Thiohalorhabdus methylotropha sp. nov., an extremely halophilic autotrophic methylotiotroph from hypersaline lakes

Sorokin, Dimitry Y.; Merkel, Alexander; Gebbie, William; Kalyuzhnaya, Marina G.

DOI
10.1016/j.syapm.2025.126602

Publication date
2025

Document Version
Final published version

Published in
Systematic and Applied Microbiology

Citation (APA)
Sorokin, D. Y., Merkel, A., Gebbie, W., & Kalyuzhnaya, M. G. (2025). Thiohalorhabdus methylotropha sp. nov., an extremely halophilic autotrophic methylotiotroph from hypersaline lakes. *Systematic and Applied Microbiology*, *48*(3), Article 126602. https://doi.org/10.1016/j.syapm.2025.126602

Systematic and Applied Microbiology 48 (2025) 126602

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.com/locate/syapm

Thiohalorhabdus methylotropha sp. nov., an extremely halophilic autotrophic methylotiotroph from hypersaline lakes

Dimitry Y. Sorokin [a,b,*], Alexander Y. Merkel [a], William Gebbie [c], Marina G. Kalyuzhnaya [c]

[a] *Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia*
[b] *Department of Biotechnology, Delft University of Technology, Delft, The Netherlands*
[c] *Department of Biology, San Diego University, CA 92182, USA*

ARTICLE INFO

Keywords:
Methylotrophic
Sulfur-oxidizing
Extremely halophilic
Thiohalorhabdus
Hypersaline lakes

ABSTRACT

So far, there have been no reports of trimethylamine (TMA)-utilizing extremely halophilic microorganisms in hypersaline habitats. Our aerobic enrichments at 4 M total Na^+ with 5 mM TMA inoculated with surface sediments from hypersaline soda (at pH 9.5) or chloride-sulfate (at pH 7) lakes in southwestern Siberia were successful only for the latter. The initial enrichment included both bacteria and haloarchaea but only the bacterial component was able to grow as a pure culture with TMA. Strain Cl-TMA forms a new-species lineage within the genus *Thiohalorhabdus* which includes extremely halophilic and obligate lithoautotrophic sulfur-oxidizing gammaproteobacteria. Cl-TMA can grow methyloautotrophically utilizing TMA, dimethylamine (DMA) and methanol (MeOH) as the electron donors or chemolithoautotrophically with thiosulfate. Mixotrophic growth was also observed with the three methyl compounds and thiosulfate. Carbon is assimilated autotrophically via the Calvin-Benson-Basham pathway. Unlike the type species of *Thiohalorhabdus*, *T. denitrificans*, Cl-TMA was incapable of anaerobic growth via denitrification. The isolate belongs to extreme halophiles growing between 2.5 and 5 M NaCl with an optimum at 3–3.5 M. Genome analysis identified two gene clusters coding for PQQ-dependent methanol dehydrogenases (MxaFI and XoxF), four homologues of the formaldehyde activating enzymes (Faes), a TMA/DMA oxidation locus, and two cluster of genes encoding an *N*-methylglutamate dehydrogenase pathway (NMGP) for methylamine oxidation. The first steps of C_1-subtrate conversions are followed by the tetrahydrofolate (THF)-linked and tetrahydromethanopterin (H4MPT)-linked formaldehyde oxidation pathways and two formate dehydrogenases. All of those signatures of methylotrophy were absent in *T. denitrificans*. In contrast, genes for two key sulfur oxidation enzymes, thiosulfate dehydrogenase TsdAB and sulfide dehydrogenase FccAB, that are present in the type species are missing in Cl-TMA. Thiosulfate is oxidized to sulfate by a combination of an incomplete Sox cycle and an sHdr system. Strain Cl-TMA^T (JCM 35977 = UQM 41915) is proposed to be classified as *Thiohalorhabdus methylotrophus* sp. nov.

Introduction

Anoxic sediments of hypersaline aquatic habitats such as inland lakes, marine solar salterns and temporary-spatially isolated marine lagoons in evaporative climate zones are a source of C_1-methylated compounds (methylamines and methylsulfides). These compounds are produced during anaerobic degradation of the methylated osmolytes glycine betaine and dimethylsulfoniopropionate released from cells of halophilic microbes upon hypoosmotic shock or dead cells lysis (Heijthuijsen and Hansen, 1989; Mouné et al., 1999; Oren, 1990; Zhuanga et al., 2017; Jones et al., 2019; Christman et al., 2020; Welsh, 2000). C_1-methylated amines diffusing into aerobic zone can be utilized either as carbon and energy source by a specialized group of methylotrophic bacteria or by methylotrophic autotrophs as electron donors (Chistoserdova and Lidstrom, 2013; Chistoserdova and Kalyuzhnaya, 2018).

So far, only a few methylotrophic bacteria have been shown to be able to use reduced sulfur from mercaptans or inorganic sulfur compounds as an energy source. These include several species in the genera *Methylophaga* (de Zwart et al., 1996; Kröber and Schäfer, 2019), *Methylobacterium* (Anandham et al., 2007, 2009) and *Methylovirgula* (Gwak et al., 2022). Among them, only the *Methylophaga* species are

* Corresponding author at: Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia
E-mail addresses: soroc@inmi.ru, d.sorokin@tudelft.nl (D.Y. Sorokin).

https://doi.org/10.1016/j.syapm.2025.126602
Received 25 January 2025; Received in revised form 6 March 2025; Accepted 7 March 2025
Available online 14 March 2025

moderately salt-tolerant and mostly found in marine habitats. On the other hand, recent comparative genomic analysis identified the presence of thiosulfate-oxidizing Sox cycle genes and quinone-dependent sulfide dehydrogenase (Sqr) in many genomes of methanotrophic bacteria suggesting that a combination of methylotrophic and sulfur-based lithotrophic metabolisms might occur more widely than is currently recognized (Gwak et al., 2022).

In our previous work (Sorokin et al., 2007), two moderately salt-tolerant aerobic methylotrophic gammaproteobacteria were enriched from hypersaline lakes with MeOH, the neutrophilic *Methylohalomonas lacus* from salt lakes and alkaliphilic *Methylonatrum kenyense* from a soda lake (grown optimally at 2 M NaCl and 1 M total Na^+ as carbonates, respectively). However, so far, aerobic methylotrophs growing optimally in hypersaline brines are missing in pure culture. In this work we describe the isolation, phenotypic properties and results of functional genomic analysis of the first extremely halophilic, autotrophic, (thio) methylotrophic gammaproteobacterium enriched from hypersaline lakes in southwestern Siberia belonging to a new species of the thioautotrophic genus *Thiohalorhabdus* (Sorokin et al., 2008a; Sorokin et al., 2020).

Materials and methods

Inoculum, enrichment conditions and isolation of pure culture

The top 1–2 cm layer of oxic sediments and near-bottom brines (1:4 *v*/v) were collected from three hypersaline salt lakes (Lomovoe, Crimson, Hummocky) in southwestern Siberia (Kulunda Steppe, Altai region, Russia) in July 2022. The salt concentration of the brines ranged from 230 to 360 g l^{-1} and the pH from 7.5 to 8.0. Before inoculation, the samples from different lakes were mixed in equal proportions in a 50 ml Falcon tube, homogenized by vortexing and centrifuged for 10 s at minimal speed to remove course sand particles. The residual fine particle suspension was decanted and used as an inoculum (5 % v/v).

Enrichment base salt medium included 4 M NaCl, 5 g l^{-1} KCl, 2.5 g l^{-1} K_2HPO_4 and 4 mM NH_4Cl and the final pH was adjusted to 7 with a 0.5 M KH_2PO_4 solution. After sterilization, the medium was supplemented with 2 mM Mg sulfate and 1 ml each of trace metal and vitamin mix (Pfennig and Lippert, 1966). Finally, 5 mM TMA chloride was added as substrate. One of the incubations was also supplemented with a mixture of three antibiotics (ampicillin, streptomycin and vancomycin; 100 mg l^{-1} each) to suppress bacterial growth in favor of haloarchaea. 30 ml medium portions in 115 ml screw cap bottles were incubated on a rotary shaker at 120 rpm and 35 °C until visible evidence of bacterial growth (turbidity and microscopy) after removal of sediment particles by a 1 min centrifugation in 2 ml Eppendorf tubes at 4000 rpm. The positive initial enrichment was transferred two times at 1:100 dilution to obtain a sediment-free culture. At that stage, the culture was dominated by long flexible motile rods and flat polymorphic coccoids (typical for haloarchaea), with or without antibiotics. Therefore, further purification was attempted without antibiotics by serial dilutions in the same medium. Plating into soft TMA agar (0.8–1 %) with 4 M NaCl resulted in the growth of red haloarchaeal colonies but none of them grew in liquid culture with TMA. Therefore, we focused on purifying the rod-shaped component. It did not form colonies and serial dilutions (positive up to 10^9) also did not eliminate the TMA-negative haloarchaea. Decrease in salinity to a minimal of 2.5 M (at which growth of the mixed culture was still possible) depressed growth of haloarchaea and numerical domination of the (apparently) bacterial component allowed its purification in a single dilution series. The isolate was designated strain Cl-TMA. The culture purity was verified by light microscopy, 16S rRNA gene and ultimately by genome sequencing.

Microscopy and chemotaxonomy

Phase contrast microscopy (Zeiss Axioplan Imaging 2 microscope, Göttingen, Germany) was used for routine checks and electron microscopy microscopy (Jeol JEM-1400 electron microscope, Japan) - to examine flagellation after fixing the cells with paraformaldehyde in 3 M NaCl (final concentration 2 %, *v*/v) at 4 °C for 12 h and positively staining with 1 % (*w/v*) uranyl acetate. The preparations were examined in Jeol JEM-1400 electron microscope (Japan).

