Systematic and Applied Microbiology
September 2020, Volume 43 Issue 5 Pages 126107 (8p.)
https://doi.org/10.1016/j.syapm.2020.126107
https://archimer.ifremer.fr/doc/00635/74744/

Methanohalophilus profundi sp. nov., a methylotrophic halophilic piezophilic methanogen isolated from a deep hypersaline anoxic basin

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Abstract :

A novel anaerobic methylotrophic halophilic methanogen strain SLHTYROT was isolated from a deep hypersaline anoxic basin called "Tyro" located in the Eastern Mediterranean Sea. Cells of SLHTYROT were motile cocci. The strain SLHTYROT grew between 12 and 37 °C (optimum 30 °C), at pH between 6.5 and 8.2 (optimum pH 7.5) and salinity from 45 to 240 g L−1 NaCl (optimum 135 g L−1). Strain SLHTYROT was methylotrophic methanogen able to use methylated compounds (trimethylamine, dimethylamine, monomethylamine and methanol). Strain SLHTYROT was able to grow at in situ hydrostatic pressure and temperature conditions (35 MPa, 14 °C). Phylogenetic analysis based on 16S rRNA gene and mcrA gene sequences indicated that strain SLHTYROT was affiliated to genus Methanohalophilus within the order Methanosarcinales. It shared >99.16% of the 16S rRNA gene sequence similarity with strains of other Methanohalophilus species. Based on ANIb, AAI and dDDH measurements, and the physiological properties of the novel isolate, we propose that strain SLHTYROT should be classified as a representative of a novel species, for which the name Methanohalophilus profundi sp. nov. is proposed; the type strain is SLHTYROT (=DSM 108854 = JCM 32768 = UBOCC-M-3308).

Keywords : *Methanohalophilus*, *Methanosarcinaceae*, *Methanosarcinales*, Methylotrophic, Piezophilic, Deep hypersalin anoxic basin (DHAB)

INTRODUCTION

Tyro basin was the first deep hypersaline anoxic basin discovered [13]. Between 5.6 and 5.3 million years ago, the Messinian salinity crisis was characterized by cyclic desiccation of the Mediterranean Sea causing deposition of massive evaporites [5,7]. The subsequent dissolution of these ancient evaporite rocks resulted in formation of brines that filled most depressions along the seafloor. These contemporaneous formations occur now at a depth of more than 3,000 m below the sea level (bsl) and are known as deep-sea hypersaline anoxic basins (DHABs) [32]. So far, eight deep hypersaline anoxic lakes Tyro, Bannock, Discovery, Atalante, Urania, Medee, Thetis, and Kryos have been discovered and studied for microbiology in the Eastern Mediterranean Sea [8,9,32,36,37]. Molecular analysis of the microbial diversity inhabiting these basins revealed the presence of numerous uncultivated phylogenetic clades. Based on the geochemistry indicating the biogenic methane production in these basins and the simultaneous detection of uncultivated *Archaea* closely related to known methanogenic clades named MSBL1 (Mediterranean Sea Brine Lake), it was speculated that the members of this MSBL1 clade were the methane producers [32,36]. However the production of methane by members of the MSBL1 is still under debate because no representative strain have been isolated in pure culture and metagenomics studies still failed to demonstrate the presence of enzymes required for methane production from the 15 metagenomes of the MSBL1 [22,35].

Interestingly, methyl coenzyme M reductase transcripts of the *mcrA* genes, specific common phylogenetic markers for the detection of methanogens, were recovered from several hypersaline anoxic basins and formed a tight cluster affiliated with *Methanohalophilus* [34], suggesting these microorganisms could be active methanogens. To date, the genus *Methanohalophilus* comprises four species, all isolated from terrestrial environment, *Methanohalophilus mahii, M. halophilus, M. portucalensis* and *M. levihalophilus* [2,14,25,33]. On the basis of 16S rRNA sequence analysis and rRNA probe hybrization, it was also proposed to rename *Methanococcoides euhalobius*, a methanogen isolated from deep shale reservoir, to *Methanohalophilus euhalobius* [10,24]. Recently, the analysis of the genome of a new *Methanohalophilus* strain RSK isolated from the Kebrit deep Brine in the Red Sea showed a highly conserved genomic core among the *Methanohalophilus* genus [12].

In the present study, we describe a novel methylotrophic, halophilic, piezophilic, anaerobic archaeon belonging to the genus *Methanohalophilus* and isolated from the deep halophilic anoxic basin "Tyro" extending our knowledge of the presence of this genus as the possible source of biogenic methane in the deep-sea hypersaline anoxic lakes.

Materials and Methods

The deep hypersaline anoxic basin Tyro (33.52419°N; 26.21956°E) at a depth of 3,350 m has been sampled during the oceanographic cruise "MAMBA C" in 2011. Depth-stratified brine samples were collected on board the RV Urania. Sampling was performed with 12 L Niskin bottles, housed on a rosette provided by SBE-911 plus CTD sensors (Sea-Bird Electronics, Bellevue, WA, USA). The interface was captured and fractionated as described elsewhere [34]. Salinity measurements at the top and bottom of the Niskin bottle indicated the fraction sampled of the interface collected. Samples for cation analysis were collected in 2 mL tubes and stored at -80°C directly on board. Sodium, methylated amines (methylamine, dimethylamine, trimethylamine, choline, N,N-dimethylethanolamine, N-methylethanolamine), ammonium and ethanolamine were analysed using a Dionex ICS-2000 Reagent-Free™ Ion Chromatography System (Dionex, Camberley UK) fitted with a AS50 autosampler and an IonPac CS16 column maintained at 60°C coupled with a CSRS 300 of 4 mm suppressor and a DS6 conductivity detector (35°C). The gradient program was: 14 mmol L^{-1} methanesulphonic acid (MSA) for 30 min, increase 5.33 mmol L^{-1} MSA min^{-1} to 46 mmol L^{-1} (0 min), decrease 68 mmol L^{-1} MSA min^{-1} to 12 mmol L^{-1} (8.5 min), increase 5.8 mmol L^{-1} MSA min^{-1} to 70 mmol L^{-1} (5 min), decrease 11.2 mmol L^{-1} MSA min^{-1} to 14 mmol L^{-1} (5 min). Sub-samples for microbiology cultures were collected in 1 L Pyrex bottle flushed with nitrogen and closed with butyl rubber stopper. Sub-samples for cation measurements and microbiology cultures were taken at the same time.

