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EXPERIMENTAL ARTICLES

Microbial Utilization of Glycine Betain in Hypersaline Soda Lakes

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Abstract—Glycine betaine (GB) is a biologically important compound for microbial communities living in saline habitats most commonly employed as an organic osmolyte. At fluctuating salinity, halophilic microbes producing GB excrete it into environment making it available for heterotrophic/methylotrophic community members as a source of carbon, energy and nitrogen. Although many halolalkaliphilic bacteria have a potential for synthesis of GB as the main osmolyte, so far there was no targeted investigation of its microbial mineralization at soda lake conditions. In this work GB was used as substrate to enrich for GB-utilizing bacteria and archaea from sediments of hypersaline soda lakes located in southwestern Siberia. Aerobic enrichments at moderate and soda-saturated conditions (pH 10) resulted in isolation of several gammaproteobacterial strains identical in its 16S RNA gene to each other and to the known species *Halomonas alkalicola*. These isolates grew equally well with several methylated compounds: methylglycine (sarcosine), dimethylglycine (DMG), GB and choline (trimethylethanolamine). No growth was observed in aerobic hypersaline enrichments in presence of antibiotics indicating that bacteria are the main mineralizers of GB in hypersaline soda lakes at oxic conditions. In contrast, an anaerobic enrichment at 4 M Na^+ and pH 9.7 targeting GB-utilizing haloalrchea was positive with sulfur as electron acceptor and resulted in isolation of a pure natronarchaeal culture belonging to the previously described genus of sulfur-reducing haloarchaea *Halalkaliarchaeum*. Anaerobic enrichments with GB at fermentative conditions were positive at salinities 2–4 M total Na^+ (pH 10) and consisted of a bacterial component forming trimethyamine (TMA) and methylotrophic methanogens consuming the latter. In both cases the bacterial component belonged to the genus *Natroniella* (*Halanaerobiia*), while the methanogenic partner at 2 M Na^+ was identified as *Methanosalsum natronophilum* and at 4 M Na^+/48°C—as members of the methyl-reducing genus *Methanonatronarchaeum*.

Keywords: glycine betaine, sarcosine, dimethylglycine, soda lakes, haloalkaliphilic, trimethylamine (TMA)

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Glycine betaine (GB, trimethylglycine) is the most widespread organic osmolyte utilized by halophilic bacteria and a few methanogenic archaea employing the so called "salt-out" osmoprotection mechanism. Many obligate halophiles can synthesize GB *de novo* either by choline oxidation or by 3-step sequential methylation of glycine, while halotolerant organisms import the GB from external source using a range of ABC transporter or Na^+-dependent symporters (da Costa et al., 1998; Oren, 1990; Robberts, 2001; Robertson et al., 1990; Roeûler M and Müller, 2001).

Microbial mineralization of GB is best studied under anoxic conditions and can be fermentative or with an external reductant, either leading finally to formation of acetate and methylamines (Hormann and Andreesen, 1981; Möller et al., 1984; Müller et al., 1981; Visser et al., 2016) or to its complete utilization by methylotrophic acetogens and methanogens (Creighbaum et al., 2019; Ticak et al., 2014, 2015; Watkins et al., 2014; Visser et al., 2016). Furthermore, anaerobic GB mineralization can be linked to dissimilatory sulfodogenesis by either a partial oxidative demethylation (*Desulfobacterium*) to DMG or fermentation to TMA and acetate with a partial C_2 backbone oxidation to CO_2 (*Desulfuromonas*) (Heijthuijsen and Hansen, 1989a, 1989b).

Since the GB-producing halophiles release GB into environment when salinity decreases, which often occurs in saline habitats seasonally and also after cell death/autolysis, this can be considered as an important substrate for the organic carbon mineralization process. Until now, its microbial mineralization has been addressed only in hypersaline chloride-sulfate habitats with neutral pH, but for soda lakes there have been no targeted studies on this topic. At neutral hypersaline habitats the documented cases include three extremely halophilic members of the class *Halanaerobiia*: (1) *Acetohalobium arabaticum*—a methy-

lotrophic acetogen breaking down GB to acetate and TMA and further converting the latter to acetate and ammonium (Zhilina and Zavarzin, 1990); (2) *Halanaerobacter salinarius*, performing reductive cleavage of GB to acetate and TMA with external *e*-donors, such as H_2 or serine (Mouné et al., 1999); (3) *Halanaerobium* sp. from hypersaline Gulf of Mexico fermenting GB (implicated from the presence of GB reductase genes in the metagenome which are also present in many other *Halanaerobium* metagenomes) (Christman et al., 2020). Same outcome can be achieved without the external reductant by a partial fermentation of the C_2 backbone to produce either NAD(P)H or thioredoxine as a reductant used for a further 3-step reductive deamination by the selenocystein reductases consisting of the GrdIHBD complex (betaine reductase, DMG reductase and sarcosine reductase) (Meyer et al., 1995; Visser et al., 2016). Although such pathway has not yet been demonstrated in any pure culture of extreme halophilic or haloalkalaliphilic anaerobes, these genes homologues are present in metagenomes of several *Halanaerobiales* members (Christman et al., 2020; Daly et al., 2016). Partial degradation of GB is the most likely pathway for anaerobic sediments of neutral saline habitats, when halophilic bacteria would degrade GB to acetate and TMA followed by its methanation in a consortium with halophilic methylotrophic methanogens (Christman et al., 2020; Daly et al., 2016; Jones et al., 2019; La Cone et al., 2015; Nigro et al., 2016, 2020; Yakimov et al., 2013).