The fatty acid composition was analyzed using a gas chromatograph-mass spectrometer 7890B + 5977B (Agilent Technologies, USA). The freeze-dried cells were saponificated (3.75 M NaOH/MeOH, 100 °C, 30 min), then subjected to acidic methanolysis (6 N HCl/MeOH, 80 °C, 10 min) and the products were extracted with hexane:methyl tert-butyl ether (1:1 *w*/w) followed with alkali treatment (0.3 M NaOH, 5 min). The final products were separated on a 5 % phenyl-methyl silicone capillary column HP-5MS (0.25 mm × 30 m) in a temperature gradient from 45 to 300 °C at 40 °C min^{-1}. Fatty acids were ionized by electron impact and analyzed in the scan mode. The compounds were identified using the NIST17 mass spectrometer library. The relative abundance was determined as the percentage of the total ion current peak area.

Growth physiology

Growth with different substrates was investigated in the above mentioned mineral medium containing 3 M NaCl. The following C1 methyl compounds were tested: TMA and DMA (5 mM), methylamine (10 mM), MeOH (20 mM), tetramethylammonium (5 mM) and glycine betaine (5 mM). Other organic compounds (alcohols and carbonic acids) were used at 5 mM concentration as well. For thioautotrophic growth, either thiosulfate (5–10 mM) or sulfide (2 mM with O_2 reduced in the gas phase to 2 %) + 10 mM filter-sterilized $NaHCO_3$ as the inorganic carbon source. The pH was adjusted periodically within the range of 6.5–7.0 by addition of either bicarbonate or sterile CO_2 into the gas phase within the range from 6.5 to 7. Anaerobic growth was tested either with MeOH or thiosulfate as the energy source and nitrate (5 mM) or N_2O (20 mM) as acceptors. Salinity tolerance was examined with TMA as substrate within the range of NaCl from 1 to 5 M. Incubation temperature in all tests was 35 °C.

Resting cell activity experiments

To test the metabolic potential of Cl-TMA expressed during growth with various electron donors, cells were pregrown with three C_1-methyl compounds and thiosulfate, individually or in combination, harvested by centrifugation, washed two times and finally resuspended in 3 M NaCl with 50 mM K-P buffer, pH 7. The cells were diluted in the same buffer to a cell protein concentration of 0.1 mg ml^{-1} and their substrate-oxidizing potential was investigated in two ways: 1 – by substrate-dependent dO_2 consumption using a Biological Oxygen Monitor (Yellow Spring Instruments, USA) with 100 μM substrate addition; 2 – by measuring CO_2 production rates from oxidation of C_1 compounds (5 ml cell suspensions incubated in 23 ml serum bottles closed with butyl rubber stoppers). Results were compared with substrate-free (endogenous) controls.

Genome sequencing, phylogenomic analysis and functional genomics

For the primary identification, the genomic DNA from strain Cl-TMA was extracted by alkaline hydrolysis at 60 °C and purified using Wizard (R) MiniColumns (Promega, USA). The 16S rRNA gene was amplified with 3 primers (11f, 530f and 926f) and sequenced by the Sanger method. For the whole genome sequencing, genomic DNA was extracted with the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). A shotgun WGS library preparation and sequencing were performed using KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, UK) and NovaSeq 6000 system (Illumina, San Diego, CA, USA). The genome was assembled with SPAdes version 4.00 (Prjibelski et al., 2020) and submitted for automatic annotation to the Procaryotic Genome Annotation

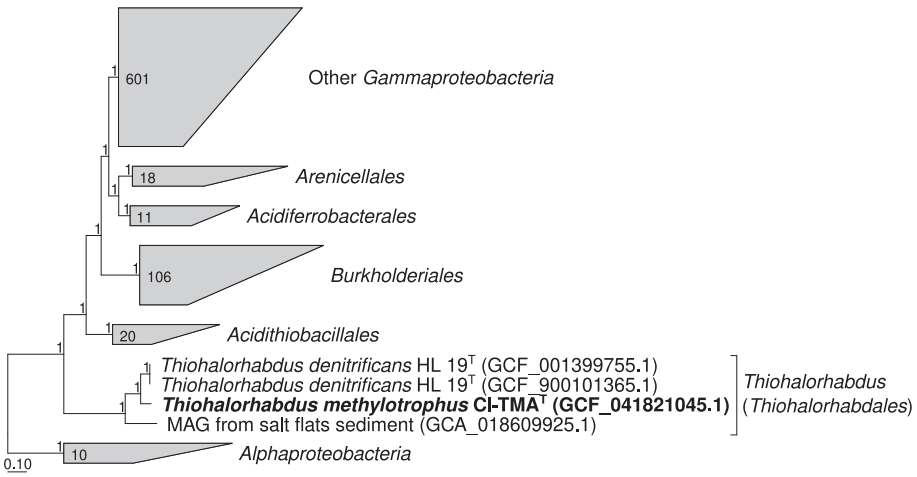


Fig. 1. Phylogenetic placement of strain Cl-TMA within the class *Gammaproteobacteria* based on concatenated amino acid sequences of 120 bacterial single copy conserved marker proteins. The length of the alignment is 23,192 aa. Bootstrap consensus tree is shown with values placed at the nodes. Bar, 0.1 change per position.

Pipeline (Tatusova et al., 2016) in GenBank. The draft genome (coverage x 277) of strain Cl-TMA is 4.6 Mbp and consisted of 32 contigs, 3931 genes encoding 3826 proteins and a single set of rRNA genes. The genome assembly accession number in the GenBank is GCF_041821045.

The genome-based phylogeny was reconstructed using 120 single copy conserved bacterial marker proteins according to the Genome Taxonomy Database (Rinke et al., 2021). These were aligned with GTDB-Tk v2.4.0 (Chaumeil et al., 2022) and trimmed with trimAl 2.rev0 build 2019-08-05 using "-automated1" and "-gt 0.99" modes (Capella-Gutiérrez et al., 2009) producing 23,192 aa length fragments. The tree was built with the IQ-TREE2 program v2.2.0.3 (Minh et al., 2020) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation (Minh et al., 2013) as well as approximate likelihood-ratio test for branches (Anisimova and Gascuel, 2006). The whole genome comparison included Average Nucleotide Identity (ANI), using Pyani 0.2.12 (Pritchard et al., 2016); Average Amino acid Identity (AAI) by the EzAAI v1.1 (Kim et al., 2021) and digital DNA-DNA hybridization (DDH) by the Genome-to-Genome Distance Calculator 3.0 online tool (http://ggdc.dsmz.de/ggdc.php) using recommended formula - identities/high-scoring segment pairs (HSP) length.

Functional genome analysis of key metabolic markers in strain Cl-TMA included methylotrophy, sulfur oxidation, autotrophy and halophilic adaptation using reference proteins encoded in *Methylophilus methylotrophus* and the type species of *Thiohalorhabdus* for Blast search in the UniProt.

Analyses

Biomass growth was monitored by measuring OD_{600}. Cell protein in the cell suspension experiments was determined by the Lowry method (Lowry et al., 1951). Thiosulfate and tetrathionate concentrations were quantified by spectrophotometry at 460 nm after cyanolytic conversion to thiocyanate and reaction with Fe(III) (Kelly et al., 1969). Sulfate was determined by a turbidimetric method following conversion to $BaSO_4$ at acidic conditions after complete oxidation of thiosulfate. CO_2 production was measured by gas chromatography using Chromatek 5000.1 GC (Ufa, Russia) equipped with a methanator, flame ionization detector and a Hayesep column (80–100 mesh 2 m × 3 mm, 40 °C; carrier gas Ar).

Results and discussion

Phylogenetic analysis and classification

Sanger sequencing of 16S rRNA gene identified Cl-TMA as a member of the genus *Thiohalorhabdus* (class *Gammaproteobacteria*) (98.8 % sequence identity to the type strain of the genus) (Supplementary Fig. S1). Until now, this genus included multiple closely related isolates of

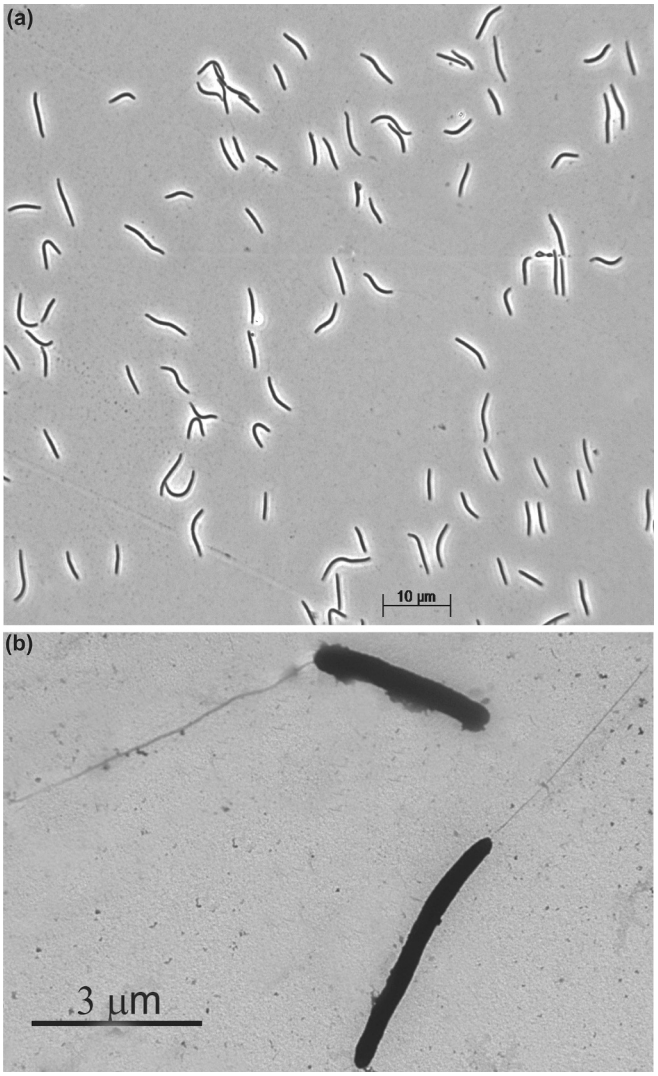


Fig. 2. Cell morphology of strain Cl-TMA grown with trimethylamine at 3 M NaCl. (**a**), phase contrast microphotograph; (**b**), transmission electron microscopy showing polar flagellation.