In the laboratory, brine samples were used as inoculum for anaerobic enrichments. The medium that was used for the enrichment of the strain had the following composition (per liter): 200 g NaCl, 4 g $MgCl_2$. $6H_2O$, 3.75 g $MgSO_4$. $7H_2O$, 0.25 g NH_4Cl, 0.33 g KCl, 0.14 g $CaCl_2$. $2H_2O$, 10 mL trace element solution (DSMZ medium 141), 2 g $NaHCO_3$, 0.2 g yeast extract, 0.2 g tryptone, 0.2 g casamino acids, 1.91 g

trimethylamine (TMA) and 0.14 g KH_2PO_4. The pH was adjusted to 7.0 at room temperature. The medium was boiled under N_2/CO_2 (80/20) gas, cooled down prior to addition of 0.5 g of cysteine-hydrochloride. The medium was dispensed into 50 mL serum bottles in 20 mL portions. The vessels were sealed with butyl rubber stoppers and aluminum crimp caps. The medium was autoclaved (121°C for 20 min). Thereafter, 1 mL of brine sample was transferred to a 50 mL serum bottle containing 20 mL of medium reduced with 0.004 M Na_2S from a sterile anoxic stock solution. A pure culture was obtained from single colonies on enrichment medium solidified with 0.8% gellan gum (Phytagel™, Sigma-Aldrich) in Wolf's flask incubated at 20°C. After the third transfer on Wolfe's flask, a single white colony was picked, and designated as strain SLHTYRO^T. The purity of the strain was determined on the one hand, by inoculation of a methyl-free medium and on the other hand, by sequencing the 16S rRNA and mcrA genes amplified from DNA extracted from SLHTYRO^T cultures.

Pressure experiments were done in serum glass vials of 20 mL totally filled with liquid medium and closed with butyl rubber stoppers and aluminium caps. Vials were then transferred into stainless steel pressure vessel-incubators that were heated in ovens, custom-built by Top Industrie (Vaux le Penil, FRANCE). Each vessel-incubators was pressurized to one of the following specific pressures: 10, 20, 35, 50 and 70 MPa and heated to 30°C [21]. Hydrostatic pressure was generated using a hydraulic pump (Top Industrie S.A.), and cold tap water served as hydraulic fluid. Bourdon® pressure gauges (100 MPa) monitored the pressure in each pressure vessel [21]. Atmospheric-pressure controls were prepared in the same conditions and were incubated in an oven at 30°C (0.1 MPa). In a second round of experiment, incubations were also performed at the *in situ* temperature (14°C) and pressure (35 MPa) by placing the pressure-vessel incubator in an air-conditioned room.

Genomic DNA of strain SLHTYRO^T was isolated by using the procedure described by Charbonnier & Forterre (1994) [6]. Precipitated DNA was collected using a sterile glass rod, washed in 70% ethanol, dried at room temperature for 10 to 15 min. Finally, DNA was suspended in TE (Tris-EDTA) overnight at 4°C. The complete genome was sequenced with a combination of two sequencing approaches. A 100 bp paired-end library sequenced on an Illumina® HiSeq (Illumina, Beckman Coulter Genomics, Danvers, MA) and a PacBio® RS library (Genotoul, Toulouse). The 49,403,100 paired reads of 100 bp were quality trimmed (Q30)

and *de novo* assembled into scaffolds with the 117,638 PacBio reads using SPAdes version 3.10.0 [23]. The 27 resulting scaffolds were then reorganized using MeDuSa scaffolder and *Methanohalophilus halophilus* DSM 3094^T as reference genome [4]. A final gap-closing step was added using the scaffolded genome and the Illunina reads with the GapClose software included in SOAPdenovo2 leading to a final assembly with 4 scaffolds (including 2 genomic scaffolds > 1,000 bases) [20].

The annotation was performed on the MicroScope Microbial Genome Annotation and Analysis Platform (MaGe) at the genoscope [30,31]. Average nucleotide identity (ANIb) and Average amino acid identity (AAI) scores were calculating using the phylogenomic pipeline EDGAR 2.3 [1]. In silico DNA-DNA hybridization (DDH) estimations were calculated using the genome-to-genome distance calculator (GGDC2.1). In pour case, the GGDC results were based on the recommended formula 2 which is independent of genome size and thus is robust when comparing genomes of different sizes.

The 16S rRNA and *mcrA* genes were amplified and sequenced as described previously [18]. The *mcrA* gene sequence was translated to the amino acid sequence *in silico* and aligned with those of related taxa using *ClustalW* implemented in *ARB* package [19].

The 16S rRNA gene sequences obtained in this study were imported into the version 132 of Silva database [26]; (http://www.arb-silva.de) using the *ARB* software package [19]. Sequences from other studies that were not included in Silva were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences were automatically aligned against the entire database by using the “Integrated Aligner” of ARB, and the alignments were refined manually. The final consensus sequence was assembled from three independent sequencing from each primer used. Positions that had not been sequenced in one or more reference organisms were omitted. Phylogenetic trees (1,226 nucleotides) including sequence from strain RSK were calculated using RaxML, a maximum-likelihood method [29], with rate distribution model GTRGAMMA and a weighting mask based on positional variability of archaeal reference sequences. The outgroup-rooted (*Methanopyrus kandleri*) phylogenetic tree topology and node support (1,000 bootstraps) were tested.

The *mcrA* gene sequences were imported into a seed alignment. The *mcrA* sequences were translated into amino acid sequences, hand-aligned and then by using the Fast Aligner in ARB. Trees were calculated at the amino acid level (228 amino acids) using a maximum likelihood RaxML method [27] with amino acids

rate distribution model PROTGAMMA and substitution model DAYHOFF implanted in ARB package. The outgroup-rooted (*Methermicoccus shengliensis* and *Methanosaeta harundinacea*) phylogenetic tree topology and node support (1,000 bootstraps) were tested.

Results &discussion

Tyro is a funnel-shaped anoxic basin, diameter *c.a.* 4 km, located at the western end of the Strabo trench at a depth of 3,350 meters in the Eastern Mediterranean Sea [7]. The Tyro basin is a thalassic lake, characterised by salinity close to saturation (315 ‰), high concentrations of sodium (5,300 mM) and chloride (5,350 mM). Between the deep anoxic brine and the overlying seawater a sharp interface of few meters exhibiting a steep salinity gradient ranging from 39 ‰ at its upper face to 315 ‰ at its lower face. DHAB interfaces are hot spots for wide range of metabolically active microorganisms mainly revealed by molecular surveys [8,9,32,35]. These studies also reported the presence of numerous uncultivated lineages identified as MSBL candidate divisions. It was even suggested that MSBL1 could be a group of methanogens [3,32] responsible for the production of the biogenic methane in DHABs. In order to try to elucidate the metabolism of MSBL1 archaea, numerous enrichment cultures were performed from Tyro DHAB brine samples using a range of methanogenic substrates (MMA, DMA, TMA, DMEA, choline, glycine betaine, DMS, methanol, formate, acetate, H_2/CO_2). The only cultivated methanogen was a methylotrophic halophilic archaeon (strain SLHTYRO^T) closely affiliated with *Methanohalophilus* genus.