For the aerobic GB mineralization, there is only a single direct evidence describing this potential in halophilic microorganisms, namely, in a moderately halophilic *Chromohalobacter salexigens*, which can grow with choline, GB and DMG as sole source of carbon and energy converting GB to DMG via betaine monooxygenase BmaAB (Shao et al., 2017, 2018) and sarcosine to glycine via sarcosine oxidase soxBDAG (genomic evidence). An indirect conclusion from genome analyses also suggests that many members of the genus *Halomonas*, the most abundant and metabolically versatile moderately halophilic genus of gammaproteobacteria widespred in saline habitats, can potentially completely mineralize GB using the pathway of complete oxidative demethylation (Wargo, 2013). This includes GB demethylation to DMG by the GB demethylase GbcAB, DMG demethylation to sarcosine by the DMG dehydrogenase DgcAB and sarcosine demethylation to glycine by the sarcosine oxidase soxBDAG.

In this work we tested whether GB can serve as carbon and energy source for halolalkaliphilic microbial communities of hypersaline soda lakes located in southwestern Siberia (Altai, Russia).

MATERIALS AND METHODS

Two composite samples of near-bottom brines-sediments collected from several hypersaline soda lakes in Kulunda Steppe (Altai region, Russia) in 2015–2016 were used to enrich for haloalkaliphilic GB-utilizing microorganisms: for aerobic enrichments the top oxidized 1 cm sediment layer and the near-bottom brine were collected by a 50 mL syringe with attached silicon tube hose into a 50 mL Falcon tubes; for anaerobic incubations, reduced sulfidic sediments 5–15 cm deep were obtained by a sediment corer. The subsamples from different lakes were mixed in equal proportion as 1 : 1 sediment-brine slurries, homogenized by extensive vortexing and separated from the course sandy fraction by a brief low-speed centrifugation resulting in a fine colloidal particle fraction containing most of the microbial cells. 1 mL of this final preparation was mixed with 99 mL liquid media and distributed in 10 mL portions into 50 mL (aerobic incubations) or 23 mL (anaerobic incubations) serum bottles for primary enrichments.

The enrichment media varied in salinity from 0.6 to 4 M total Na^+ at pH 9.7–10. The 0.6–2 M Na^+ mineral bases were prepared from a mixture of sodium bicarbonate/carbonate containing 0.1–0.2 M of NaCl with the final pH 10. The 4 M Na^+ medium was either based on sodium carbonates and has a final pH of 10, or contained 2 M NaCl and 2 M Na^+ as carbonates with the final pH 9.7 to enrich for natronoarchaea. Both were supplied with 1 g/L of K_2HPO_4 before sterilization and with 4 mM NH_4Cl, 1 mM Mg sulfate and 1 mL/L of acidic and basic trace metal and vitamin mixtures after sterilization (Pfennig and Lippert, 1966; Plugge, 2005). The GB or precursors were added at 5 mM concentrations in the primary enrichments and 10 mM to the sediment-free cultures.

For anaerobic incubations of liquid cultures, full medium was assembled aerobically in serum bottles with butyl rubber stoppers, 0.5 mM of filter-sterilized sulfide was added as a first reductant and the bottles were subjected to 3 cycles of sterile evacuation-argon flushing. The final medium reduction was achieved by injecting 1 drop of 10% dithionite in 1 M $NaHCO_3$. For colony formation, the above-mentioned liquid media were mixed 3 : 2 with 4% washed a gar at 55°C with salinity compensation for haloarchaea by adding solid NaCl before mixing with agar (up to a final concentration of 4 M Na^+). The colonies from anaerobic cultures were produced by agar shake dilutions poured into petri dishes which were incubated anoxically in a 3.5 L anaerostat (Oxoid) under argon. The incubation temperature in enrichment cultures and in pure *Halomonas* cultures was 30°C, except for an enrichment specifically targeting *Methanonatronoarchaeum*, in which case it was 48°C. The pure haloarchaeal sulfur-reducing culture was grown at 37°C.