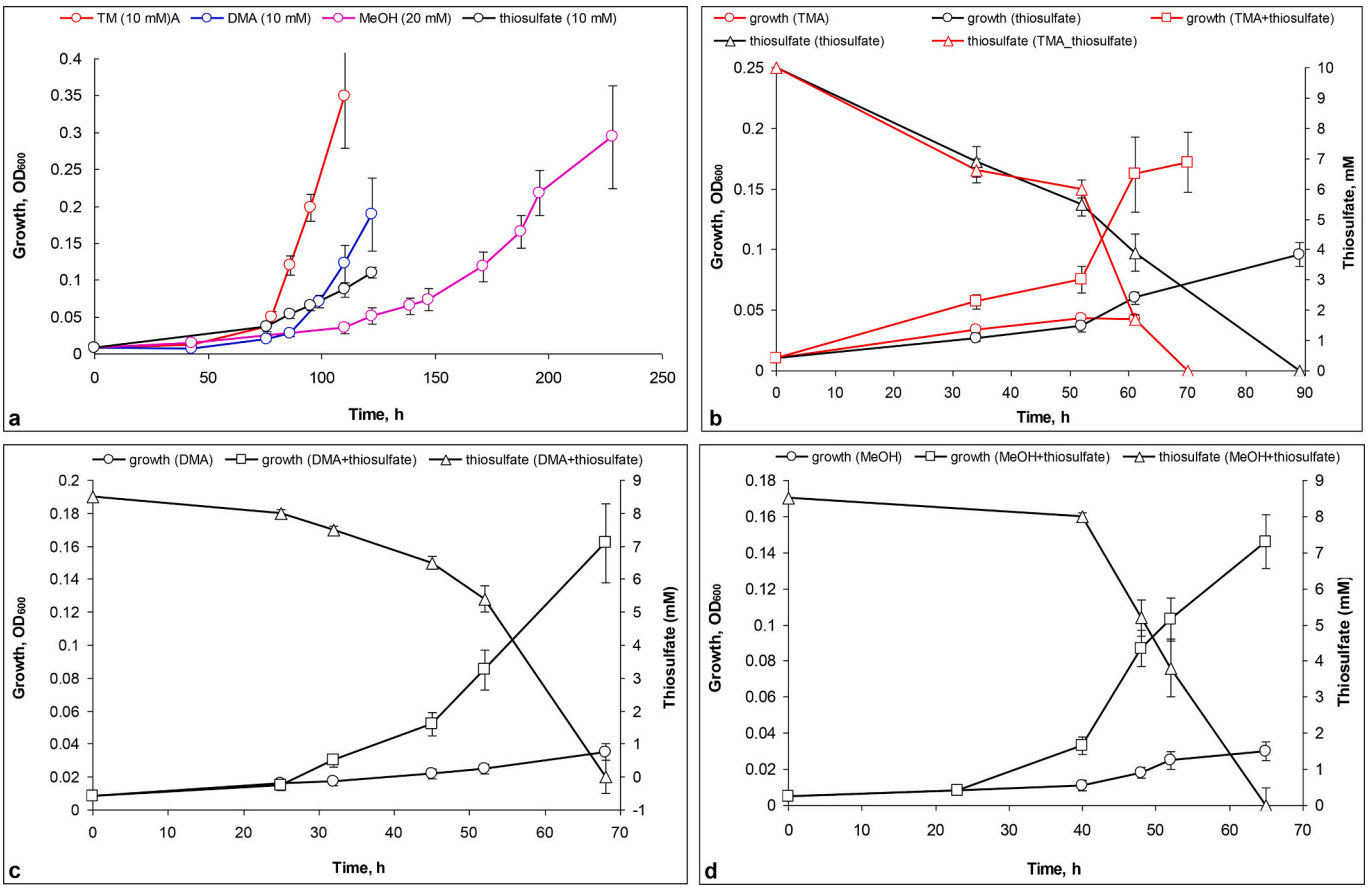


Fig. 3. Growth dynamics of strain Cl-TMA at 3 M NaCl and 35 °C. (**a**), comparative growth dynamics of methylo-autotrophic and thio-autotrophic growth; (**b-d**) – comparative growth dynamic at mixotrophic conditions with 3 C_1-methyl compounds (2 mM) and thiosulfate (8.5–10 mM). The pH was controlled within 6.5–7.0 by periodic addition of $NaHCO_3$. The presented results are from 3 replicate experiments.

extremely halophilic and obligately thioautotrophic bacteria from hypersaline lakes and solar salterns forming the type species *T. denitrificans* (Sorokin et al., 2008a; Sorokin et al., 2020). Among the 90 16S rRNA gene sequences assigned to the genus *Thiohalorhabdus* in the Silva 138.2 database (Quast et al., 2013), only one clone (GQ894431) found in the sediments of a hypersaline lake (Hollister et al., 2010) had more than 99 % identity (in its 422 bp fragment) to the 16S rRNA gene of Cl-TMA. Phylogenetic reconstruction based on 120 bacterial conserved protein markers (Parks et al., 2018) confirmed that the new isolate belongs to the genus *Thiohalorhabdus* at the level of a new species (Fig. 1). The estimated values of ANI, AAI and the DDH with *Thiohalorhabdus denitrificans* HL 19^T were 85.84, 80.01 and 25.10 %, respectively.

Cell morphology and chemotaxonomy

Cells of Cl-TMA are relatively long slender rods, sometimes twisted or even looped and actively motile with a single polar flagellum (Fig. 2). The latter differentiates the isolate from the type species of the genus *Thiohalorhabdus* whose cells are nonmotile straight rods. The dominant polar lipid fatty acids included (in order of abundance) 10Me-C17:0, C16:0 and *iso*C12:0 (Supplementary Table S1). The major difference to the type species was in the methylated compound identified as 10Me-C16:0 in *T. denitrificans* HL19 (Sorokin et al., 2008a). Interestingly, the same type of variation was observed between closely related strains of extremely halophilic sulfur-oxidizing gammaproteobacteria belonging to the genus *Thiohalospira*: in the type strain HL3 the dominant compound was identified as 11Me-C17:1, while in strain HL4 – 10Me-C16:0 (Sorokin et al., 2008b). Notwithstanding the variations, the dominance of methylated C16-C17 FA in polar lipids in this bacteria seems to be correlating with their extreme halophily.

Growth physiology

Since Cl-TMA was enriched on TMA as the only carbon and energy source, the pure culture was first examined for its methylotrophic potential with other C_1 methyl compounds, common for saline habitats, including, DMA, MA, tetramethylammonium, MeOH, and formate. Growth was observed with TMA, DMA and MeOH (in order of growth rate) (Fig. 3a). A much slower growth was also detected with MA, but it was not stable in transfers and, therefore, was not investigated further. Judging from the biomass formed per one mM of methyl group (calculated for MeOH), more than 2 methyls from TMA and more than 1 methyl from DMA should have been utilized. None of the other tested organic compounds (see Methods) supported growth, indicating that Cl-TMA is an obligate methylotroph.

Next, a chemolithoautotrophic potential of Cl-TMA was investigated (as a member of the sulfur-oxidizing genus *Thiohalorhabdus*), using thiosulfate and sulfide (at microoxic conditions) as the electron donors and HCO_3^- as the only carbon source. The results were positive for thiosulfate (Fig. 3a), although, in contrast to the type species *T. denitrificans*, thiosulfate was oxidized to sulfate directly without intermediate tetrathionate formation.

From the above results, the most interesting question was whether methylated compounds and thiosulfate could be used by Cl-TMA simultaneously for mixotrophic growth, which had never been shown before for hypersaline conditions. The results demonstrate that it depended on the concentration of methyl compound (Fig. 3b–d). At 10 mM for all three methylated compounds supporting growth of Cl-TMA,