Strain SLHTYRO^T was isolated from a sample (TY11Barch-14) characterized by a high salinity (215 ‰) collected at the interface of the Tyro basin. After 8 months of incubation at 20 °C, motile cocci that fluoresced blue-green under UV (420 nm) were enriched in a medium containing 200 g L^{-1} of NaCl. Subculturing of this enrichment revealed that the optimal salinity was close to 135 g L^{-1} of NaCl. After 2 months of incubation at 20°C, mustard-coloured colonies with a diameter of 1-2 mm were observed in the Wolfe's flask. Once isolated, cells of strain SLHTYRO^T appeared as regular slightly motile cocci, with a diameter of 0.7 to 1.2 µm (**Fig. 1**).

The comparison of the physiological characteristics between strain SLHTYRO^T and species of the *Methanohalophilus* genera are presented in **Table 1,** in summary it shows that SLHTYRO^T strain is the only motile, piezophilic and the most halophilic species of the *Methanohalophilus* genus.

To resolve the phylogeny of SLHTYRO^T, 16S rRNA and *mcrA* gene sequences were compared to a comprehensive set of sequences from public databases. Based on 16S rRNA gene sequences, the closest neighbors of the strain SLHTYRO^T were *Methanohalophilus* species. The highest similarity was observed with the species *M. halophilus* strain Z7982^T (99.83%) and *M. portucalensis* strain FDF-1^T (99.83%) and the lowest with *M. euhalobius* strain 283 (99.23%). The 16S rRNA gene sequence from SLHTYRO^T showed 99.66% similarity with *Methanohalophilus* strain RSK, isolated also from a deep hypersaline anoxic environment [12] (**Table S1**). The 16S rRNA and *mcrA* gene phylogenetic trees including *Methanohalophilus* spp. confirmed that strain SLHTYRO^T grouped within genus *Methanohalophilus* (**Fig. 2 and 3**). These topologies were supported by bootstrap values (> 70% for 16S rRNA and > 50% for *mcrA*) with maximum likelihood methods (**Fig. 2 and 3**). Moreover, an environmental RNA-derived 16S rRNA sequence, recovered from the same brine sample, was closely related to the type strain SLHTYRO^T within the *Methanohalophilus* genus (data not shown), suggesting these communities were metabolically active and could be involved either directly or indirectly in the methane cycling process. In addition, environmental *mcrA* gene sequences recovered from Discovery, L'Atalante, Kryos and Thetis deep hypersaline anoxic basins were included in the analysis. Strain SLHTYRO^T was closely related to one *mcrA* sequence collected from the Thetis hypersaline brine (HQ658638) suggesting that methane in hypersaline basin might be produced by methanogens belonging to *Methanohalophilus* genus (data not shown).

SLHTYRO^T whole-genome sequencing results were distributed among 4 scaffolds representing a total size of 1,830,088 bp (with 898 unspecified bases) and an average coverage of approximately 5,300X. This genome has a GC content (42.7 %) in the range of all other *Methanohalophilus* species (41.9 to 44%; **Table 1**). The genome of SLHTYRO^T harbours a total of 2,027 open-reading frames that were identified using the MaGe platform [30,31] **(Table 1)**. These values were also in the range to those predicted for all other *Methanohalophilus* spp (1,990 to 2,124**; Table 1)**. The core genome of strain SLHTYRO^T and close relatives

of *Methanohalophilus* spp has 1219 protein-coding genes which represents 57 to 61% of their predicted proteomes. The proportion of unique proteins present in the SLHTYRO^T genome (7.3%, n=149) is also in the range of other *Methanohalophilus* spp (6.1 to 9.3%, n=125 to 192) suggesting they could represent genomic traits specific to each species of *Methanohalophilus*. Moreover, most these unique proteins in SLHTYRO^T genome were assigned as hypothetical proteins (58%) which could later uncover specific traits for adaptation to DHABs.

Analysis of 16S rRNA sequences has a limited resolution to discriminate between closely related species. DNA-DNA digital hybridization (dDDH) were performed between strain SLHTYRO^T and the closest validate references *M. portucalensis* FDF-1^T (58%), *M. mahii* SLP^T (45%) and *M. halophilus* Z-7982^T (51%) calculated using the genome-to-genome distance calculator GGDC2.1 [11]. All dDDH values obtained were below the threshold value of 70 % DNA–DNA relatedness generally accepted for the definition of a novel species [28]. Average nucleotide identity (ANI) among described *Methanohalophilus* species and *Methanohalophilus* strain RSK, calculated using the ANI calculator of [11], varies between 90.96 and 94.34% (**Fig. S1**). The value of 94.34% is obtained between strain SLHTYRO^T and *M. portucalensis*. Average amino acid identity (AAI) among *Methanohalophilus* spp., calculated using the AAI calculator of the phylogenomic EDGAR platform, varies between 93.11% and 95.82% which is also below the threshold value of 96% [16] (**Fig. S2**). Thus, based on an ANI threshold of ~95%, AAI threshold of ~96%, and dDDH results below 70% which are currently being operationally used for species delineation[15–17], our results indicate that strain SLHTYRO^T represents a novel species in the genus *Methanohalophilus*.

Growth of SLHTYRO^T under high hydrostatic pressure was also investigated as Tyro basin samples were collected at a depth of 3,350 m. We observed that the strain SLHTYRO^T was able to grow under hydrostatic pressure at 30°C with an optimum at 35 MPa which was close to the *in situ* hydrostatic pressure (**Fig. 4**). *M. halophilus*, *M. portucalensis* and *M. mahii* were not able to grow under hydrostatic pressure (data not shown). Growth of the isolate at *in situ* pressure (35 MPa) and temperature (14°C) was also demonstrated as cell densities reached 5 x 10^8 cells mL^{-1} after two months of incubation. Furthermore, high concentrations

(< 0.816 µM) of N-methyl compounds such as trimethylamine were also detected in the upper interface of the Tyro brine lake indicating that methanogenic substrates were also available *in situ.*

Conclusion

Strain SLHTYRO^T could be metabolically active *in situ*, as it can grow at *in situ* temperature (14°C), hydrostatic pressure (35 MPa), salinity (215‰) and utilize the high concentrations of *N*-methyl compounds present at the Tyro basin interface as catabolic substrates. Moreover, 16S rRNA and *mcrA* gene sequences transcripts were closely affiliated to the genus *Methanohalophilus* and were detected in the same interface or others DHABs suggesting that the strain SLHTYRO^T could be one of the microbial partners involved in the methane production in the Tyro basin.

Novel strain SLHTYRO^T is the first piezophilic, highly halophilic microorganism isolated from deep-sea belonging to the *Methanohalophilus* genus. Phenotypical, phylogenetic and genomic features indicate that it represents a novel species of the genus *Methanohalophilus* for which the name *Methanohalophilus profundi* sp. nov., is proposed, in reference to the depth of its source location.

Description of *Methanohalophilus profundi* sp. nov.

