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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA *rrnA* and *rpoB'* gene sequences of strain SWO25^T are HQ844527 and KJ644779, respectively.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

Abbreviations: PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PGS, phosphatidylglycerol sulfate; TGD-2, triglycosyl diether; DGD, diglycosyl diether

Abstract

One halophilic organism, SWO25 was isolated from salted water sampled in Algeria in the salt lake (sebkha) of Ouargla. The novel strain stained Gram-negative, and cells were pleomorphic with a red pigmentation. Strain SWO25 grew optimally at 35-45°C, at pH 6.0-8.0 and 0.05-0.25 M $MgCl_2$ concentrations. Cells were extremely halophilic, with an optimal growth at 4.3-5.1 M NaCl. The predominant membrane polar lipids were C20C20 glycerol diether derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, and triglycosyl diether and diglycosyl diether. The major respiratory menaquinone component was MK-8. Cells were highly tolerant to the presence of decane and isooctane in the growth medium. Chemotaxonomic properties supported the assignment of strain SWO25 to the genus *Haloarcula*. The DNA G+C content was 61.1 mol%. DNA-DNA hybridization and phylogenetic analyses of the 16S rRNA and *rpoB'* genes showed that strain SWO25 is distinct from known *Haloarcula* species.

Based on phenotypic, chemotaxonomic, genotypic and phylogenetic data, we describe a novel species of the genus *Haloarcula* for which the name *Haloarcula sebkhae* sp. nov. is proposed. The type strain is SWO25^T (=CIP 110583^T=JCM 19018^T).

Extremely halophilic archaea of the class *Halobacteria* Grant *et al.* 2002 thrive in hypersaline environments worldwide, such as salt lakes or solar salterns. At the present time, many of the 59 genera belonging to the *Halobacteria* (*Haloadaptatus, Halapricum, Haloarchaeobius, Haloarcula, Halobaculum, Haloferax, Halomicroarcula, Halomicrobium, Halopiger, Haloquadratum, Halorubellus, Halorubrum, Halorussus, Halosimplex, Halostella, Haloterrigena, Natrialba, Natrinema, Natronoarchaeum*) have been reported to harbour multiple copies of 16S rRNA genes [1-7]. This feature both impairs phylogenetic studies and introduces a bias in molecular-based diversity evaluation of environmental samples, as the 16S rRNA gene is considered the standard marker for these applications [8]. While 1-1.3% nucleotide substitution between the 16S rRNA genes from two strains is the threshold generally accepted for distinguishing species [9], one study indicates that intragenomic heterogeneity remains below 1% for 87.5% of the 952 prokaryotic genomes displaying such feature [8]. A few genera belonging to the family *Haloarculaceae* Gupta *et al.* 2016 (*Haloarcula* Torreblanca *et al.* 1986 and the closely related genera *Halomicroarcula* Zhang and Cui 2014, *Halomicrobium* Oren *et al.* 2002 and *Halosimplex* Vreeland *et al.* 2003) [10, 11], display several copies of the 16S rRNA gene, with a heterogeneity of 3-9.4% [12-14].

Intragenomic 16S rRNA gene heterogeneity was first detected in *Haloarcula marismortui* Oren *et al.* 1990 [15, 16], and then shown as a characteristic common to all *Haloarcula* strains, some displaying up to four copies of the gene [17]. In fact, sequencing of the genome of *Har. marismortui* ATCC 43049 has revealed the presence of three copies, named *rrnA*, *rrnB*, and *rrnC* [18]. A phylogenetic analysis of *Haloarcula* and *Halomicrobium* 16S rRNA genes has demonstrated that two monophyletic clusters could be defined, type I including *Har. marismortui rrnA* gene, and type II, corresponding to *Har. marismortui rrnB* and *rrnC*

[12]. Moreover, the finding that the *pyrD* gene encoding dihydroorotate oxidase was found immediately upstream all type I sequences, led to the hypothesis that type I sequences represented the orthologous gene derived from the ancestral gene, while paralogous type II sequences could have arisen through gene duplication [4]. The use of the orthologous 16S rRNA type I *rrnA* gene may resolve the problem in the phylogenetic analyses of *Haloarcula* species.