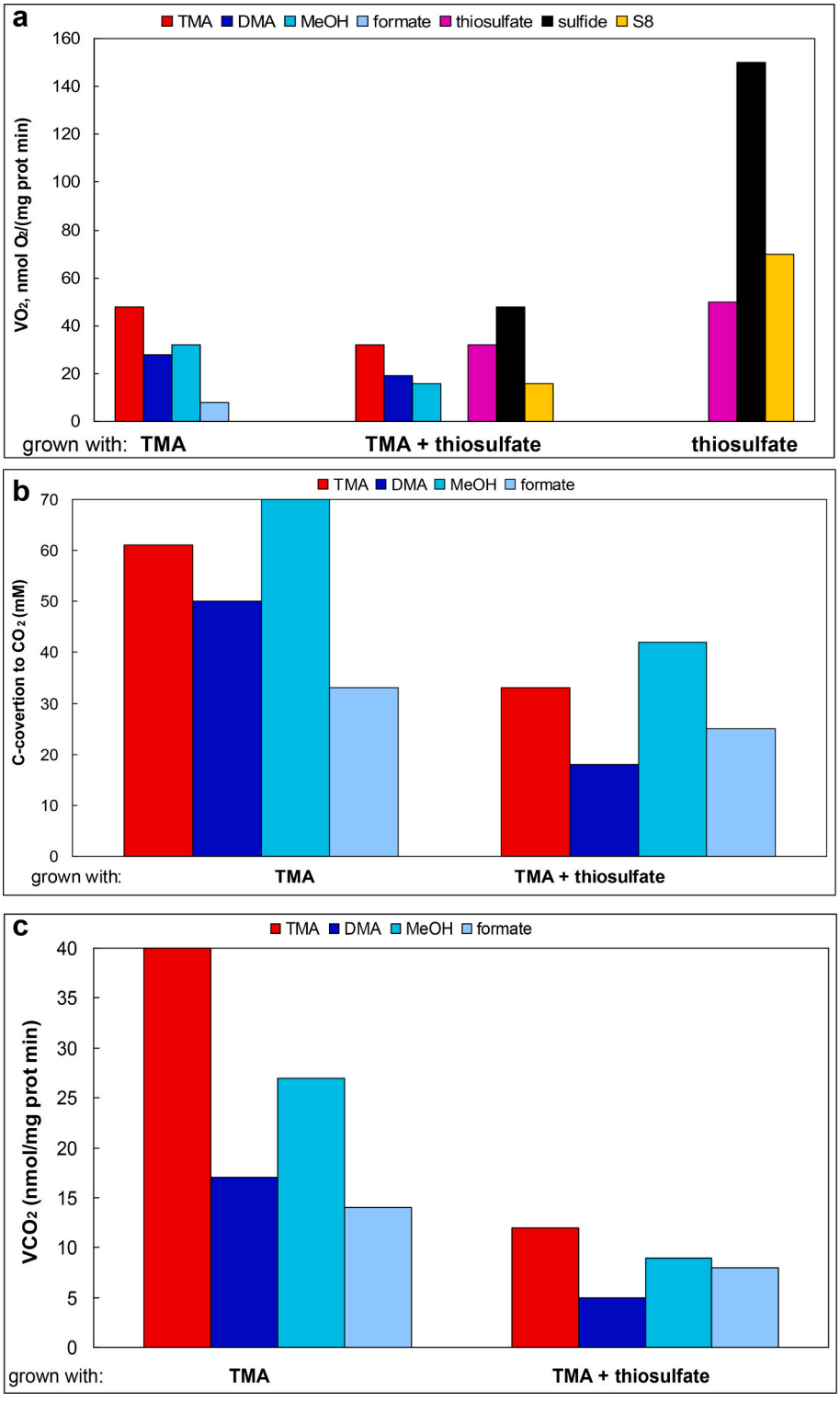


Fig. 4. Metabolic potential of washed cells of strain Cl-TMA at 3 M NaCl, pH 7 and 35 °C pregrown with different substrates. (**a**) – activity of substrate-dependent dO_2 consumption of cells grown methyl-autotrophically with TMA, lithoautotrophically with thiosulfate and mixotrophically with TMA and thiosulfate. There was no activity for sulfur compounds oxidation in the cells grown methylotrophically with either TMA or DMA, and, vice versa – no activity for oxidation methyl compounds in the cells grown with thiosiulfate. (**b-c**) – mineralization activity (CO_2 release) during oxidation of C_1 compounds by cells grown either with TMA +/−thiosulfate (**b**) or DMA +/−thiosulfate (**c**). Incubation time – 51 h, cell protein concentration – 0.1 mg ml^{-1}. The endogenous control values were subtracted. The respiration test was a single run, and the CO_2 formation activity tests are mean values from 2 replicate experiments, with the deviations ranged from 4 to 18 %.

Table 1
Properties of strain Cl-TMA comparative to *Thiohalorhabdus denitrificans* HL19^T.

Property	**Cl-TMA**	*Thiohalorhabdus denitrificans*
Cell morphology	Long motile flexible rods 0.3–0.5 × 3–10 μm	Long nonmotile flexible rods 0.3–04 × 3–12 μm
Thioautotrophic growth with:		
thiosulfate	+ (thiosulfate>sulfate)	+ (thiosulfate>tetrathionate>sulfate)
sulfide	-	+ (sulfur is the main product)
thiocyanate	-	+* (w) (CNS^- > sulfate)
Methyloautotrophic growth	+ [TMA, DMA, MA (w), MeOH]	-*
Denitrification	–	+ (NO_3^- > NO_2^-; N_2O > N_2) with thiosulfate or sulfide as *e*-donors
Autotrophy	obligate (CBB cycle)	obligate (CBB cycle)
Salinity range (opt.) M NaCl	2.5–5.0** (3.0–3.5)	2.0–5.0 (3.0)
Predominant polar lipid fatty acids	10Me-C17:0, C16:0, *iso*C12:0	10Me-C16:0, C16:0, C16:1ω7
Genome size (Mbp)	3.7	2.9
G + C (%, whole genome)	67.0	68.9
Habitat	hypersaline salt lakes	hypersaline salt lakes and solar salterns

(w), weak growth; TMA, trimethylamine; DMA, dimethylamine; MA, monomethylamine.
* This work.
** max. NaCl for growth on MeOH.

thiosulfate oxidation was initially blocked and only commenced when the methylated substrate was consumed, resulting in a diauxic, rather than mixotrophic growth. However, when the concentration of methyl substrate was reduced to 2 mM, thiosulfate oxidation started during the early exponential growth phase and, judging from the final biomass yield, resulted in simultaneous thio-methylotrophy. This was in contrast to what was reported for the mixotrophic growth of *Methylobacterium* species on MeOH + thiosulfate and for the methanotrophic *Methylovirgula*, whereby the C_1 methyl compound did not inhibit thiosulfate oxidation even at high concentrations (Anandham et al., 2007, 2009; Gwak et al., 2022). However, Cl-TMA also showed one important difference in its mixotrophy from the methylotrophs mentioned above: at limited concentration of methyl compounds, the growth yield on two substrates exceeded the sum of two individual yields (from 16 % on MeOH to 24 % on TMA-DMA) (Fig. 3b–d). This indicated that at low concentrations of C_1 compounds Cl-TMA was energy-limited. Translating to *in-situ* conditions, such behavior would be more important for a mixotrophic organism than a simple metabolic variability in competition with either obligate methylotrophs or sulfur-oxidizing lithoautotrophs.

To test the mixotrophic potential of Cl-TMA further, the catabolic activity of washed cells pre-grown with various substrates alone or in combination was investigated, either by measuring their substrate dependent respiration for both methyl and sulfur electron donors in cells grown on TMA and thiosulfate or TMA+ thiosulfate (Fig. 4a), or carbon mineralization to CO_2 from the C_1 compounds in cells grown with TMA and DMA alone in comparison to cell grown mixotrophically (Fig. 4b–c). Both methods showed that cells grown with solely methyl compounds did not express sulfur-oxidizing potential, and vice versa, while cells grown mixotrophically were active with both C_1-methylated and sulfur compounds, albeit both types of activity were lower in comparison with the cells grown with one type of the respective substrates. The cells grown with TMA were inactive with MA, despite that slow growth with this compound did happened. The TMA-grown cells showed a low level of formate-dependent O_2-consumption which was absent in the mixotrophic cells (Fig. 4a), while in the CO_2-production tests both TMA and TMA + thiosulfate grown cells showed substantial CO_2 formation from formate (Fig. 4b–c). Perhaps, in the absence of a membrane-bound respiratory FDH, this extremely halophilic bacterium does not generate enough energy for formate-dependent autotrophic growth.

The *T. denitrificans* strains all can grow anaerobically with nitrate or N_2O as the electron acceptors. Therefore, Cl-TMA was also tested for such ability using nitrate or N_2O as acceptors with TMA, MeOH or thiosulfate as the electron donors. The results were negative in all combinations indicating another profound difference in physiology of the new *Thiohalorhabdus* species from the type species.

Salinity profiling with MeOH or TMA as substrates demonstrated that strain Cl-TMA belongs to extreme halophiles with the NaCl range for growth between 2.5 and 4.75–5.0 M and an optimum at 3.0–3.5 M, thus representing a first extremely halophilic bacterium among the so far described aerobic methylotrophs (Supplementary Fig. S2). At 3 M NaCl and MeOH as substrate, it grew within the pH range from 6.2 to 8.0 with an optimum at 6.8–7.2. The maximum temperature for MeOH-dependent growth at 3 M NaCl and pH 7 was 43 °C.

Phenotypic comparison of Cl-TMA and the type species of the genus *Thiohalorhabdus* is presented in Table 1.

Functional genome analysis

Methylotrophy

We identified four clusters encoding methyl-oxidizing potential in the genome of Cl-TMA (which are lacking in its closest relative *T. denitrificans*). Two of them are responsible for MeOH oxidation to formaldehyde, one – for TMA/DMA oxidation to formaldehyde and the fourth one – for oxidation of formaldehyde to CO_2 (Table 2). The methanol oxidation loci code for two different types of methanol dehydrogenases and their assemblage chaperons: a two-subunit Ca-dependent methanol dehydrogenase (MxaFI) and a monosubunit lanthanide-dependent methanol dehydrogenase (known as XoxF). However, an equimolar mixture of four light lantanides (La^{3+}/Ce^{3+}/Nd^{3+}/Sm^{3+}) supplementation did not show any stimulating effect during growth on MeOH, while the growth was clearly stimulated by addition of Ca^{2+} (Supplementary Fig. S3). The TMA/DMA oxidation locus includes a FAD-containing TMA/DMA dehydrogenase (Tmd/Dmd) and the membrane-bound EtfAB complex transferring electrons from Tmd/Dmd to quinone. This cluster is highly similar to those present in the genome of methylated amines-utilizing facultatively methylotrophic betaproteobacteria – *Methylophilus methylotrophus* (Lin et al., 2021) and *Methyloversatilis* spp. (Latypova et al., 2010). The genes for Cu-dependent methylamine dehydrogenase are absent in Cl-TMA. Instead, the TMA/DMA utilization gene cluster preceded and followed by a set of genes known to encode the *N*-methylglutamate pathway for methylamine oxidation (NMGP), which has been well characterized in beta- and alpha-proteobacterial methylotrophs (Latypova et al., 2010; Chen et al., 2010; Nayak and Marx, 2014). The pathway included the *N*-methylglutamate synthetase (MgsABC), *N*-methylglutamate dehydrogenase (MgdABCD) and gamma-glutamylmethylamide synthetase (GmaS), followed by a putative ammonia transporter (Amt), predicted to transport methylamine or export an access of ammonium. The genome also includes an additional NMGP cluster, and a cluster gene

Table 2
Functional genome comparison of methylo-thiotrophic *Thiohalorhabdus* Cl-TMA and thiotrophic *Thiohalorhabdus denitrificans* HL19^T. H4MPT -tetrahydromethanopterin; THF – tetrahydrofolate. The key catalytic subunits are in bold.