Methanohalophilus profundi (pro.fun'di. L. gen. n. *profundi* of the depth of the sea).

Cells are regular cocci (diameter 0.7-1.2 µm), slightly motile and occur singly or in pairs. Colonies are mustard-coloured, circular and convex. Obligately anaerobic. Growth occurs on methylated compounds (TMA, DMA, MMA, methanol).

Optimal growth occurs at 30°C, pH 7.5 and 13.5 % (w/v) NaCl. Piezophilic, growing optimally under 35 MPa (pressure range: 0-50 MPa).

The type strain SLHTYRO^T (=DSM 108854= JCM 32768= UBOCC-M-3308) was isolated from the Tyro deep-sea hypersaline anoxic basin located at a depth of 3,350 m in the Eastern Mediterranean Sea closed to the Strabo trench. (33.52419°N; 26.21956°E).

The DNA G+C content of this strain is 42.7 mol %.

Description of the *Methanohalophilus profundi* sp. nov. is given in **Table 2**.

Acknowledgements

This study was funded by the European Union program MaCuMBA (grant agreement 311975) and the MAMBA project (FP7-KBBE-2008–226977). Research was supported by UBO, CNRS and Ifremer. We thank the captain and crew members of the *RV* URANIA for assistance in collecting samples.

Références

[1] Blom, J., Albaum, S.P., Doppmeier, D., Pühler, A., Vorhölter, F.J., Zakrzewski, M., Goesmann, A. (2009) EDGAR: A software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics 10, Doi: 10.1186/1471-2105-10-154.

[2] Boone, D.R., Mathrani, I.M., Liu, Y., Menaia, J.A.G.F., Mah, R.A., Boone, J.E. (1993) Isolation and characterization of *Methanohalophilus portucalensis* sp. nov. and DNA reassociation study of the genus *Methanohalophilus*. International Journal of Systematic Bacteriology 43(3), 430–7, Doi: 10.1099/00207713-43-3-430.

[3] Borin, S., Brusetti, L., Mapelli, F., D'Auria, G., Brusa, T., Marzorati, M., Rizzi, A., Yakimov, M., Marty, D., De Lange, G.J., Van der Wielen, P., Bolhuis, H., McGenity, T.J., Polymenakou, P.N., Malinverno, E., Giuliano, L., Corselli, C., Daffonchio, D. (2009) Sulfur cycling and methanogenesis primarily drive microbial colonization of the highly sulfidic Urania deep hypersaline basin. Proceedings of the National Academy of Sciences 106(23), 9151–6, Doi: 10.1073/pnas.0811984106.

[4] Bosi, E., Donati, B., Galardini, M., Brunetti, S., Sagot, M.F., Lio, P., Crescenzi, P., Fani, R., Fondi, M. (2015) MeDuSa: a multi-draft based scaffolder. Bioinformatics 31(15), 2443–51.

[5] Camerlenghi, A., Cita, M.B.B. (1987) Setting and tectonic evolution of some Eastern Mediterranean deep-sea basins. Marine Geology 75(1–4), 31–55, Doi: 10.1016/0025-3227(87)90095-8.

[6] Charbonnier, F., Forterre, P. (1994) Comparison of DNA topology among mesophilic and thermophilic Eubacteria and Archaebacteria. Journal of Bacteriology 176(5), 1251–9, Doi: 10.1128/jb.176.5.1251-1259.1994.

[7] Cita, M.B. (2006) Exhumation of Messinian evaporites in the deep-sea and creation of deep anoxic brine-filled collapsed basins. Sedimentary Geology 188–189(0), 357–78, Doi: http://dx.doi.org/10.1016/j.sedgeo.2006.03.013.

[8] La Cono, V., Smedile, F., Bortoluzzi, G., Arcadi, E., Maimone, G., Messina, E., Borghini, M., Oliveri, E., Mazzola, S., L'Haridon, S., Toffin, L., Genovese, L., Ferrer, M., Giuliano, L., Golyshin,

P.N.P.N., Yakimov, M.M.M.M.M. (2011) Unveiling microbial life in new deep-sea hypersaline Lake Thetis. Part I: Prokaryotes and environmental settings. Environmental Microbiology 13(8), 2250–68, Doi: 10.1111/j.1462-2920.2011.02478.x.

[9] Daffonchio, D., Borin, S., Brusa, T., Brusetti, L., van der Wielen, P., Bolhuis, H., Yakimov, M.M., D'Auria, G., Giuliano, L., Marty, D., Tamburini, C., McGenity, T.J., Hallsworth, J.E., Sass, A.M., Timmis, K.N., Tselepides, A., de Lange, G.J., Hubner, A., Thomson, J., Varnavas, S.P., Gasparoni, F., Gerber, H.W., Malinverno, E., Corselli, C., Garcin, J., McKew, B., Golyshin, P.N., Lampadariou, N., Polymenakou, P., Calore, D., Cenedese, S., Zanon, F., Hoog, S., Biodeep Sci, P. (2006) Stratified prokaryote network in the oxic-anoxic transition of a deep-sea halocline. Nature 440(7081), 203–7, Doi: 10.1038/nature04418.

[10] Davidova, I.A., Harmsen, H.J.M., Stams, A.J.M., Belyaev, S.S., Zehnder, A.J.B. (1997) Taxonomic description of *Methanococcoides euhalobius* and its transfer to the *Methanohalophilus* genus. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 71(4), 313–8, Doi: 10.1023/a:1000103618451.

[11] Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., Tiedje, J.M. (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57, 81–91, Doi: 10.1099/ijs.0.64483-0.

[12] Guan, Y., Ngugi, D.K., Vinu, M., Blom, J., Alam, I., Guillot, S., Ferry, J.G., Stingl, U. (2019) Comparative Genomics of the Genus *Methanohalophilus*, Including a Newly Isolated Strain From Kebrit Deep in the Red Sea. Frontiers in Microbiology 10(APR), 839, Doi: 10.3389/fmicb.2019.00839.

[13] Jongsma, D., Fortuin, A.R., Huson, W., Troelstra, S.R., Klaver, G.T., Peters, J.M., Vanharten, D., Delange, G.J., Tenhaven, L. (1983) Discovery of an anoxic basin within the Strabo trench, Eastern Mediterranean. Nature 305(5937), 795–7, Doi: 10.1038/305795a0.

[14] Katayama, T., Yoshioka, H., Mochimaru, H., Meng, X.Y., Muramoto, Y., Usami, J., Ikeda, H., Kamagata, Y., Sakata, S. (2014) *Methanohalophilus levihalophilus* sp nov., a slightly halophilic, methylotrophic methanogen isolated from natural gas-bearing deep aquifers, and emended description

of the genus *Methanohalophilus*. International Journal of Systematic and Evolutionary Microbiology 64(PART 6), 2089–93, Doi: 10.1099/ijs.0.063677-0.