Production of CH_4 and TMA in the gas phase of anaerobic cultures (the presented results are mean values from duplicate cultures) was detected by the GC using the Varian 3800 GC (Agilent Technology, NL) equipped with a FID detector operated at 200°C with H_2 flow was 30 mL/man and air flow was 300 mL/min. For CH_4, a CPSil5CB 5 (50 m × 0.32 mm) capillary column was used isothermically at 100°C at a constant He flow rate of 10 mL/min. For TMA, the column was capillary Carbowax fused-silica [30 m × 0.25 mm, film thickness 0.25 μm (Supelco)] with the carrier gas N_2 at 1 mL/min, oven temperature program 40°C (5 min) ramped at 25°C/min to 180°C (0 min)]. Ammonium was determined by the phenol-hypochlorite method according to Weatherburn (1967).

The 16S rRNA gene sequences obtained in this work were deposited in the GenBank under the accession numbers MW970321–MW970323.

RESULTS AND DISCUSSION

GB Utilization at Aerobic Conditions

Enrichments for aerobic GB-utilizing natronoarchaea at 4 M total Na^+ and pH 9.7 with a mixture of antibiotics (kanamycin/streptomycin/vancomycin, 100 mg/L each) was negative, indicating that GB is probably not a substrate for the aerobic extremely haloalkaliphilic members of *Halobacteria* living in hypersaline soda lakes. In contrast, aerobic enrichments at 0.6, 2.0 and 4.0 M total Na^+ and pH 10 without antibiotics resulted in stable positive cultures already after one week of incubation. After three successive 1 : 100 transfers, the cultures were plated onto solid media and the dominant colony types from each salinity were isolated and regrown in liquid culture with GB. All three cultures were able to grow within the whole tested salinity spectrum from 0.3 to 4 M total Na^+ in sodium carbonate buffer not only with GB, but also with sarcosine, DMG, choline, glycine and acetate. The cultures appear to have completely mineralized GB and its precursors to ammonia, CO_2, and biomass, as only traces of TMA were detectable in the gas phase in the fully grown cultures (Fig. 1). The 16S rRNA gene sequence analysis showed that the isolates were identical to each other and closely related to the known *Halomonas* species—*H. alkalicola*, isolated from an alkaline soap-producing plant in China, although this species is reported as a much less salt-tolerant than our isolates (maximum is equivalent to 1.2 M total Na^+) (Tang et al., 2017).

GB Utilization at Anaerobic Conditions

No growth with GB was observed at low salt concentration of 0.6 M Na^+, while three types of anaerobic enrichment cultures at hypersaline conditions were positive: one culture at 2 M Na^+ and pH 10 and two cultures at 4 M Na^+, pH 9.7.

The GB enrichment culture at 2 M Na^+ (pH 10) was dominated by long flexible motile rods and it was also transferable on sarcosine and DMG, but not on choline. The culture developed in two phases. At first, the long rods prevailed, which later began to form sphaeroplasts concomitant with accumulation of TMA. Then flat coccoid cells started to take over (showing blue fluorescence characteristic of methanogens) leading to accumulation of methane in the gas phase (Fig. 2). The latter morphotype was purified by subculturing in dilution series with TMA and identified as *Methanosalsum natronophylum*—high-salt methylotrophic methanogen typical for hypersaline soda lakes (Soroklin et al., 2015). However, the isolate did not grow with GB, DMG or sarcosine. On the other hand, the bacterial component was further purified with GB as the only substrate in serial dilutions with addition of BES (methanogen inhibitor, 10 mM). Strain ANB-GB1 was identified as a member of the genus *Natroniella* (98.0–98.7% 16S rRNA gene identity to its two known species). Interestingly, the methylotrophic metabolism was not reported before in members of this genus. ANB-GB1 also grew with sarcosine and DMG, but with much lower activity and the cultures quickly lost their viability. High concentrations of TMA detectable in the gas phase indicated that GB was most likely fermented into TMA and acetate.

An enrichment at 4 M Na^+/pH 9.7 and 48°C with GB as methyl substrate and formate as an external *e*-donor was originally aimed to find out if the methyl-reducing methanogens *Methanonatronarchaeum* recently discovered in hypersaline soda lakes (Sorokin et al., 2017) would be able to use GB as a methyl-group acceptor. Indeed, such an enrichment was positive and after a serial dilution it contained two organisms: a long rod similar to ANB-GB1 and tiny cocci, similar to *Methanonatronarcheum* (Fig. 3). In fact, this culture behaved similar to the one obtained at 2 M Na^+, i.e. forming TMA as an intermediate and methane as the final product. The addition of antibiotics completely arrested growth, indicating that the primary GB degrader was the long bacterial rods, while *Methanonatronarchaeum* utilized the released TMA as an acceptor and formate as an *e*-donor. Indeed, a bacteria-free methanogenic culture, "*Methanonatronarchaeum* AMET-GB", was isolated from the initial consortium using TMA + formate and it contained two organisms with 100% identity of their 16S rRNA gene to the previously described strains of *Methanontronarchaeum thermophilum*: the type strain AMET1^T and the most divergent strain AMET6-2 (Sorokin et al., 2017, 2018). The bacterial component was isolated in a pure culture with GB alone and at 30°C (the conditions suppressing growth of "AMET-GB"). The 16S rRNA gene analysis of strain ANB-