Enzyme	Protein	Locus	
		Cl-TMA WP_37365+	**HL19**
	Methylotrophy		
Methanol dehydrogenase cluster 1:			–
methanol dehydrogenase maturation	MoxJ1	5854	
methanol dehydrogenase	XoxF	5856	
MDG-PQQ biogenesis locus	DsbL/SoxZY/PggBCD	5857–5864	
Methanol dehydrogenase cluster 2:			–
methanol dehydrogenase	MxaF	6980	
methanol dehydrogenase maturation	MoxJ2	6981	
cytochrome *c* (L) (*e*-acceptor)	MoxG	6982	
MA/DMA/TMA utilization clusters:			–
MA-utilization (1):			
N-methylglutamate dehydrogenase (1)	MgdABCD (2)	4326–4329	
γ-glutamylmethylamide synthase (1)	GmaS (1)	4330	
N-methylglutamate synthase (1)	MgsABC (1)	4331–4333	
ammonium/MA transporter	Amt	4334	
MA-utilization (2):			
γ-glutamylmethylamide synthase (2)	GmaS (2)	5997	
N-methylglutamate dehydrogenase (2)	MgdABCD (2)	5998–6000/6007	
MA-utilization (3):			
γ-glutamylmethylamide synthase (3)	GmaS (3)	5511	
N-methylglutamate synthase (3–4)	MgsC (3–4)	5512/6068	
DMA/TMA utilization:	EtfAB	6004–6005	
electron transfer flavoproteins (electron acceptor)	Dmd/Tmd (1)	6008	
DMA/TMA dehydrogenase (formaldehyde-forming)	CntB	6009	
TMA-forming carnitine oxygenase (reductase)			
DMA/TMA dehydrogenase (formaldehyde-forming)	Dmd/Tmd (2)	7237	
	C_1 pathways		
formaldehyde activating enzyme (H4MPT-dependent)	Fae	4828/5871/5973/6954	–
H4MPT-C1-transfer pathway	Mch	5976	
methenyl-H4MPT cyclohydrolase	PylC	5977	
H4MPT-C_1 transfer protein	MtdB	5978	
NAD(P)-dependent methylene-H4MPT dehydrogenase	FhcABCD	5980–5983	
Formyltransferase/hydrolase	Pcd	5984	
4a-hydroxy-H4MPT dehydratase (H4MPT biosynthesis)			
	FolD	4763	
THF-C1-transfer pathway	PurU	4276	
methylene-THF dehydrogenase/methenyl-THF cyclohydrolase	MTHFS	7210	
formyl-THF deformylase	MetF	6237	
5-formyl-THF cycloligase/5,10-methenyl-THF synthetase			
NAD(P)-dependent methylenetetrahydrofolate reductase			
NAD-dependent W-formate dehydrogenase	FwdAB	5985–5986	–
cytoplasmic formate dehydrogenase	FdsABCDG	6609–6614	
	Autotrophy (CBB cycle)		
RuBisCO activator	CbbQ	5490	SCX74879
RuBisCO (1)	CbbSL(1)	5488–5489	SCX74886-894
carboxysomal shell proteins	CcsA1A2	5497–5498	SCX74900-912
carboxysomal proteins	CcsAB	5499–5500	SCX74922-928
carboxysomal shell proteins	CcsS2S3	5501–5502	SCX7493-941
RuBisCO (2)	CbbSL(2)	5503–5504	SCX75038-048
bicarbonate transporter (high affinity)	DabAB(1)	5492–5493	SCX74853-864
	DabAB(2)	7227; 7229	SCX74976-986
sulfate/bicarbonate transporter (low affinity)	SulP	4887	SCY22057; SCY64866
	Sulfur oxidation		
thiocyanate dehydrogenase	TcDH	–	SCY46626
tetrathionate-forming thiosulfate dehydrogenase	TsdAB	–	SCX94285-286
sulfide dehydrogenase (cytochrome *c* dependent)	FccAB	–	SCY46923-963
sulfide-quinone reductase cluster1	Sqr1/TusA/DsrEFH	3541/3555–56	SCY34869-871
sulfide-quinone reductase cluster2	Sqr2/quinol oxidase CydAB	4813–4815	SCY63817/835-860
thiosulfate oxidation proteins, incomplete Sox cycle	SoxAXYZB	4640–4644	SCY37851-8051
sulfite dehydrogenase (quinone-dependent)	SoeABC	4652–4654	SCY37501/7533-75619
sHDR/lipamide sulfur oxidation locus	GlpE/TusA/DsrEFH/HdrB1AC1B2/DsrE/HdrH	4655–4673	SCY37115-SCY37501
	LipS1S2T/DLDH/LplAJ/HdrC2	4842	

(*continued on next page*)

Table 2 (*continued*)

Enzyme	Protein	Locus	
		Cl-TMA WP_37365+	HL19
	Denitrification		
membrane dissimilatory nitrate reductase cluster 1	NarG1H1I1/J1L/K1K2	–	SCY35030-134
membrane dissimilatory nitrate reductase cluster 2	NarG2H2I2/J2	–	SCY35179-229
nitric oxide reductase (cytochrome *c*)	NorBC/EQD	5612–5616	SCY61379-471
nitrous oxide reductase (clade II) cluster	NosZ/LYFD/R	4820–4825	SCY63944-4054

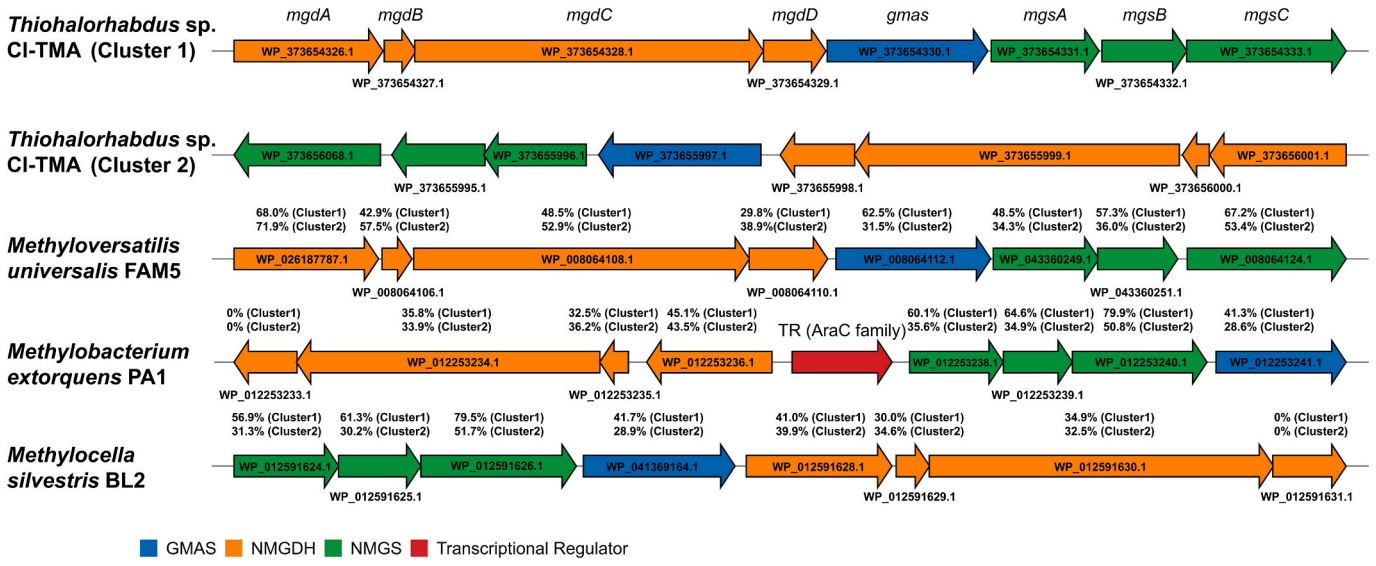


Fig. 5. Synteny among the *N*-methylglutamate pathway gene clusters in *Thiohalorhabdus* Cl-TMA and other methylotrophic bacteria, including *Methyloversatilis universalis* FAM5, *Methylobacterium extorquens,* and *Methylocella silvestris*. Contrary to other studied methylotrophs, the genome of C1-TMA encodes two similar but not identical clusters genes. NMGDH – *N*-methylglutamate dehydrogenase; NMGS – *N*-methylglutamate syntethase; GMAS - γ-glutamylmethylamide synthase.

homologues encoding glutamate and glutamine synthetases. The structure of both NMGP gene clusters in C1-TMA are identical to *Methyloversatilis universalis* FAM5 (Fig. 5). The NMGP generates methynyltetrahydrofolate (THF), which is further oxidized to CO_2 via the THF mediated C1-transfer.

The genes essential for the oxidative version of the THF pathways were also identified, including the bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase (FolD) and the formyltetrahydrofolate deformylase (purU) Genes essential for oxidation of formaldehyde via the H4MPT-dependent cascade are also present. The formate oxidation machinery comprised of a cytoplasmic formate dehydrogenase FdsABCDG and a tungsten-dependent formatted dehydrogenase (FwdAB) (Table 2).

The genome of Cl-TMA does not contain markers for two known methylotrophic pathways of formaldehyde assimilation (such as the ribulose monophosphate and the serine cycles). Instead, Cl-TMA assimilates CO_2 via a carboxysomal variant of the CBB cycle, similar to the lithoautotrophic *T. denitrificans* (Table 2).

The inorganic sulfur oxidation potential of strain Cl-TMA is similar to what is present in the nonmethylotrophic type species *T. denitrificans* except for the absence of three key enzymes: cytochrome *c*-dependent sulfide dehydrogenase (FccAB), tetrathionate-forming thiosulfate dehydrogenase (TsdAB) (explaining the absence of tetrathionate as an intermediate product of thiosulfate oxidation) and thiocyanate dehydrogenase (TcDH) (Table 2). The latter presence was overlooked during the original characterization of *T. denitrificans* type strain HL19, while in this work we confirmed that it can indeed grow chemolithoautotrophically with thiocyanate as the energy source after gradual adaptation via mixotrophy on thiosulfate + thiocyanate. Overall, the sulfur oxidation pathway in Cl-TMA includes oxidation of sulfide to sulfur via a sulfide-quinone oxidoreductase Sqr, followed by sulfur oxidation to sulfite via a sHdr system (Koch and Dahl, 2018), and final sulfite oxidation to sulfate by a cytoplasmic quinone-dependent SoeABC. Oxidation of thiosulfate to sulfate and sulfur proceeds via the incomplete Sox cycle.