In a previous study on extremely halophilic archaea from Algerian hypersaline environments, we isolated thirteen strains of *Haloarcula* spp. A phylogenetic analysis of the two copies of the 16S rRNA gene showed they form a distinct cluster from the currently nine validated species of *Haloarcula*. Moreover, sequence alignments revealed nucleotide substitutions very specific to this cluster, which includes the two solvent-tolerant strains OHF-1 and OHF-2 isolated from French commercial salt [19-21]. This suggests that these *Haloarcula* strains may constitute a new species. The aim of this study is a description of an Algerian strain belonging to this new species, *Haloarcula sebkhae* sp. nov, which represents a novel species within the family *Haloarculaceae*. The characterization of this strain was achieved by following a polyphasic approach according to the minimal standards for the description of new taxa in the order *Halobacteriales* Grant and Larsen 1989 [22].

A new strain named SWO25 was isolated from salted water (pH = 8.1, 4.8 M NaCl) sampled in 1997 in the sebkha of Ouargla, a salt lake located in the Algerian Sahara (31°57'N, 5°20'E) [19]. Strain SWO25 was isolated in 1% (w/v) peptone (Merck), 0.1% (w/v) meat extract (Merck), 0.2% (w/v) yeast extract (Merck), 4.3 M NaCl, pH 7.2, following incubation at 37°C. The medium was solidified with 2% agar. Strain SWO25 as well as *Har. quadrata* JCM11927^T, *Har. argentinensis* DSM 12282^T, *Har. salaria* JCM15759^T, *Har. amylolytica* JCM 13557^T and *Har. tradensis* JCM15760^T, were routinely grown aerobically in DSMZ *Halobacteria* medium 372 (http://www.dsmz.de) at 37°C [19].

Cell morphology and motility were examined by phase-contrast microscopy in exponentially growing liquid cultures. Gram staining was performed according to Dussault [23], and pigmentation was determined by observing colonies on medium 372 agar plates after incubation at 37°C for 1-2 weeks. Growth range and optima for NaCl and Mg^{2+} concentrations, temperature, and pH were examined in medium 372. Anaerobic growth in the presence of L-arginine, $NaNO_3$ or DMSO (each at 5 g L^{-1}) was tested for 15 days in the dark in completely filled 20 mL tubes containing medium 372, using a BBL Gaspak anaerobic jar (Becton, Dickinson and Cie, Sparks, MD, USA). Single-carbon source utilization tests were performed in BC medium and BC medium with low yeast extract (1 and 0.1 g L^{-1} respectively) [24] supplemented with appropriate carbon source (500 µg mL^{-1}) in microtiter plates. Following incubation for 10 days, phenol red (85 µg mL^{-1}) was added to the medium to detect the production of acid from the different carbon sources. Growth was determined by monitoring the turbidity at 600 nm of 10 mL-liquid cultures incubated at 37°C. Growth in the presence of organic solvents (isooctane, cyclooctane, decane) was tested at 30°C for 13 days as described by Usami *et al*. [20, 21] in tubes containing 2 mL medium 372 and 1 mL solvent or sterile paraffin as control.