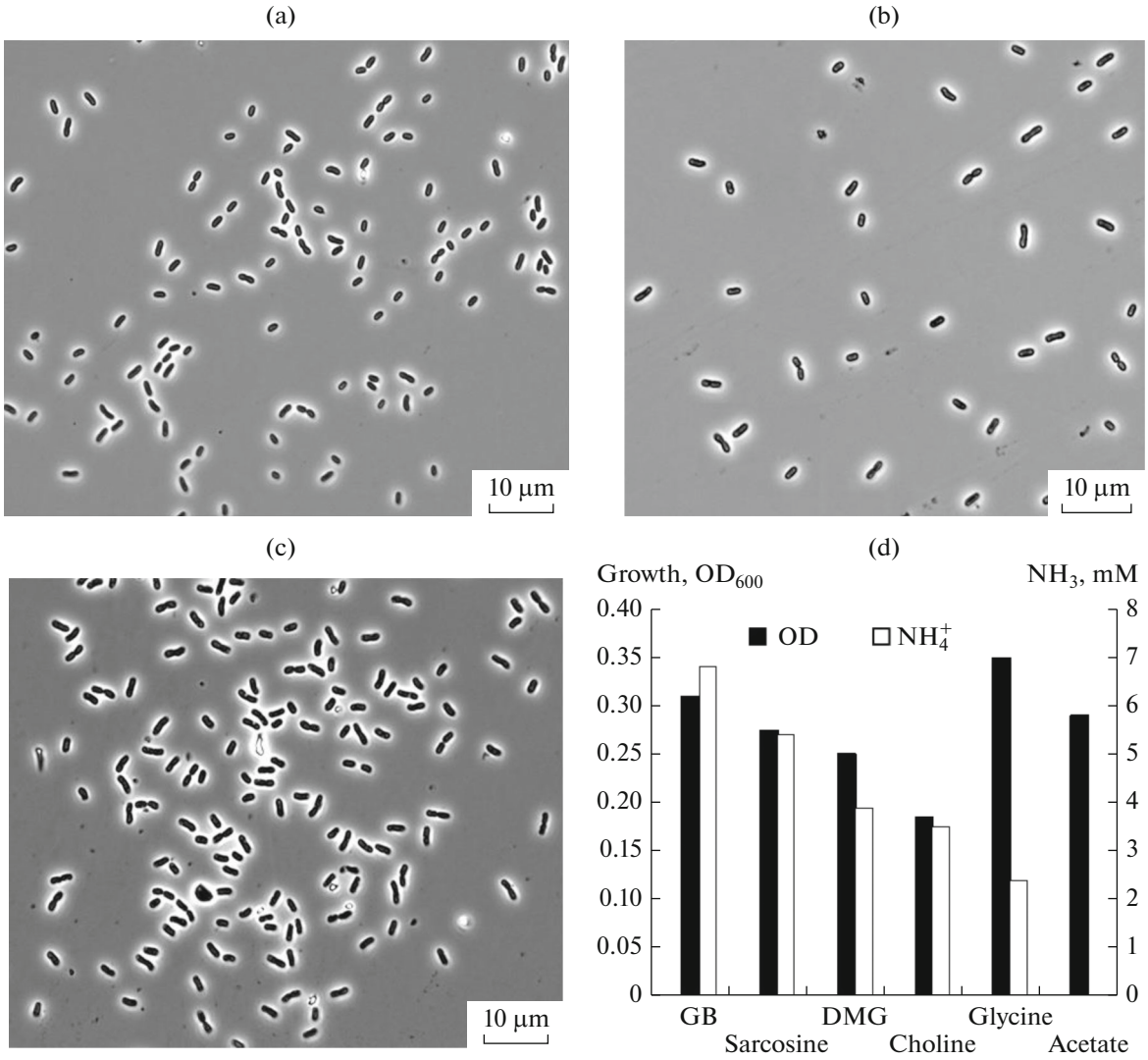

Fig. 1. Aerobic utilization of GB and precursors by *Halomonas* sp. AB-GB1 enriched from hypersaline soda lakes with GB. (a–c) Phase contrast microphotographs of cells grown at 0.6, 2.0 and 4.0 M total Na^+ and pH 10 with GB; (d) maximum biomass yield and ammonium formation in AB-GB1 culture (43 hours, 30°C) with GB and precursors (10 mM) at 2 M Na^+.

GB2 showed 100% identity to *Natroniella* ANB-GB1 enriched at 2 M Na^+/30°C.

These results demonstrated that anaerobic degradation of GB without added acceptor in soda lakes is active at the conditions of hypersaline soda lakes through fermentative GB cleavage to TMA and most likely acetate by *Halanaerobiales* members followed by TMA conversion to methane by extremely salt-tolerant natronophilic methylotrophic methanogens, similar to that observed in marine sediments (Oren, 1990; Creighbaum et al., 2019; Jones et al., 2019).