Cl-TMA tested negative for anaerobic growth by denitrification with nitrate, either with MeOH or thiosulfate as the electron donors. This result is in line with the absence of genes for known dissimilatory nitrate and nitrite reductases, while the type species of *Thiohalorhabdus* has two membrane-bound NarGHIJ loci and can grow anaerobically with nitrate. Furthermore, despite the presence of a N_2O-reductase operon, Cl-TMA was also unable to grow anaerobically with N_2O.

For aerobic respiration, the isolate has four different terminal oxidases, including 2 quinol oxidases (a type *bd* in tandem with one of the two copies of Sqr, and a type ba_3), and two heme-copper cytochrome *c* oxidases encoded in a single genomic locus, of the aa_3 and the cbb_3 families. The genomes of both Cl-TMA and *T. denitrificans* HL19 feature a full repertoire of genes for ubiquinone UQ8 biosynthesis pathway (UbiACDEFGHIX).

The genomic markers for potential halophilic adaptation of Cl-TMA are listed in Supplementary Table S2. The cation transporters include three multisubunit proton antiporters, two for Na^+ and one for K^+; four single-subunit sodium/potassium:proton antiporters and a Ca^{2+}:Na^+ antiporter. The osmotic balance is apparently based on glycine betaine, either exported by three types of single-subunit transporters and a 3-subunit ABC transporter, or synthesized *de novo* by a sequential methylation of glycine via sarcosine and dimethylglycine by two *N*-methyltransferases (Nyyssölä et al., 2001). Those are also present in the type species *T. denitrificans,* where production of glycine betaine was confirmed by direct chemical analysis (Sorokin et al., 2008a).

Table 3
Description of *Thiohalorhabdus methylotropha* sp. nov.

Parameter	
Genus name	*Thiohalorhabdus*
Species name	*methylotropha*
Status	sp. nov.
Type species	no
Description of a new taxon	*methylotropha* [me.thy.lo.tro'pha. N.L. neut. n. *methylum*, methyl radical; N.L. neut. n. *methyl*, pertaining to the methyl radical; Gr. masc. adj. *trophos*, feeds; N.L. fem. adj. *methylotropha*, methyl-consuming] Cells are Gram-negative long motile rods, sometimes curved, 0.3–0.5–10.0 μm, motile by a single polar flagellum. The major polar lipid fatty acids include 10Me-C17:0, C16:0, and *iso*C12:0. Strictly aerobic autotroph utilizing either thiosulfate or C_1-methylated compounds (trimethylamine, dimethylamine methylamine and methanol) as the electron donors. Can grow mixotrophically with methyl compounds and thiosulfate. Extremely halophilic, with the NaCl range for growth from 2.5 to 4.75–5.0 M (optimum at 3.0–3.5 M), neutrophilic (pH optimum at 7.0) and mesophilic (max. growth temperature at 43 °C). The G + C content of the genomic DNA is 67.0 % (whole genome sequence). The type strain, Cl-TMA (JCM 35977 = UQM 41915), was isolated from a mix sample of surface sediments and brines from hypersaline salt lakes in Kulunda Steppe (Altai, Russia).
Type strain	Cl-TMA
Culture collection numbers	JCM 35977; UQM 41915
Genome status	Draft
GenBank genome assembly	GCF_041821045
Genome size (Mbp)	4.6
16S-rRNA	PQ638356
Country of origine	Russian Federation
Region	Altai
Source of isolation	Surface sediments from soda lakes
Latitude	N52°06'/N51°37'/N51°40'
Longitude	E79°09'/E79°50'/ E79°54'
Sampling date	July 2022
pH of the samples	7.5–8.2
Salinity of the sample	230–340
Number of strains in study	1

Conclusion

Strain Cl-TMA represents the first example of extremely halophilic aerobic TMA-utilizing methylotroph isolated from a hypersaline habitat. Its most interesting property is the ability to grow autotrophically by oxidation of C_1-methylated and inorganic sulfur compounds alone or in combination, resulting in a methylo-thio-mixotrophy. Our simultaneous attempts to enrich such methylotrophs from hypersaline soda lakes at 4 M total Na^+ (as carbonates) at pH 9.5 failed, probably because of the acute toxicity of TMA in its unprotonated form at highly alkaline conditions. Another negative outcome is the failure to enrich methylotrophic haloarchaea even after several pretreatments of the inoculum with antibiotics. They might still be there, but a different approach needs to be considered to tease them out in front of the more competitive extreme halophilic gammaproteobacteria, for example by using salinity above 5 M limit of extremely halophilic methylotrophic bacteria like *Thiohalorhabdus* Cl-TMA.

On the basis of distant phylogenetic position and unique phenotypic properties, the extremely halophilic methylothiotrophic strain Cl-TMA is proposed to be classified in the genus *Thiohalorhabdus*, as *Thiohalorhabdus methylotropha* sp. nov. The new species protologue is presented in Table 3.

Amended description of the genus *Thiohalorhabdus* Sorokin et al. 2008

In addition to the original description of the genus *Thiohalorhabdus* Sorokin et al. (2008), some of the strains can use thiocyanate and C_1-methylated compounds as the electron donors for autotrophic growth.

Funding information

DYS and AYM were supported by the Russian Ministry of Higher Education and Science. DYS was also supported by the Gravitation-SIAM Program of the Dutch Ministry of Education and Sciences (grant 24002002). MK received support from U.S. Department of Energy (DOE), contract DE-SC0024289.

CRediT authorship contribution statement

Dimitry Y. Sorokin: Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Alexander Y. Merkel:** Writing – original draft, Methodology, Investigation, Formal analysis. **William Gebbie:** Methodology. **Marina G. Kalyuzhnaya:** Writing – original draft, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2025.126602.

Data availability

The sequences are deposited in the GenBank open to public

References

Anandham, R., Indiragandhi, P., Madhaiyan, M., Kim, K., Yim, W., Saravanan, V.S., et al., 2007. Thiosulfate oxidation and mixotrophic growth of *Methylobacterium oryzae*. Can. J. Microbiol. 53, 869–876.
Anandham, R., Indiragandhi, P., Madhaiyan, M., Chung, J., Ryu, K.Y., Jee, H.J., 2009. Thiosulfate oxidation and mixotrophic growth of *Methylobacterium goesingense* and *Methylobacterium fujisawaense*. J. Microbiol. Biotechnol. 19, 17–22.
Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.
Capella-Gutiérrez, S., Silla-Martínez, J.M., Gabaldón, T., 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics (Oxford, England) 25, 1972–1973.
Chaumeil, P.A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2022. GTDB-Tk v2: memory friendly classification with the genome taxonomy database. Bioinformatics (Oxford, England) 38, 5315–5316.
Chen, Y., Scanlan, J., Song, L., Crombie, A., Rahman, M.T., Schäfer, H., et al., 2010. γ-Glutamylmethylamide is an essential intermediate in the metabolism of methylamine by *Methylocella silvestris*. Appl. Environ. Microbiol. 76, 4530–4537.
Christman, G.D., León-Zayas, R.I., Summers, Z.M., Biddle, J.F., 2020. Methanogens within a high salinity oil reservoir from the Gulf of Mexico. Front. Microbiol. 11, 570714.
Gwak, J.-H., Awala, S.I., Nguyen, N.-L., Yu, W.-J., Yang, H.-Y., von Bergenb, M., 2022. Sulfur and methane oxidation by a single microorganism. PNAS 119, e2114799119.
Heijthuijsen, J.H.F.G., Hansen, T.A., 1989. Anaerobic degradation of betaine by marine *Desulfobacterium* strains. Arch. Microbiol. 152, 393–396.
Hollister, E.B., Engledow, A.S., Hammett, A.J., Provin, T.L., Wilkinson, H.H., Gentry, T. J., 2010. Shifts in microbial community structure along an ecological gradient of hypersaline soils and sediments. ISME J. 4, 829–838.
Jones, H.J., Kröber, E., Stephenson, J., Mausz, M.A., Jameson, E., Millard, A., et al., 2019. A new family of uncultivated bacteria involved in methanogenesis from the ubiquitous osmolyte glycine betaine in coastal saltmarsh sediments. Microbiome 7, 120.
Kalyaanamoorthy, S., Minh, B.Q., Wong, T., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589.