[15] Konstantinidis, K.T., Rosselló-Móra, R., Amann, R. (2017) Uncultivated microbes in need of their own taxonomy. ISME Journal, 2399–406, Doi: 10.1038/ismej.2017.113.

[16] Konstantinidis, K.T., Tiedje, J.M. (2005) Towards a genome-based taxonomy for prokaryotes. Journal of Bacteriology 187(18), 6258–64, Doi: 10.1128/JB.187.18.6258-6264.2005.

[17] Konstantinidis, K.T., Tiedje, J.M. (2005) Genomic insights that advance the species definition for prokaryotes. Proceedings of the National Academy of Sciences of the United States of America 102(7), 2567–72, Doi: 10.1073/pnas.0409727102.

[18] L'Haridon, S., Chalopin, M., Colombo, D., Toffin, L. (2014) *Methanococcoides vulcani* sp nov., a marine methylotrophic methanogen that uses betaine, choline and N,N-dimethylethanolamine for methanogenesis, isolated from a mud volcano, and emended description of the genus *Methanococcoides*. International Journal of Systematic and Evolutionary Microbiology 64(PART 6), 1978–83, Doi: 10.1099/ijs.0.058289-0.

[19] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar., Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H. (2004) ARB: a software environment for sequence data. Nucleic Acids Research 32(4), 1363–71, Doi: 10.1093/nar/gkh293.

[20] Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., He, G., Chen, Y., Pan, Q., Liu, Y., Tang, J., Wu, G., Zhang, H., Shi, Y., Yu, C., Wang, B., Lu, Y., Han, C., Cheung, D.W., Yiu, S.M., Peng, S., Xiaoqian, Z., Liu, G., Liao, X., Li, Y., Yang, H., Wang, J., Lam, T.W. (2012) SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. GigaScience 1(1), 1–18.

[21] Marteinsson, V.T., Birrien, J.L., Reysenbach, A.L., Vernet, M., Marie, D., Gambacorta, A., Messner, P., Sleytr, U.B., Prieur, D. (1999) *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal

vent. International Journal of Systematic Bacteriology 49, 351–9.

[22] Mwirichia, R., Alam, I., Rashid, M., Vinu, M., Ba-Alawi, W., Anthony Kamau, A., Kamanda Ngugi, D., Göker, M., Klenk, H.-P., Bajic, V., Stingl, U. (2016) Metabolic traits of an uncultured archaeal lineage -MSBL1- from brine pools of the Red Sea. Scientific Reports 6, 19181, Doi: 10.1038/srep19181http://www.nature.com/articles/srep19181#supplementary-information.

[23] Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A., Prjibelski, A.D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S.R., Woyke, T., McLean, J.S., Lasken, R., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2013) Assembling Single-Cell Genomes and Mini-Metagenomes From Chimeric MDA Products. Journal of Computational Biology 20(10), 714–37, Doi: 10.1089/cmb.2013.0084.

[24] Obraztsova, A., … O.S.-., 1987, undefined. (n.d.) Properties of the coccoid methylotrophic methanogen, *methanococcoides-euhalobius* sp-nov. 233 SPRING ST, NEW YORK, NY ….

[25] Paterek, J.R., Smith, P.H. (1988) Methanohalophilus mahii gen. nov., a methylotrophic haophilic methanogen. International Journal of Systematic Bacteriology 38(1), 122–3.

[26] Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35(21), 7188–96, Doi: 10.1093/nar/gkm864.

[27] Saitou, N., Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4), 406–25.

[28] Stackebrandt J., E.& E. (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33, 152–5.

[29] Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21), 2688–90, Doi: 10.1093/bioinformatics/btl446.

[30] Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., Le Fevre, F., Longin, C., Mornico, D., Roche, D., Rouy, Z., Salvignol, G., Scarpelli, C., Smith, A.A.T., Weiman, M., Medigue, C. (2013) MicroScope-an integrated microbial resource for the curation and comparative analysis of

genomic and metabolic data. Nucleic Acids Research 41(D1), E636–47, Doi: 10.1093/nar/gks1194.

[31] Vallenet, D., Labarre, L., Rouy, Z., Barbe, V., Bocs, S., Cruveiller, S., Lajus, A., Pascal, G., Scarpelli, C., Medigue, C. (2006) MaGe: a microbial genome annotation system supported by synteny results. Nucleic Acids Research 34(1), 53–65, Doi: 10.1093/nar/gkj406.

[32] van der Wielen, P., Bolhuis, H., Borin, S., Daffonchio, D., Corselli, C., Giuliano, L., D'Auria, G., de Lange, G.J., Huebner, A., Varnavas, S.P., Thomson, J., Tamburini, C., Marty, D., McGenity, T.J., Timmis, K.N., BioDeep Sci, P. (2005) The enigma of prokaryotic life in deep hypersaline anoxic basins. Science 307(5706), 121–3, Doi: 10.1126/science.1103569.

[33] Wilharm, T., Zhilina, T.N., Hummel, P. (1991) DNA-DNA hybridization of methylotrophic methanogenic bacteria and transfer of *Methanococcus halophilus* VP to the genus *Methanohalophilus* as *Methanohalophilus halophilus* comb. nov. International Journal of Systematic Bacteriology 41(4), 558–62.

[34] Yakimov, M.M., La Cono, V., Denaro, R., D'Auria, G., Decembrini, F., Timmis, K.N., Golyshin, P.N., Giuliano, L. (2007) Primary producing prokaryotic communities of brine, interface and seawater above the halocline of deep anoxic lake L'Atalante, Eastern Mediterranean Sea. Isme Journal 1(8), 743–55, Doi: 10.1038/ismej.2007.83.

[35] Yakimov, M.M., La Cono, V., Slepak, V.Z., La Spada, G., Arcadi, E., Messina, E., Borghini, M., Monticelli, L.S., Rojo, D., Barbas, C., Golyshina, O. V., Ferrer, M., Golyshin, P.N., Giuliano, L. (2013) Microbial life in the Lake Medee, the largest deep-sea salt-saturated formation. Scientific Reports 3, Doi: 355410.1038/srep03554.

[36] Yakimov, M.M., La Cono, V., Spada, G.L., Bortoluzzi, G., Messina, E., Smedile, F., Arcadi, E., Borghini, M., Ferrer, M., Schmitt-Kopplin, P., Hertkorn, N., Cray, J.A., Hallsworth, J.E., Golyshin, P.N., Giuliano, L. (2015) Microbial community of the deep-sea brine Lake Kryos seawater-brine interface is active below the chaotropicity limit of life as revealed by recovery of mRNA. Environmental Microbiology 17(2), 364–82, Doi: 10.1111/1462-2920.12587.

