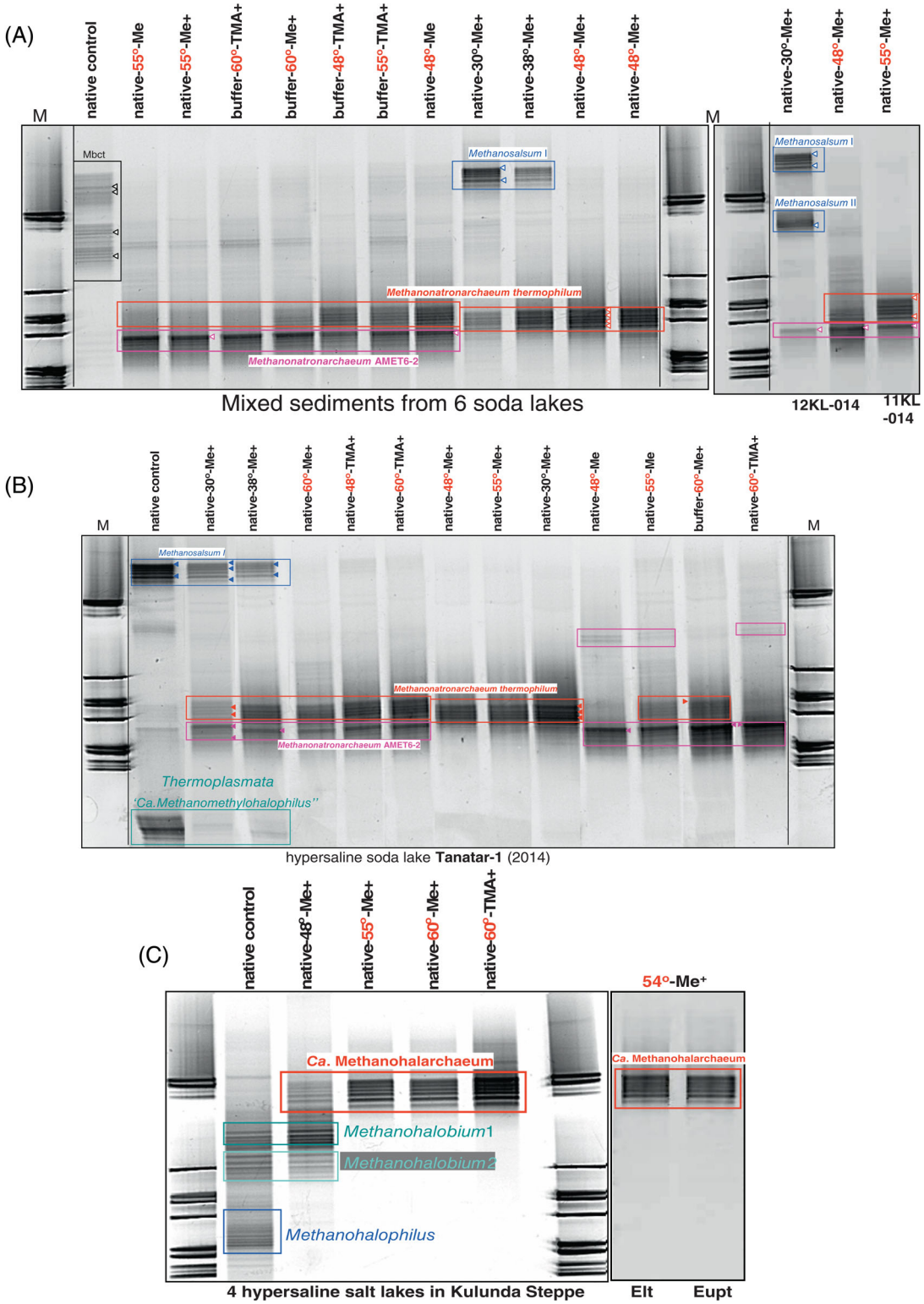

FIGURE 5 Results of *mcrA*-DGGE analysis of methanogenic populations developing in sediment slurry incubations from hypersaline soda (A, B) and salt (C) lakes at classical methylotrophic conditions with methanol (Me) or trimethylamine (TMA) and methyl-reducing conditions with formate addition (marked as Me^+ and TMA^+) and at different temperatures. At conditions used, the *mcrA* genes from single organisms resolved in bundles of 2–6 separate bands with identical sequences. The *mcrA* band bundles belonging to two different *Methanonatronarchaeum* species (A, B) are framed in red for the type species *M. thermophilum*-like and in purple for a putative new species *Methanonatronarchaeum* sp. AMET6-2. The results were obtained from duplicate sediment incubations pooled together for DNA extraction. Salt lakes abbreviations in (C): Elt—lake Elton; Eupt—Eupatorian solar saltern

conditions, in addition to high temperature, were more favourable for *Ca*. *Methanohalarchaeum* (Plain script for Methanohalarchaeum).

Attempts to determine a basic abundance of *Methanonatronarchaeia* in native sediments of hypersaline lakes using 16S rRNA gene amplicon sequencing showed, expectedly, a low representation, ranging from 0.3%–0.4% (soda lakes) to 0.6%–2% (salt lakes) of the total archaeal communities dominated by haloarchaea. An alternative method based on *q*PCR with an AMET1-specific 16S rRNA gene primer set showed that *Methanonatronarchaeum* consisted of 1%–1.1% of the total archaeal population in two sediment samples from hypersaline soda lakes in Kulunda Steppe (a mixed sample from four lakes and a sample from Tanatar-1). However, in both types of hypersaline lakes, it only took 2–4 weeks of incubation at methyl-reducing and moderately thermophilic conditions for *Methanonatronarchaeia* to become a dominant archaeal population, ranging from 13% to 62% in salt lakes (Supplementary Table S2) and up to nearly 100% in soda lakes (Figure 4), according to the 16S rRNA amplicon sequencing results. In soda