111 Colonies formed on agar plates were red-pigmented with a circular regular shape (diameter
112 2-6 mm) after 1-2 weeks of growth. The cells of strain SWO25 were stained Gram-negative
113 and pleomorphic with small coccoid, triangular, and short rod-like morphotypes (1-3 µm)
114 when grown in liquid medium. Growth occurred from 1.7-5.1 M NaCl, with optimal growth
115 at highest NaCl concentrations (4.3-5.1 M). The organisms had an optimal growth temperature
116 between 35 and 45°C, and could not grow below 25°C or above 55°C. Mg^{2+} was not required
117 for growth, but optimal growth occurred at 0.05-0.25 M $MgCl_2$ concentrations. The optimum
118 pH for growth was between 6.0 and 8.0 with growth occurring in the range 5.0 to 9.0.
119 Anaerobic growth was possible in the presence of nitrate and DMSO but not L-arginine.
120 Growth occurred in the presence of isooctane and decane, but not with cyclooctane in a growth
121 medium containing 3.4 or 5.1 M NaCl. High tolerance to decane was previously observed by
122 Usami and co-workers for *Haloarcula* strains OHF-1, OHF-2 and *Har. argentinensis* [20,21].
123 Strain SWO25, like OHF-1 and OHF-2, was more tolerant to isooctane than *Har.*
124 *argentinensis*, whatever the NaCl concentration. Therefore, tolerance to the presence of
125 isooctane in the growth medium may constitute a differential characteristic of the novel
126 species.

127 All biochemical tests were performed aerobically. Assays for catalase and oxidase
128 activities, nitrate reduction with gas production, formation of indole, and hydrolysis of gelatin,
129 Tween-80, starch, and casein, were performed in 372 agar medium according to standard or
130 modified procedures previously described by Oren *et al.* [22, 25]. Oxidase and catalase tests
131 were positive. Gelatin, starch and Tween 80 were hydrolyzed, but not casein. Nitrate reduction
132 occurred with gas production; no indole formation was detected. Utilization of D-glucose, D-
133 galactose, D-xylose, D-ribose, glycerol and sorbitol occurred with acid production, but
134 utilization of sucrose, D-trehalose, D-fructose, cellobiose and L-lysine resulted in growth
135 without acid production. Growth with mannose, D-lactose, L-aspartic acid or D-raffinose as

sole source of carbon was not possible. Sensitivity to antimicrobial agents was determined on 372 agar plates by using filter-sterilized antibiotics. Strain SWO25 was found to be sensitive to novobiocin (5 µg) and anisomycin (50 µg) but resistant to ampicillin (30 µg), bacitracin (10 µg), erythromycin (15 µg), neomycin (30 µg), chloramphenicol (30 µg), and rifampicin (5 µg). Detailed results of the phenotypic tests and the nutritional features of strain SWO25 are given in the species description. Table 1 shows the differential characteristics of strain SWO25 in comparison with those of type strains belonging to closely related species of the *Haloarcula* genus, *Har. argentinensis* [24], *Haloarcula japonica* Takashina *et al.* 1991 [26], *Har. salaria* and *Har. tradensis* [27].

Polar lipids were extracted from 40 mL culture pellets, using methanol/chloroform/aqueous phase (1:1:0.9, v/v) as described by Bligh and Dyer [28]. Dried lipids extracts were dissolved in methanol/chloroform (2:1, v/v). Polar lipids were separated by one- and two-dimensional TLC by using silica gel plates (Merck silica gel 60 F_{254}) and visualized as described [29], except for glycerol-containing lipids which were revealed with anthrone [30]. The major polar lipids as determined by one and two-dimensional TLC (Supplementary Fig. S1 and Supplementary Fig. S2) were the C20C20 glycerol diether derivatives of phosphatidylglycerol, methyl-phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether, diglycosyl diether. Analysis of respiratory quinones was carried out by the DSMZ Identification Service, (Braunschweig, Germany). Respiratory lipoquinones were extracted from freeze dried cells as described by Tindall *et al.* [31], purified by TLC and analysed by reversed-phase HPLC. The major respiratory lipoquinone was the menaquinone MK-8 (92%), while other lipoquinones were found less abundantly (MK-7: 5% and MK-8(H_2): 3%). These results are in accordance with those previously published for other *Haloarcula* species [32].