[37] Yakimov, M.M., Giuliano, L., Cappello, S., Denaro, R., Golyshin, P.N. (2007) Microbial community of a hydrothermal mud vent underneath the deep-sea anoxic brine Lake Urania (eastern

Mediterranean). Origins of Life and Evolution of Biospheres 37(2), 177–88, Doi: 10.1007/s11084-006-9021-x.

[38] Zhilina, T.N. (1983) New obligate halophilic methane producing bacterium. Microbiology 52(3), 290–7.

Fig. 1.

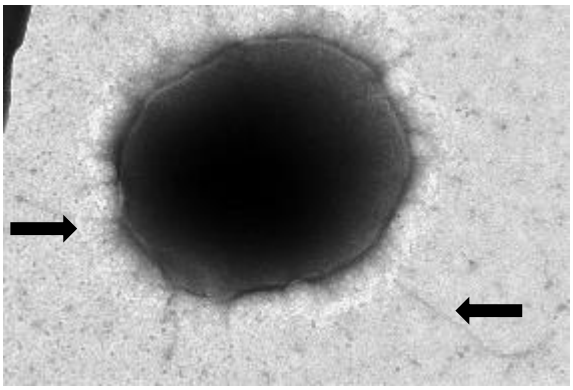

Fig. 1. Transmission electron micrograph of negatively stained cell of SLHTYRO^{T}. The arrows indicated the presence of two flagella.

Fig. 2.

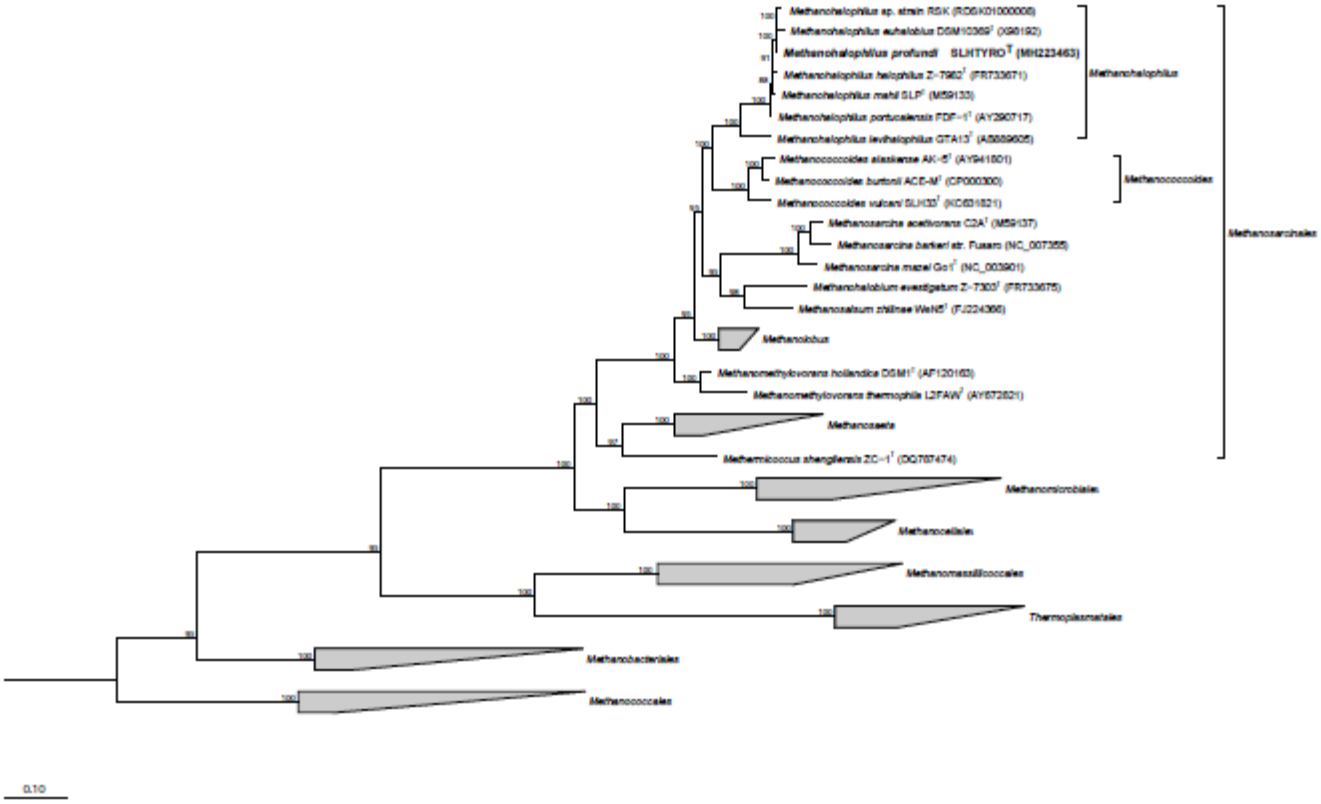

Fig. 2. Maximum likelihood tree based on 16S rRNA gene sequences of strain SLHTYRO^{T} and the type strains of related taxa within the *Methanosarcinaceae*. *Methanopyrus kandleri* served as outgroup. Numbers at node indicate branches with bootstrap values >70% (1,000 replicates) in maximum likelihood trees (RaxML and PROTGAMMA-DAYHOFF rate distribution model analysis of 1,226 nucleic acid positions). The sequence obtained in this study is marked in bold type. Bar. 0.1 substitutions per nucleotide.

Fig. 3.