lakes the amplicons belonged only to the genus *Methanonatronarchaeum*, while in salt lakes in addition to *Ca*. Methanohalarchaeum thermophilum HMET1 amplicons related to several uncultured lineages of the SA1 group (Eder et al., 2002) from various hypersaline systems were also detectable in significant proportion with sequence identity to *Ca*. Methanohalarchaeum from 91% to 93% (Supplementary Table S2). Nevertheless, all our highly enriched methyl-reducing methanogenic cultures from neutral hypersaline lakes were dominated by the HMET1-like populations. One such highly enriched methyl-reducing culture from the salt lake Baskunchak was also analysed by classical cloning of 16S rRNA gene to obtain nearly full-length sequences. This culture consisted of 60% *Ca*. Methanohalarchaeum (99%–100% sequence identity to HMET1) and 40% of *Methanohalobium* (98.2%–100% to *M*. *evestigatum*), which was also evident from microscopy (Supplementary Figure S2). In addition, two more enriched cultures dominated by the HMET1-like methyl-reducers were obtained from salt lake Elton located in the same area as lake Baskunchak and from a solar saltern in Eupatoria (Crimea) (Supplementary Figure S3). The fact that in the potential activity tests from salt lakes, especially those from lake Elton, a presence of several SA1 phylogenetic lineages different from *Ca*. Methanohalarchaeum were detected in methanogenically active sediment incubations but never developed into sediment-free enrichments might have two possible explanations: 1—that they belonged to non-methanogenic extremely halophilic archaea (similar to the single-cell-derived SA1 archaeon metagenome from the Red Sea) (Ngugi & Stingl, 2018); 2—that the medium used for cultivation was highly selective for the HMET1-like *Ca*. Methanohalarchaeum. The prevalence of those distant lineages in the lake Elton incubations in contrast to the ones from lake Baskunchak suggests that it might have something to do with the basic difference in their brine composition (low Mg in Baskunchak vs. high Mg in Elton).