When anaerobic GB enrichments at 2–4 M total Na^+ were additionally supplemented with a sulfur-based electron acceptor (sulfur, thiosulfate and sulfate) in an attempt to obtained sulfur-respiring GB utilizers similar to those described by Heijthuijsen et al. (1989a, 1989b), the only positive culture was with elemental sulfur at 4 M total Na^+, pH 9.7 and 30°C. The initial enrichment contained several rod-shaped members and a morphotype represented by flat polymorphic cells typical for haloarchaea. The addition of antibiotics did not effect the activity of GB-dependent sulfur reduction in furher transfers. After plating a maximum serial dilution, small red anaerobic colonies were growing inside agar surrounded by a zone of polysulfide clearance (polysulfide is formed by chemical reaction of sulfide and sulfur and is stable at highly alkaline anoxic conditions), indicating that the colonies belonged to a sulfur-reducer capable of utilizing GB as the *e*-donor (Figs. 4a–4c). The colonies were regrown in liquid culture with GB + sulfur (but not without sulfur) and also with sarcosine, but not with DMG or choline. The isolate AArc-GB was identified as a member of the know sulfur-reducing haloalka-

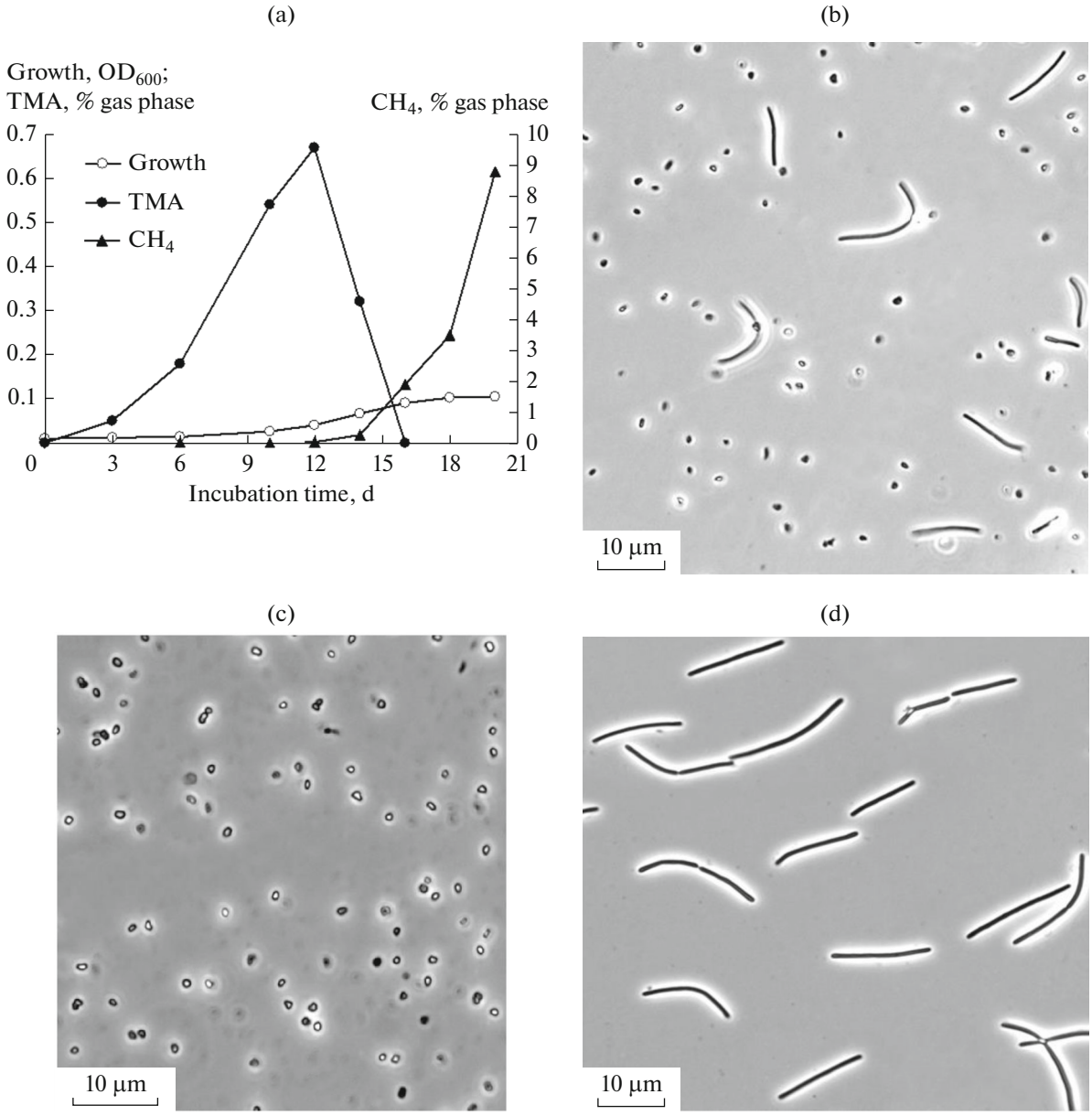

Fig. 2. Growth dynamic (a) and cell morphology (b) in a methanogenic consortium grown with GB (10 mM) at 2.0 M total Na^+ and pH 10 (30°C) from hypersaline soda lakes. (c) Cell morphology of pure culture of the TMA-utilizing *Methanosalsum natronophilum*; (d) pure culture of the GB-fermenting *Natroniella* sp. ANB-GB1.