Kelly, D.P., Chambers, L.A., Trudinger, P.A., 1969. Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal. Chem. 41, 898–901.
Kim, D., Park, S., Chun, J., 2021. Introducing EzAAI: a pipeline for high throughput calculations of prokaryotic average amino acid identity. J. Microbiol. (Seoul, Korea) 59, 476–480.
Koch, T., Dahl, C., 2018. A novel bacterial sulfur oxidation pathway provides a new link between the cycles of organic and inorganic sulfur compounds. ISME J. 10, 2479–2491.
Kröber, E., Schäfer, H., 2019. Identification of proteins and genes expressed by *Methylophaga thiooxydans* during growth on dimethylsulfide and their presence in other members of the genus. Front. Microbiol. 10, 1132.
Latypova, E., Yang, S., Wang,Y.S., Wang, T., Chavkin, T.A., Hackett, M., et al., 2010. Genetics of the glutamate-mediated methylamine utilization pathway in the facultative methylotrophic beta-proteobacterium *Methyloversatilis universalis* FAM5. Mol. Microbiol. 75, 426–439.
Lin, N., Tao, Y., Gao, P., Xu, Y., Xing, P., 2021. Comparative genomics revealing insights into niche separation of the genus *Methylophilus*. Microorganisms 9, 1577.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Minh, B.Q., Nguyen, M.A., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195.
Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37, 1530–1534.
Mouné, S., Manac'h, N., Hirschler, A., Caumette, P., Willison, J.C., Matheron, R., 1999. *Haloanaerobacter salinarius* sp. nov., a novel halophilic fermentative bacterium that reduces glycine-betaine to trimethylamine with hydrogen or serine as electron donors; emendation of the genus *Haloanaerobacter*. Int. J. Syst. Bacteriol. 49, 103–112.
Nayak, D.D., Marx, C.J., 2014. Methylamine utilization via the *N*-methylglutamate pathway in *Methylobacterium extorquens* PA1 involves a novel flow of carbon through C1 assimilation and dissimilation pathways. J. Bacteriol. 196, 4130–4139.
Nyyssölä, A., Reinikainen, T., Leisola, M., 2001. Characterization of glycine sarcosine *N*-methyltransferase and sarcosine dimethylglycine *N*-methyltransferase. Appl. Environ. Microbiol. 67, 2044–2050.
Oren, A., 1990. Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments. Antonie Van Leeuwenhoek 58, 291–298.
Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.A., Hugenholtz, P., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat. Biotechnol. 36, 996–1004.
Pfennig, N., Lippert, K.D., 1966. Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. 55, 245–256.
Pritchard, L., Glover, R.H., Humphris, S., Elphinstone, J.G., Toth, I.K., 2016. Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. Anal. Methods 8, 12–24.
Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. 2020. Using SPAdes De Novo Assembler. Curr. Protoc. Bioinformatics, 70(1), e102.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.
Rinke, C., Chuvochina, M., Mussig, A.J., Chaumeil, P.-A., Davín, A.A., Waite, D.W., Whitman, W.B., Parks, D.H., Hugenholtz, P., 2021. A standardized archaeal taxonomy for the genome taxonomy database. Nat. Microbiol. 6, 946–959.
Sorokin, D.Y., Trotsenko, Y.A., Doronina, N.V., Tourova, T.P., Muyzer, G., 2007. *Methylohalomonas lacus* gen. Nov., sp. nov. and *Methylonatronum kenyi* gen. Nov., sp. nov., new methylotrophic gammaproteobacteria from hypersaline lakes. Int. J. Syst. Evol. Microbiol. 57, 2762–2769.
Sorokin, D.Y., Tourova, T.P., Galinski, E.A., Muyzer, G., Kuenen, J.G., 2008a. a. *Thiohalorhabdus denitrificans* gen. Nov. sp. nov., an extremely halophilic sulfur-oxidizing deep-lineage gammaproteobacterium from hypersaline habitats. Int. J. Syst. Evol. Microbiol. 58, 2890–2897.
Sorokin, D.Y., Tourova, T.P., Muyzer, G., Kuenen, J.G., 2008b. b. *Thiohalospira halophila* gen. Nov., sp. nov., and *Thiohalospira alkaliphila* sp. nov., novel obligately chemolithoautotrophic, halophilic, sulfur-oxidizing gammaproteobacteria from hypersaline habitats. Int. J. Syst. Evol. Microbiol. 58, 1685–1692.
Sorokin, D.Y., Merkel, A.Y., Muyzer, G., 2020. Thiohalorhabdus. In: Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Inc. https://doi.org/10.1002/9781118960608.gbm01940
Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L., Lomsadze, A., Pruitt, K.D., Borodovsky, M., Ostell, J., 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 44, 6614–6624.
Welsh, D.T., 2000. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol. Rev. 24, 263–290.
Zhuanga, G.-C., Lina, Y.-S., Bowlesa, M.W., Heuera, V.B., Leverb, M.A., Elverta, M., 2017. Distribution and isotopic composition of trimethylamine, dimethylsulfide and dimethylsulfoniopropionate in marine sediments. Mar. Chem. 196, 35.
de Zwart, J.M.M., Nelisse, P.N., Kuenen, J.G., 1996. Isolation and characterization of *Methylophaga sulfidovorans* sp. nov.: an obligately methylotrophic, aerobic, dimethylsulfide oxidizing bacterium from a microbial mat. FEMS Microbiol. Ecol. 20, 261–270.

Thiohalorhabdus methylotrophus sp. nov.*, an extremely halophilic autotrophic methylotiotroph from hypersaline lakes.*

Dimitry Y. Sorokin[a,b*], Alexander Y. Merkel[a], William Gebbe[c] and Marina G. Kalyuzhnaya[c]

[a] *Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia*

[b] *Department of Biotechnology, Delft University of Technology, Delft, The Netherlands*
[c] *Department of Biology, University of San Diego, CA, USA*

Supplementary data

Table S1. Polar lipids fatty acid profile of strain Cl-TMA in comparison with *Thiohalorhabdus denitrificans* HL19^T grown either with TMA or thiosulfate, respectively at 3 M NaCl, pH 7 and 35^{o}C. Only compounds with the relative abundance>0.5% are shown.

Table S2. pH-ion homeostasis/osmoprotection proteins encoded in the genome of strain Cl-TMA.

Fig. S1. Phylogenetic placement of strain Cl-TMA within the class *Gammaproteobacteria* based on 16S rRNA gene sequences. The tree was built with the IQ-TREE2 program v2.2.0.3 (Minh et al., 2020) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation (Minh et al., 2013) as well as approximate likelihood-ratio test for branches (Anisimova and Gascuel, 2006). Best-fit model: GTR+F+I+I+R10 chosen according to BIC (Bayesian Information Criterion). Bootstrap consensus tree is shown with values placed at the nodes. Bar, 0.1 change per position.

Fig. S2. Influence of NaCl on methyloautotrophic growth of strain Cl-TMA with either MeOH or TMA at 35^{o}C and pH 6.5-6.8. Results are mean values from a duplicate experiment.

Fig. S3. Influence of Ca^{2+} (0.3 mM) and La^{3+} (30 μM) on growth of strain Cl-TMA at 3 M NaCl with 25 mM MeOH. Results are mean values from a duplicate experiment.

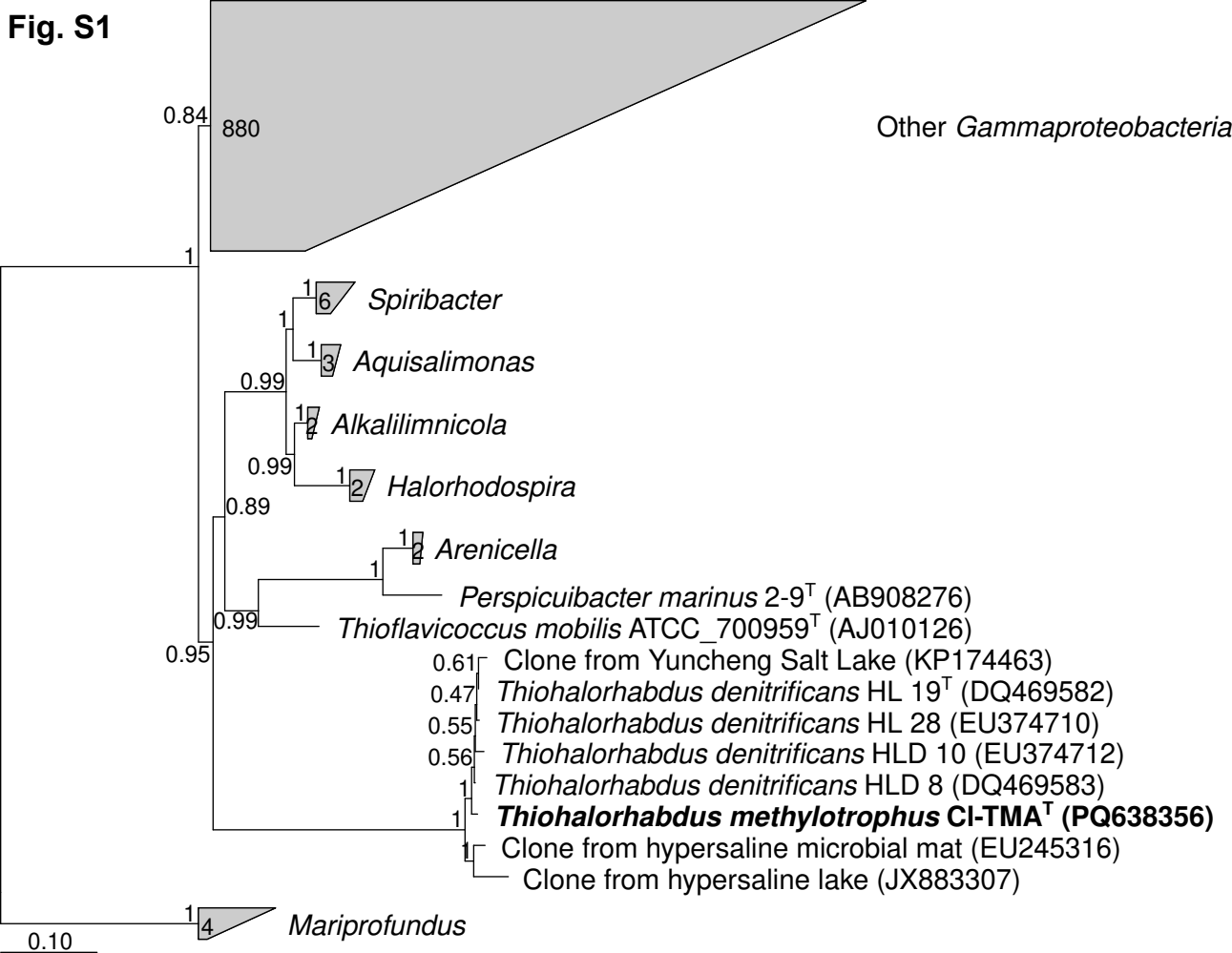
Table S1. Comparison of fatty acid composition in the polar lipids between *Thiohalorabdus* Cl-TMA and the type species of the genus *Thihalorhabdus* (above 0.5% from the total)

Fatty acid	*Thiohalorabdus* Cl-TMA	*Thihalorhabdus denitrificans* HL19
C8:0	2.2	-
C10:0	9.3	-
C12:0	-	3.6
*i*C12:0	9.6	-
C14:1	-	4.8
C15:0	-	0.6
C16:0	**35.0**	**29.4**
*i*16:1	**-**	2.3
3OH-C16:0	2.4	-
C16:1ω7	3.4	7.4
10Me-C16:0	-	**43.4**
10Me-C17:0	**36.6**	-
C17:0	**-**	1.1
C18:0	2.3	3.4
C18:1ω7	-	3.4