The *Methanonatronarchaeum* AMET-like sequences in methyl-reducing incubations from soda lakes with variable methyl acceptors showed 100% of their 16S rRNA gene sequence identity to already known pure culture AMET isolates (Sorokin et al., 2017, 2018). With MeOH and methylamines, it was the dominant group including the type strain AMET1, while on methylated sulfides (mercaptans) the populations belonged to AMET6-2—the AMET strain most distant from the group 1 representing a second putative species in the genus (98.4% 16S rRNA gene sequence identity). Indeed, two pure cultures, obtained from the mercaptan enrichments (AMET-DMS and AMET-MT) were 100% identical in their 16S rRNA gene sequences to AMET6-2. Another culture, AMET-MMP, enriched from soda lakes with methylmercaptopropionate (MMP) as the methyl acceptor, was a mix of the two *Methanonatronarchaeum* genotypes—AMET1-like and AMET6-2-like. MMP and DMS are both products of anaerobic degradation of dimethylsulfoniopropionate (DMSP)—a common osmolyte produced in marine algae by demethylation or cleavage reactions, respectively (Reisch et al., 2011). It has only recently been shown that *Methanosarcina* spp. can perform methylotrophic methanogenesis using MMP as a methyl substrate, as well as more common DMS and MT (Fu & Metcalf, 2015). However, MMP has never been tested as a substrate for methyl-reducing methanogens before. Interestingly, the apparently mercaptan-specialized strain AMET6-2 has also proliferated in enrichments in other conditions substantially different from the ones used previously (i.e. a combination of MeOH/TMA as an acceptor and formate or H_2 as donor) as demonstrated below.

Interaction of *Methanonatronarchaeum* with non-methanogenic anaerobes from soda lake sediments

In several activity incubations of hypersaline soda lake sediments at 48°C–50°C methane could slowly be formed from MeOH without addition of an external electron donor (formate or H_2), i.e. at apparently classical methylotrophic conditions. On the other hand, the only known methylotrophic methanogen active in hypersaline soda lakes is *Methanosalsum natronophilum* (Sorokin et al., 2015b) incapable of growth above 43°C, which was also evident from the *mcrA*-DGGE results (see above). Therefore, we have tried to purify further the methanogen responsible for the observed process using dilution series. After several 1:100 transfers, it

became clear that the culture is an association between an AMET-like methyl-reducer and a fermentative bacterium apparently able to produce a low amount of either H_2 or formate from the endogenous sediment organic matter, thus supplying the methyl-reducer with a necessary electron donor missing in the original medium. When this culture was finally transferred into a medium with an antibiotic mix (streptomycin/kanamycin/vancomycin, 100 mg L^{-1} each), the methane formation ceased. The addition of external formate or H_2 allowed to purify the methyl-reducer in pure culture. The isolate AMET(−) was 100% identical in its 16S rRNA gene sequence to *Methanonatronarchaeum* AMET6-2. This situation resembles those observed by Iino et al. (2013) in enrichment culture of *Ca*. Methanogranum, a methyl-reducing member of the *Methanomassiliicoccales*. This culture was dominated by fermentative clostridia supplying hydrogen, while the MeOH-reducing methanogen remained a minor fraction of the population. Recently, a role of the H_2-consuming partner has been suggested for the *Thermoplasmatota* methyl-reducing methanogens in thermophilic syntrophic associations converting fatty acids to methane in chemostat cultures inoculated by sludge from two wastewater treatment plants (Chen et al., 2020). However, this suggestion can be questioned: first, the *Methanomassiliicoccales* have never been shown to grow at high temperature and second, classical hydrogenonotrophic methanogens were also present in the reactor. In that respect an important question is arising: if the H_2/formate-utilizing methyl-reducing methanogens might act in the capacity of hydrogenotrophic partners in syntrophy with the H_2-generating bacteria, what is determining their competition with the classical hydrogenotrophic methanogens? Nobu et al. (2016) already suggested that the methyl-reducers would have an advantage over hydrogenotrophs because they only need a single H_2 molecule to form methane instead of four H_2 in hydrogenotrophic pathway. While the stoichiometry is an important factor in the overall conversion, at μM substrate concentration range dominating in natural habitats it is the high substrate affinity which decides the outcome of competition. Recently, a dedicated study performed by Feldewert et al. (2020) on three species of freshwater obligate methyl-reducing methanogens from the genera *Methanomicrococcus*, *Methanosphaera* and *Methanomassiliicoccus* demonstrated that their H_2 affinity was higher (the consumption threshold <0.1 Pa) than in hydrogenotrophic methanogens (the consumption threshold >2 Pa). It was concluded that growth of methyl-reducing methanogens is limited by the concentration of methyl acceptor rather than H_2. Another important conclusion from this work was that the affinity to MeOH in methyl-reducers is also much higher than in classical MeOH-fermenting methanogens. In case of *Methanonatronarchaeum*, the situation is more simple: it is the triple extreme conditions that select these archaea as the only methanogens outcompeting both of the two possible rivals: the hydrogenotrophic *Methanocalculus natronophilus* (both in the high temperature and extreme salinity range) and the methylotrophic *Methanosalsum natronophilum* (in the high temperature range). Below several more examples of the trophic interaction of *Methanonatronarchaeum* with anaerobic fermentative bacteria supplying either methyl acceptor or electron donor are presented.