liphilic archaeal genus *Halalkaliarchaeum* (Sorokin et al., 2017, 2018), probably representing a new species (97.7% identity of the 16S rRNA gene to the type strain AArc-Sl^T). Therefore, we also checked the ability of *H. desulfuricum* AArc-Sl^T to grow by sulfur respiration with GB, and it was positive, although, less active than in AArc-GB (Fig. 4d).

Since the genome of *H. desulfuricum* AArc-Sl^T is available, we used this opportunity to look at the potential GB degradation pathway in this archaeon, which, most probably, should be similar to that of strain AArc-GB. The results revealed a unique combination of genes organized in two genomic loci encoding a hybrid pathway known for anaerobes and aerobes including the following (Fig. 4e).

(1) Primary anaerobic demethylation by a GB-specific cobalamine-dependent nonpirrolisine methyltransferase MtgB (2 dissimilatory gene copies) forming DMG + Me-cobalamine with a highest homology to the enzymes first found in GB-oxidizing *Desulfito-*

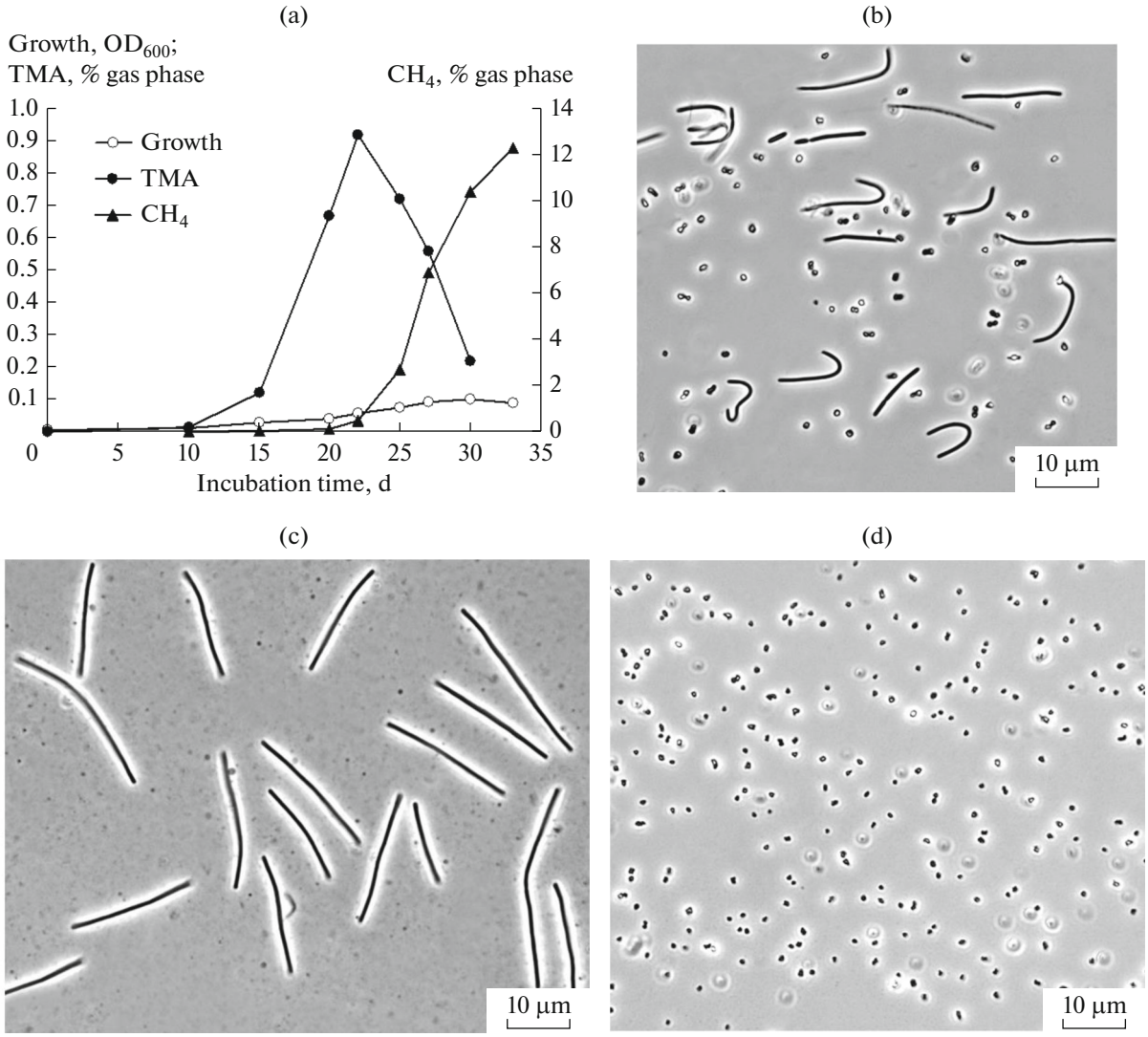

Fig. 3. Growth dynamic (a) and cell morphology (b) in a methanogenic consortium grown with GB (10 mM) at 4.0 M total Na^+ and pH 9.7 (48°C) from hypersaline soda lakes. (c) Cell morphology of pure cultures of the GB-fermenting *Natroniella* sp. ANB-GB2 and (d) cell morphology of monoarchael TMA-utilizing methyl-reducing *Methanonatronarchaeum*.