Supplementary **Table S2**

Locus tag (WP_37365+)	**Protein**	**Function**
4096-4105	MrpABCD1D2D3EFG (1)	multisubunit Na^+:H^+ antiporter
4607-4614	MrpABCD1D2D3EFG (2)	multisubunit Na^+:H^+ antiporter
6667-6674	PhaA-BCDEFGPS	multisubunit K^+:H^+ antiporter
4988; 5467	CPA2	Na^+:H^+ antiporter
4991	CPA1	K^+:H^+ antiporter
6093	NhaP	K^+:H^+ antiporter
4059-4060	TrkAH	K^+:H^+ symporter
5618; 6085; 7146	CaCA	Ca^{2+}:Na^+ antiporter
4061; 4977;	BetT	high affinity glycine betaine transporter
4989	BetL	glycine betaine:Na^+ symporter
5357; 5419	OpuD	high affinity glycine betaine transporter
4062-4064	OpuCA/CB/CC(1)	ABC glycine betaine transporter
5356	OpuCC(2)	ABC glycine betaine transporter (permease)
5365 5366	glycine betaine biosynthesis proteins	Sarcosine/dimethylglycine N-methyltransferase glycine/sarcosine N-methyltransferase

Fig. S1


0.84
880
Other *Gammaproteobacteria*
1
1
6
Spiribacter
1
1
3
Aquisalimonas
0.99
1
2
Alkalilimnicola
0.99
1
2
Halorhodospira
0.89
1
2
Arenicella
1
Perspicuibacter marinus 2-9^{T} (AB908276)
0.99
Thioflavicoccus mobilis ATCC_700959^{T} (AJ010126)
0.95
0.61
Clone from Yuncheng Salt Lake (KP174463)
0.47
Thiohalorhabdus denitrificans HL 19^{T} (DQ469582)
0.55
Thiohalorhabdus denitrificans HL 28 (EU374710)
0.56
Thiohalorhabdus denitrificans HLD 10 (EU374712)
1
Thiohalorhabdus denitrificans HLD 8 (DQ469583)
1
***Thiohalorhabdus methylotrophus* Cl-TMA^{T} (PQ638356)**
1
Clone from hypersaline microbial mat (EU245316)
Clone from hypersaline lake (JX883307)
1
4
Mariprofundus
0.10

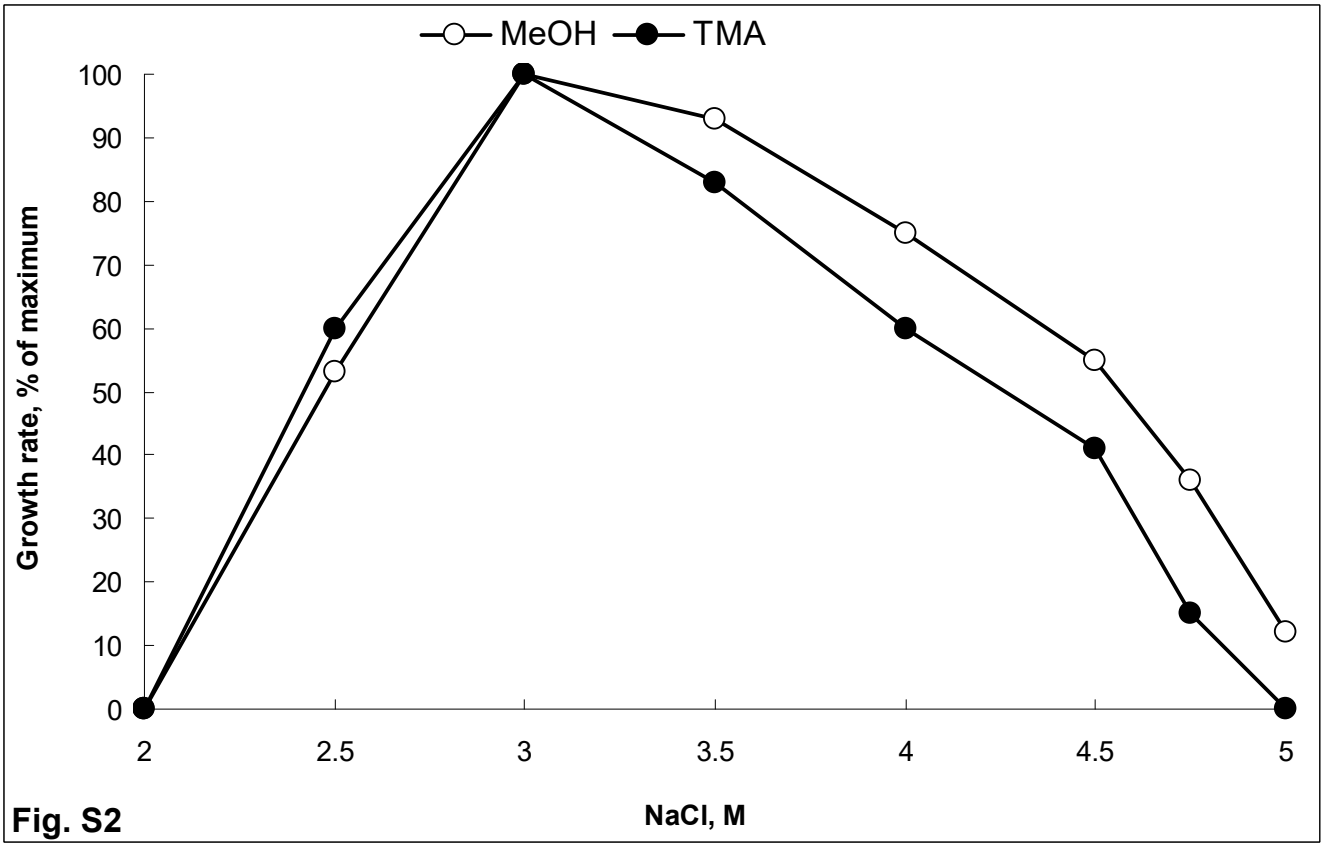

MeOH
TMA
Growth rate, % of maximum
100
90
80
70
60
50
40
30
20
10
0
2
2.5
3
3.5
4
4.5
5
NaCl, M

Fig. S2

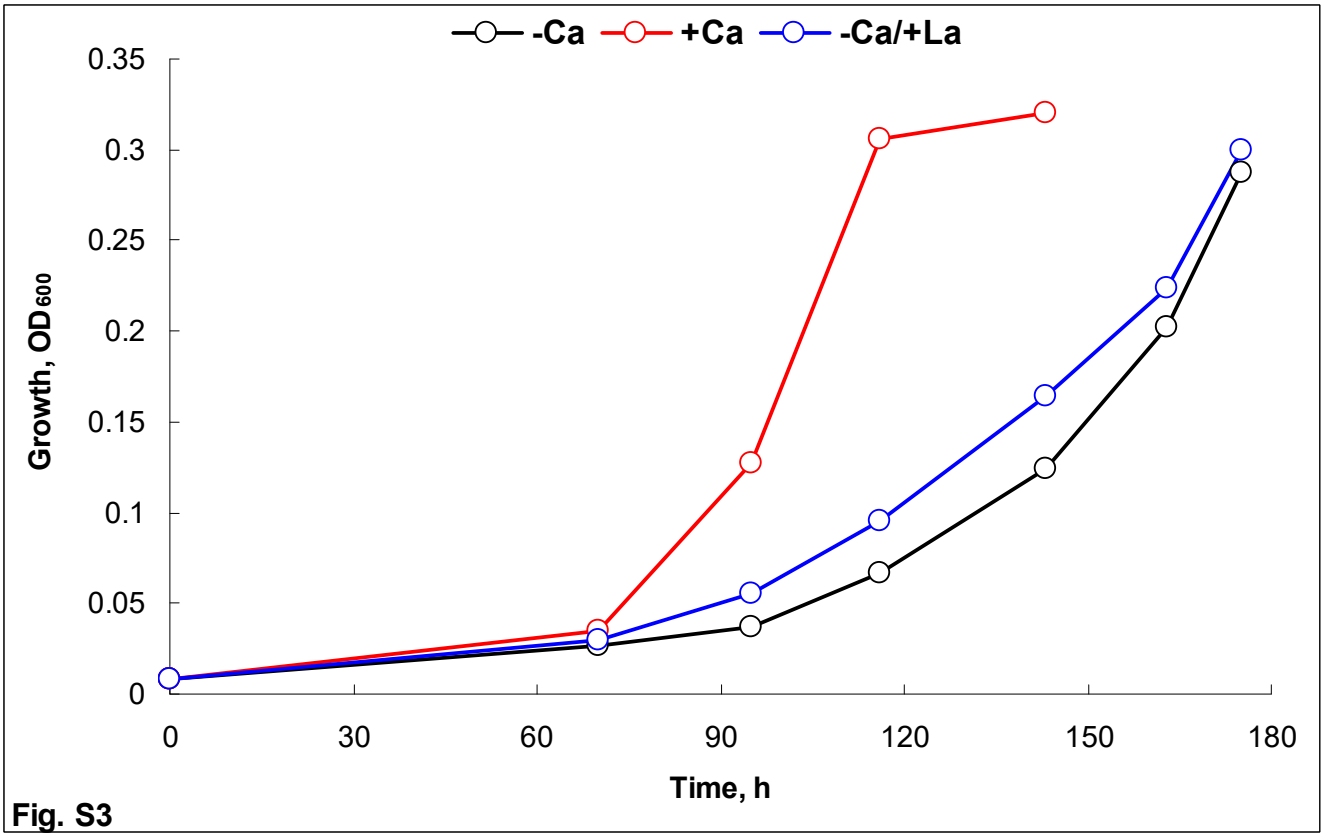

-Ca
+Ca
-Ca/+La
Growth, OD600
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
30
60
90
120
150
180
Time, h

Fig. S3