A methanogenic association was enriched from the Kulunda Steppe hypersaline soda lake sediments at 4 M total Na^+, pH 9.8 and 48°C with *n*-propanol as a possible electron donor and methanol as a methyl acceptor. The original idea was to test whether primary alcohols (ethanol, *n*-propanol or *n*-butanol) might serve as a direct electron donor for methyl-reducing methanogens at extremely haloalkaline conditions, as has been shown previously for several hydrogenotrophic methanogens (Hoedt et al., 2016; Imachi et al., 2009). Only a culture with propanol was positive for methane formation and eventually it was obtained in a sediment-free serial dilution (10^{-6}). The culture was dominated by curved rods and tiny cocci [Supplementary Figure S4(a)]. Addition of antibiotic mix completely abolished methane formation in this culture, while transfer into a medium with MeOH + H_2 restored it and further resulted in isolation of a methyl-reducing strain AMET-Pr identical in its 16S rRNA gene sequence to *Methanonatronarchaeum* AMET6-2. The conclusion was that the methyl-reducing methanogen served as an H_2 scavenger in syntrophy with the propanol-fermenting bacterium.

A third example of direct interaction of *Methanonatronarchaeum* with anaerobic bacteria at halo-alkali-thermophilic conditions was obtained in an attempt to investigate a possibility of CO utilization as the electron donor for MeOH reduction to methane. So far, although claimed for *Ca*. Methanofastidiosa on the basis of metagenomic analysis (Nobu et al., 2016), no direct proof for utilization of CO as the electron donor by methyl-reducing methanogens exist. An active methanogenesis was observed at 48°C and low concentrations of CO (5%–10% in the gas phase). It was reproducible in further sediment-free dilutions up to (10^{-8}) and developed in two phases: first CO consumption was observed, while methane formation only started when most of CO was already consumed. H_2 was only detected in trace amounts or not at all. The final active dilution was a binary co-culture consisting of curved rods and AMET-like tiny cocci [Supplementary Figure S4(b)]. Addition of antibiotics completely inhibited the co-culture proliferation, but both members were able to grow in pure culture separately. The bacterium turned out to be a primary CO-utilizing acetogen, a novel family lineage in the class *Natranaerobiia* described recently as *Natranaerofaba carboxydovora* (Sorokin et al., 2021). It converts CO mostly into acetate with formate as a minor product. Both can be used as substrates by *Methanonatronarchaeum* (acetate as the C-source,

formate as the *e*-donor). A pure methyl-reducing culture strain AMET-CO isolated from this association by using a combination of MeOH and formate was identical in its 16S rRNA gene sequence to *Methanonatronarchaeum* AMET6-2. The consortium can be reconstructed by mixing the two organisms and incubating them at the initial conditions, i.e. with MeOH + 5% CO in the gas phase. However, upon increasing CO concentration above 10%, AMET-CO was inhibited and only the CO-utilizing acetogen was able to grow.