bacterium hafniense and also present in GB-utilizing methylotrophic members of *Methanosarcinales* (Ticak et al., 2014). This enzyme is also encoded in genomes of several other secondary anaerobes (acetogens and sulfate-reducing bacteria). However, *Desulfitobacterium* is only able to oxidize a single methyl group of GB forming DMG as the final product.

(2) The tetrahydrofolate (TFT)-dependent DMG demethylation to sarcosine and Me-TFT by the DMG dehydrogenase Dmg.

(3) The TFT-dependent demethylation of sarcosine to glycine and Me-TFT by a monomeric sarcosine dehydrogenase homologous to SoxB subunit of the more common heterotetrameric SoxBDAG. The enzymes of steps 2 and 3 are found in aerobic GB-degrading bacteria, such as *Arthrobacter* and *Pseudomonas* spp. (Meskys et al., 2001; Wargo, 2013), and the corresponding genes are also present in many *Halomonas* genomes. However, the necessity for oxygen as an *e*-acceptor in case of *Halalkaliarchaeum* can, apparently, be replaced by sulfur. The large genomic locus encoding enzymes of the step 2 and 3 of GB oxidation includes two additional sets of functional genes apparently encoding the enzymes assisting in the utilization of GB. One of them is the electron transfer flavoprotein complex FixAB, which is known to be involved in the electron transfer to the quinone pool during oxidative demethylation of DMG and sarcosine in mitochondria (Augustin et al., 2016; Steenkamp and Huisain, 1982). In fact, the alternative name of the DMG dehydrogenase is *N,N*-dimethylglycine:electron-transfer flavoprotein oxidoreductase. The second set encodes a full repertoir of the C_1 metabolizing enzymes typically present in methy-