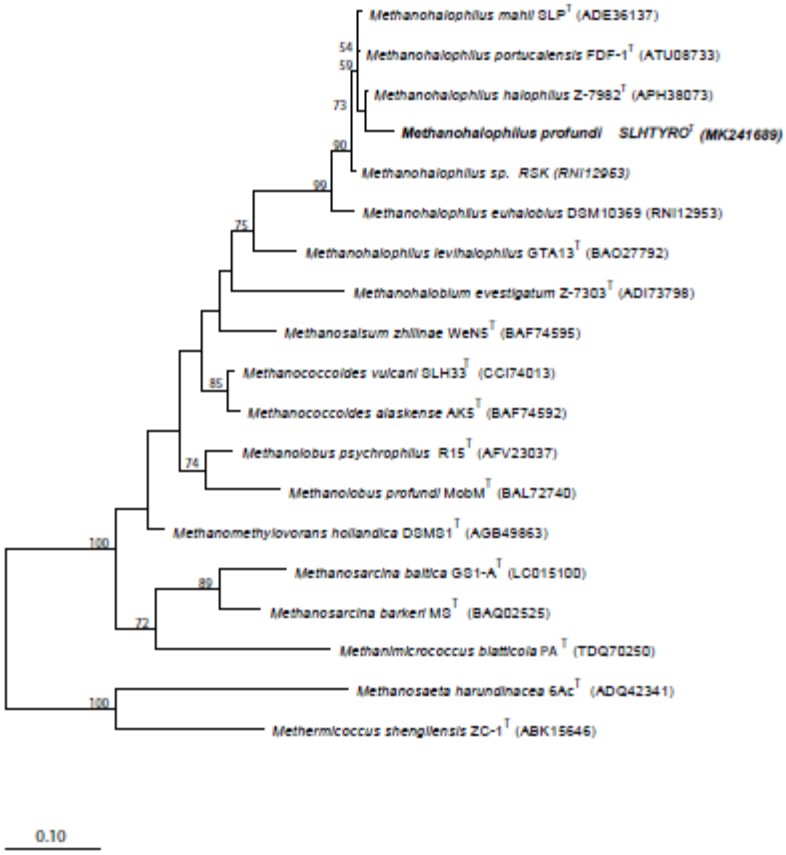

Fig. 3. Phylogenetic tree showing the relationship between strain SLHTYRO^T and methanogenic *Archaea* related to members of the genus *Methanohalophilus* in public databases, based on a Maximum Likelihood with RaxML and PROTGAMMA-DAYHOFF rate distribution model analysis of an alignment of *mcrA* genes (228 amino acid positions). The sequence obtained in this study is marked in bold type. Bootstrap values (%) are based on 1,000 replicates and are indicated at nodes for branch bootstrap support values ≥50%. Bar, 0.1 substitutions per site.

Fig. 4.

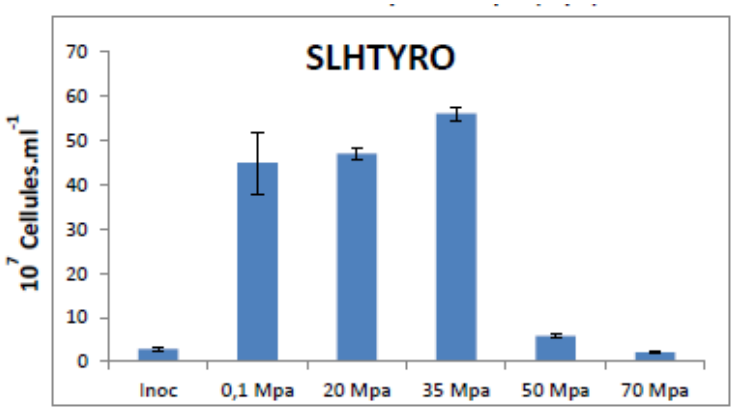

Fig. 4. Growth of strain SLHTYRO^T under hydrostatic pressure. The cells densities were measured by flow cytometry as described in the material and methods. The 0.1 MPa point correspond to a penicillin flask with a N_2 atmosphere under 0.1 MPa incubated as control. All the cultures were stopped when the control flask reached around 5 x 10^7 cells mL^{-1}.

Table 1. Characteristics differentiating strain SLHTYRO^T from *Methanohalophilus* species. Species: 1, *Methanohalophilus profundi* SLHTYRO^T (this study); 2, *M. portucalensis* FDF-1^T[2]; 3, *M. mahii* SLP^T [25]; 4, *M. halophilus* Z-7982^T [38]; 5, *M. levihalophilus* GTA13^T [14], 6, *Methanohalophilus euhalobius*[10,24] ; 7, *Methanohalophilus* strain RSK [12]. Legend: +, positive; -, negative. nd: not determined.

	1	2	3	4	5	6	7
Origin	Deep-sea Tyro basin Eastern Mediterranean Sea	Terrestrial Salinarium in Figieria da Foz Portugal	Terrestrial Great Salt Lake USA	Terrestrial Saline cyanobacterial mat Australia	Terrestrial Aquifer Japan	Saline subsurface water, Oil field Russia	Kebrit Brine-seawater interface Red Sea
Depth	3,350 m	surface	surface	surface	100-800 m	subsurface water	1468 m
Cell morphology	Cocci	Irregular cocci	Irregular cocci	Irregular cocci	Cocci	Irregular cocci	Cocci
Extracellular Structure	Flagella	-	-	-	-	-	-
Motility	+	-	-	-	-	-	-
Temperature range (°C)							
Min-(opt)-max	12-(30)-37	nd-(37-42)-nd	10-(35)-45	18-(26-36)-42	20-(35)-40	15-(28-37)-50	15-(30-33)-40
pH range							
Min-(opt)-max	6.5-(7.5)-8.2	6.6-(7.4)-7.8	6.8-(7.5)-8.2	6.3-(7.4)-7.4	6.2-(7-7.5)-8.3	5.8-(6.8-7.3)-8.0	5.5-(6.5)-7.5
NaCl concentration							
(g.L^{-1})							
Min-(opt)-max	45-(135)-240	70-(115)-175	30-(115)-175	17-(70)-150	11-(23)-75	1-(6)-14	2-(5-10)-20
Piezophilic growth	+	-	-	-	-	ND	ND
35 MPa							
Genome							
Acc. number	QBKB00000000	CP017881	CP001994	CP017921		RJJF00000000.1	RDSK00000000
DNA G+C (mol%)	42.7*	41.9*	42.6*	42.4*	44(HPLC)	42.4*	41.87*
Size (bp)	1,829,200	2,080,338	2,012,424	2,021,331	ND	1,875,790	1,969,036
Nº. of contigs	4	17	1	6		25	18
Nº of ORFs	2,027	2,124	2,016	2,047		1,990	2,053
Unique proteins&	149	166	144	125		182	192
rRNA operon	3	1	3	1		1	1
(16S-23S-5S)							
CRISPR	0	1	0	0		2	3

*: Genome analysis

&: Unique proteins within *Methanohalophilus* genus.

Table 2.

Description of *Methanohalophilus profundi* sp. nov.

Genus name	*Methanohalophilus*
Species name	*Methanohalophilus profundi*
Species status	sp. nov.
Species etymology	pro.fun'di. L. gen. n. *profundi* of the depth of the sea.
Description of the new taxon and diagnostic traits	Cells are regular cocci (diameter 0.7-1.2 µm), slightly motile and occur singly or in pairs. Colonies are mustard-coloured, circular and convex. Obligately anaerobic. Pure cultures of SLHTYRO^T grew between 12 and 37°C. No growth was detected at 7 and 40°C. Growth of isolate SLHTYRO^T was observed at range of NaCl concentrations (45 to 240 g L^{-1}; optimum at 135 g L^{-1}) representative of the *in situ* environmental conditions. No growth was observed at 30 and 255 g L^{-1} NaCl. Isolate SLHTYRO^T grew between pH 6.5 and 8.0, with an optimum pH around 7.5. No growth was detected at pH 6.0 and 8.5.. Piezophilic, growing optimally under 35 MPa (pressure range: 0-50 MPa). Growth occurs preferentially on methylated compounds (TMA, DMA, MMA, methanol) No growth was observed on DMS, glycine betaine, choline and *N*,*N*-dimethylethanolamine, formate, acetate and H_2/CO_2. When supplemented individually in the basal medium with vitamin mixture (Medium 141, DSMZ), yeast extract (0.02 %), peptone, tryptone, casamino acids stimulated the growth yield. Ammonium, nitrate, nitrite, gelatin and yeast extract were used as nitrogen sources in a nitrogen free medium (ammonium chloride and yeast extracts are removed from the medium).. Urea was not used. The presence of oxygen (>2%) in the gas phase completely inhibited growth.
Country of origin	GREECE
Region of origin	Tyro deep-sea hypersaline anoxic basin located at a depth of 3,350 m in the Eastern Mediterranean Sea closed to the Strabo trench
Date of isolation (dd/mm/yyyy)	16/10/2012
Source of isolation	Brine, deep sea hypersaline basin