Two more examples of trophic interaction of *Methanonatronarchaeum* with anaerobic fermenters were observed in enrichments from Kulunda Steppe hypersaline soda lake sediments at moderately thermophilic and extremely haloalkalophilic conditions using environmental precursors of TMA (glycine betaine and choline) as the methyl substrates and formate as the electron donor. In these cultures, the tertiary amines were first fermented by extremely halotolerant alkaliphilic bacteria with release of TMA (Sorokin, 2021). The one developing on glycine betaine was a long rod rapidly turning into sphaeroplasts [Supplementary Figure S4(c)], and the choline-fermenting bacterium was also a long rod but the cells were more stable [Supplementary Figure S4(d)]. The bacterial member of the glycine betaine co-culture (strain ANB-GB) was isolated in pure culture using glycine betaine as the only substrate and identified as a member of the genus *Natroniella* (98.7% of 16S-rRNA gene sequence identity to *N. acetigena*). It seemed to be a very narrowly specialized anaerobe utilizing exclusively glycine betaine. The bacterial member of the choline co-culture was lost and not identified. Methane in those two co-cultures was apparently formed by the AMET-like methyl-reducers utilizing TMA formed by the fermenters as the acceptor and formate as the external electron donor. Indeed, two bacteria-free methanogenic cultures purified from the consortia using a combination of TMA +- formate, AMET-GB and AMET-Chol were incapable of direct utilization of either glycine betaine or choline as the methyl acceptors for methanogenesis, in contrast to some of the classical methylotrophic methanogens (Watkins et al., 2014). Similar to AMET-MMP, both AMET-GB and AMET-Chol were a mixture of two *Methanonatronarchaeum* genetic species—AMET1-like and AMET6-2-like, but in different proportions.

The information on the newly isolated or enriched strains of *Methanonatronarchaeum* from various enrichments is given in Supplementary Table S3 and Figure S5.

Environmental metagenome of *Methanonatronarchaeum*

A single MAG belonging to the genus *Methanonatronarchaeum* has been recovered and sequenced within a metagenome sequencing program (JGI) from environmental DNA extracted from sediments of the hypersaline soda lake Tanatar-1 in Kulunda Steppe (the GenBank genome assembly GCA_004212035) (Vavourakis et al., 2018). Its metabolic reconstruction has already been published elsewhere (Borrel et al., 2019) and here we only comment on what is not mentioned there. Borrel et al. did not notice that the 16S rRNA gene fragment (937 nt) present in this MAG was 100% identical to a pure culture strain AMET6-2 which, as is shown above, is representing a second, yet undescribed, species of the genus *Methanonatronarchaeum* (Figure 6). This is confirmed by the whole-genome comparison between AMET6-2 and the type species *M. thermophilum*: the ANI, AAI and DDH values (85.9, 76.7 and 22.9%, respectively). And it is this second species which seems to be more adaptable to variable conditions, such as the ability to use toxic methylated sulfides and to grow at low concentrations of electron donors. So, it might not be accidental that it was also detected by the direct metagenomic sequencing instead of the type species.

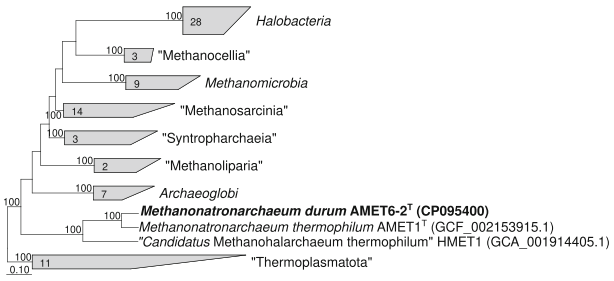

FIGURE 6 Phylogenomic placement of *Methanonatronarchaeum durum* AMET6-2 sp. nov. based on concatenated partial amino acid sequences of 122 archaeal single-copy conserved marker genes with taxonomic designations according to the GTDB (Release 07-RS207). Bootstrap consensus tree is shown with values above 90% placed at the nodes. Bar, 0.1 changes per position