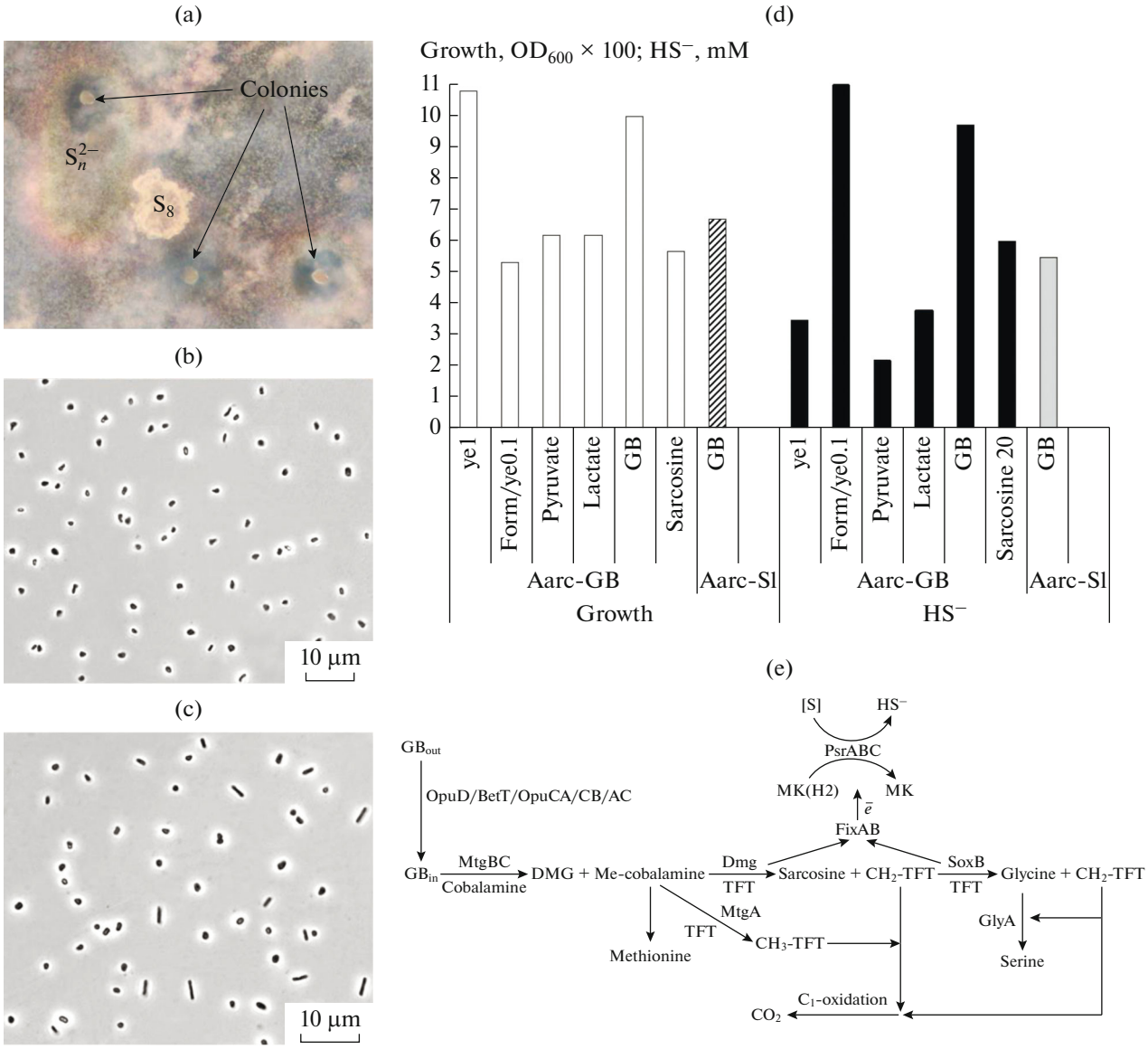

Fig. 4. *Halalkaliarchaerum* sp. AArc-GB from hypersaline lakes oxidizing GB with sulfur as electron acceptor. (a) Anaerobic colonies on GB + sulfur with a zone of polysulfide reduction; (b, c) cells of AArc-GB grown anaerobically with GB (b) or pyruvate (c) and sulfur as acceptor. (d) Maximum biomass yield and sulfide formation in the AArc-GB culture (15 d, 30°C) with GB and other substrates at 4 M Na^+, pH 9.7 and 30°C; the substrates were used at 10 mM, except for sarcosine (20 mM) and yeast extract (ye, 1 g/L). (e) Hypothetical scheme of anaerobic GB utilization by *Halalkaliarchaeum desulfuricum* AArc-Sl^T based on the genomic analysis.

lotrophs and also in the GB-utilizing *Desulfitobacterium*, which can either oxidize the TFT-bound methyl group from the demethylation steps and also channel it into assimilatory metabolism, such as the methionine biosynthesis, as has been shown for the methylotrophic *Arthrobacter* strains (Meskys et al., 2001). Moreover, the locus additionally encodes two different GB transporters: the GB-specific OpuD and a less specific choline/betaine/proline BetT. There is also a second separate copy of OpuD and a copy of the betain/choline/carnithin ABC transporter OpuCA/BD/AC. Another similarity with the *Arthrobacter* system is the presence of two copies of the glycine/serine hydroxymethyltransferase GlyA converting the final product of the GB catabolism to serine—one of the key enzymes of the methylotrophic serine cycle. Interestingly, neither the type strain AArc-Sl^T, nor AArc-GB were able to grow aerobically with GB. Most probably the reason for this is the first step of the pathway, present only in anaerobes and, hence, apparently O_2-sensitive. The full set of the proteins involved in the proposed metabolism is given in the Supplementary file.

Overall, the genus *Halalkaliarchaeum* represents

the first example of haloarchaea, and probably archaea in general, with the ability to use GB as the energy and carbon source during anaerobic sulfidogenic respiratory growth, thereby expanding the functional importance of sulfur-reducing haloarchaea in anaerobic carbon mineralization and sulfur cycling in hypersaline habitats. It is also very likely that there should be aerobic haloarchaea capable of using GB as substrate since Blast search reveal that many genes encoding aerobic GB catabolic enzymes are also present in several haloarchaeal genomes. This, however, remains to be proven on through cultivation attempts.

CONCLUSIONS

In this work a microbial network possibly involved in mineralization of GB and related compounds in hypersaline soda lakes has been uncovered. Aerobic GB degraders were represented by the genus *Halomonas* capable of complete mineralization of GB and its precursors in a wide salinity range up to soda-saturation conditions. Aerobic natronoarchaea were not enriched with GB.

Primary anaerobic GB degradation without an external *e*-acceptor was active only under hypersaline soda lake conditions (2–4 M total Na^+, pH around 10) via fermentative degradation to TMA by a member of *Halanaerobiales* (*Natroniella* sp.) with further TMA conversion to methane by extremely salt-tolerant methylotyrophic methanogens, including *Methanosalsum natronophilum* at mesophilic temperature and methyl-reducing *Methanonatronarchaeum* at moderately thermophilic conditions and in the presence of an external *e*-donor (formate or H_2). Anaerobic respiratory GB degradation was possible at salt-saturation conditions in the presence of elemental sulfur as the terminal *e*-acceptor by a novel sulfur-respiring member of the haloarchaeal genus *Halalkaliarchaeum*.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

SUPPLEMENTARY INFORMATION

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