Sampling date (dd/mm/yyyy)	17/9/2011
Latitude (xx°xx'xx"N/S)	35°1'12'' N
Longitude (xx°xx'xx"E/W)	22°0'36''E
Altitude (meters above sea level)	-
16S rRNA gene accession nr.	MH223463.
Genome accession number [RefSeq; EMBL; …]	QBKB00000000
Genome status	Complete
Genome size	1,829 Kbp
GC mol%	42.7
Number of strains in study	1
Source of isolation of non-type strains	-
Information related to the Nagoya Protocol	Hellenic republics, Ministry of Foreign affairs, No.: 6357.1/AS 37648
Designation of the Type Strain	SLHTYRO^{T}
Strain Collection Numbers	=DSM 108854=JCM 32768

11

12

Supplementary material

Methanohalophilus profundi sp. nov., a methylotrophic halophilic piezophilic methanogen isolated from a deep hypersaline anoxic basin.

Stéphane L'Haridon[a]*, Hani Haroun[a], Erwan Corre[b], Erwan Roussel[a], Chalopin Morgane[a], Patricia Pignet[a], Charlotte balière[a], Violetta la Cono[c], Mohamed Jebbar[a], Michail Yakimov[c] and Laurent Toffin[a].

Fig. S1.

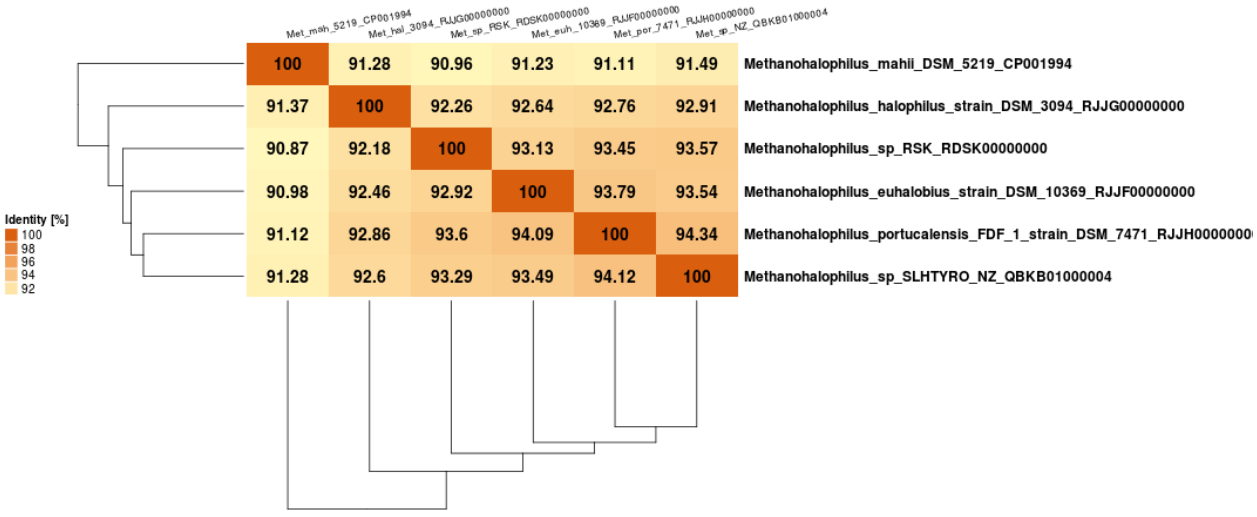

Fig. S1. Average Nucleotide Identity (ANI) among described *Methanohalophilus* spp. with available genomes. The ANI values, given in percent, were calculated using the ANI-Matrix calculator (Rodriguez-R and Konstantinidis, 2016).using the phylogenomic pipeline EDGAR 2.3.

Fig. S2.

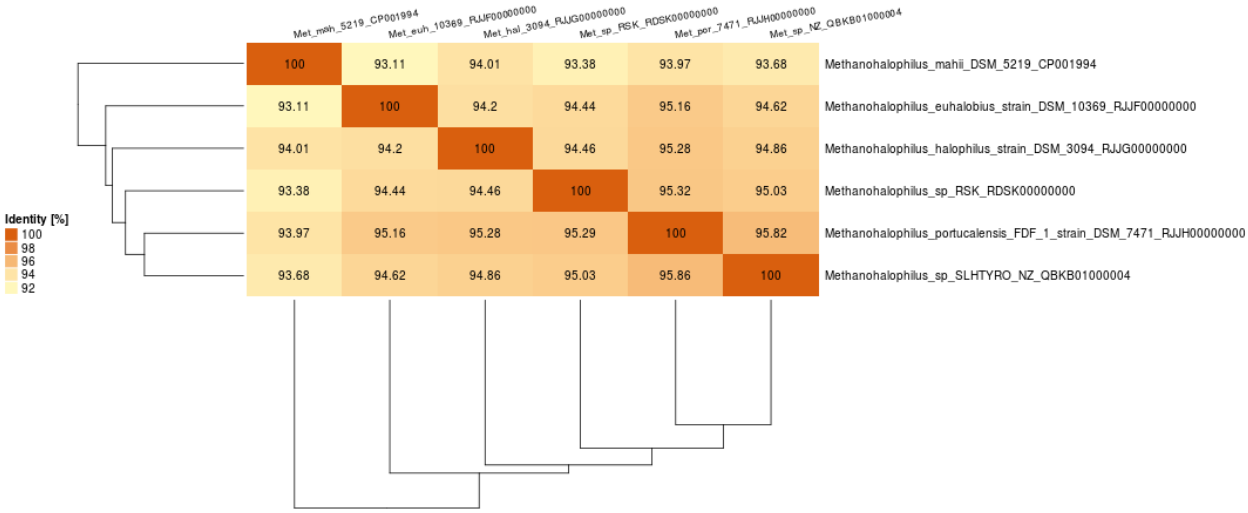

Fig. S2. Average Amino acid Identity (AAI) values among described *Methanohalophilus* spp. with available genomes. The AAI values, given in percent, were calculated using the AAI-Matrix calculator (Rodriguez-R and Konstantinidis 2016) using the phylogenomic pipeline EDGAR 2.3.