Concluding remarks

Overall, here we demonstrated that the class *Methanonatronarchaeia*, currently represented by two lineages of cultured moderately thermophilic and extremely halophilic obligate methyl-reducing methanogens, the alkaliphilic *Methanonatronarchaeum* and the neutrophilic *Ca*. Methanohalarchaeum, is widely distributed in anaerobic sediments of hypersaline soda and salt lakes, respectively. Although their basic population level is low, this group rapidly becomes a dominant archaeal lineage at a combination of moderately high temperature from 45°C to 60°C and mixotrophic conditions, i.e. availability of the C_1-methyl acceptors and H_2 or formate as the electron donors. This work extended the range of C_1-methyl

compounds known previously for both genera (MeOH and methylamines) to mercaptans, such as MT, DMS and 3-MMP. *Methanonatronarchaeum* strain AMET6-2 is taking a selective advantage in utilization of mercaptans and also proliferating in syntrophy with bacterial fermenters as an H_2-removing partner. It represents a second, yet undescribed, species in the genus *Methanonatronarchaeum*. This raises a question of how the methyl-reducing methanogens have to be classified in terms of their methanogenic pathway? For now they are considered as a (peculiar) variety of methylotrophic methanogens, but, in reality, at least in ecological perspective, they are forming a fourth, mixotrophic pathway, taking advantage of a simultaneous presence of both C_1-methyl compounds and H_2/formate. Such conditions exist, for example, in intestine of lignocellulose-feeding insects and mammalians. In hypersaline habitats, the methyl acceptors (TMA and mercaptans) are abundantly produced during anaerobic fermentation of methylated compatible solutes (glycine betaine and DMSP) released from the biomass of halophilic microorganisms, while H_2/formate can be produced in the same niche by fermentation of organic monomers, such as glycerol for example—a major compatible solute of extremely halophilic algae *Dunaliella*. However, to justify the role of methyl-reducers as both hydrogenotrophs and methylotrophs, more quantitative growth experiments need to be done in both pure and mixed cultures.

DESCRIPTION OF *METHANONATRONARCHAEUM DURUM* SP. NOV.

du'rum. L. neut. adj. *durum*, resistant, indicating the ability to deal with harsh environmental conditions

Cells are small angular non-motile cocci, 0.4–0.5 μm in diameter. The cells lyze at salinity below 2 M Na^+. The F_{420}-dependent cell blue autofluorescence is not detectable. Strictly anaerobic methyl-reducing methanogens utilizing methanol, methylamines, methanethiole, 3-methylmercaptopropionate and dimethylsulfide as the electron acceptors and formate or H_2 as the electron donors. Yeast extract or acetate can serve as the C-source. Growth depends on external CoM, FeS/or anaerobic sediments from soda lakes. Obligately alkaliphilic with a pH range for growth from 8.5 to 10.4 (optimum at pH 9.5) and extremely halophilic, growing between 3 and 5 M total Na^+ (optimum at 4 M). Growth depends on presence of both NaCl and Na-carbonates with the optimal ratio of 2 M NaCl:2 M Na^+ as carbonates. Moderately thermophilic, growing between 37°C and 62°C (optimum at 53°C–55°C). The G + C content of the genomic DNA in the type strain is 42.2%. The ANI, AAI and DDH values with the type species *M. thermophilum* AMET1 are 85.9%, 76.7% and 22.9%, respectively. In contrast to *M. thermophilum* AMET1, AMET6-2^T have only a single copy of the *mcrA* gene in the genome. The type strain, AMET6-2^T (UNIQEM U988), was isolated from sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The accession numbers of 16S rRNA gene and genome sequences of the type strain in the GenBank are KY449322 and CP095400, respectively.

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DATA AVAILABILITY STATEMENT

The 16S rRNA and mcrA gene sequences and the genome sequence of strain AMET6-2 were deposited in the GenBank.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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