For DNA G+C content and DNA–DNA hybridization experiments, the DNA was purified by chromatography on hydroxyapatite according to Cashion *et al.* [33]. The DNA G+C content and DNA–DNA hybridization were carried out by the DSMZ Identification Service. The DNA G+C content for strain SWO25, as determined by HPLC according to Mesbah *et al.* [34] was 61.1 mol%. This value is in the range of those encountered for *Haloarcula* species (60.1-64.7 mol%) [27]. DNA–DNA hybridizations were carried out according to the thermal denaturation and renaturation method [35, 36] using a Cary spectrophotometer equipped with a Peltier temperature controller (Varian). The DNA-DNA hybridization values between strain SWO25 and the type strains of other species of *Haloarcula* are indicated in Table S1. Strain SWO25 presented the highest DNA-DNA hybridization percentage with *Har. japonica* JCM 7785^{T} (68.0-67.2%), *Har. tradensis* JCM 15760^{T} (68.8-66.0%), and *Har. salaria* JCM 15759^{T} (68.0-66.0%). These values are consistent with the 65%-75% range generally accepted for belonging to the same species. DNA-DNA hybridization values with all other *Haloarcula* species were much lower (Table S1), clearly indicating that strain SWO25 does not belong to any of these species.

Genomic DNA was extracted from 20 mL exponential phase culture and purified according to the Qiagen genomic DNA extraction procedure (Qiagen, Germany). The 16S rRNA genes were amplified using specific primers, cloned and sequenced as previously described [19]. PCR-mediated amplification and sequencing of the RNA polymerase subunit B (*rpoB'*) genes were performed as described by Minegishi *et al.* [37]. The phylogenetic analyses were performed on the following website: http://www.phylogeny.fr [38], as previously described [19]. Strain SWO25 harbors two copies of the 16S rRNA gene, belonging to type I and type II sequences [19]. All the sequences used to construct the phylogenetic tree were designated *rrnA* for type I sequences, and *rrnB* for type II sequences, as defined in [19]. Phylogenetic analysis indicated that strain SWO25 is distinct from known species among the *Haloarcula*

genus (Fig.1). Regarding the orthologous *rrnA* gene; the closest strains were *Har. argentinensis* JCM 9737^T (98.8% identity), *Har. tradensis* JCM 15760^T (97.6% identity), and *Har. salaria* JCM 15759^T (95.1% identity). Similar observations can be inferred from phylogenetic analysis using the paralogous (*rrnB*) 16S rRNA genes. The *rrnB* phylogenetic analysis supports clustering of strain SWO25 with strains OHF-1 and OHF-2 (Fig.1). The phylogenetic analysis of the *rpoB'* gene (Fig. 2) confirmed these observations (95.6% identity with the *rpoB'* sequence of the three strains), but the *rpoB'* sequence of *Har. japonica* JCM 7785^T was also closely related to that of SWO25 (95.7 % identity). Finally, DNA-DNA hybridization analysis supports that strain SWO25 is closely related to the species *Har. japonica*, *Har. tradensis*and *Har. salaria*, while the phylogenetic data support that strain SWO25 is more closely related to the species *Har. argentinensis*, *Har. tradensis* and *Har. salaria*. However, biochemical data (Table 1) do not support that SWO25 is likely to belong to any of these species. Therefore taken together, chemotaxonomic (Table 1) and phylogenetic (Fig. 1) analyses indicate that strain SWO25 is distinct from other *Haloarcula* species and constitutes a novel species.

Based on the phenotypic, chemotaxonomic, genotypic and phylogenetic data presented in this paper, strain SWO25 is considered to represent a novel species of the genus *Haloarcula* into the family *Haloarculaceae* for which the name *Haloarcula sebkhae* sp. nov. is proposed.

Description of *Haloarcula sebkhae* Barreteau *et al.*, 2018 sp. nov.

Haloarcula sebkhae (N.L. gen. n. *sebkhae* of a sebkha, the Arabic name for a salt pan, defined geologically as flat expanses of ground covered with salt and other minerals, usually found in hot deserts).

Cells Gram-stain-negative, non-motile are pleomorphic with coccoid, triangular, and short rod-like morphotypes (1-3 µm), with no observable mobility. Colonies are circular with a 2-6 mm diameter and entire margin, smooth, shiny, red pigmented on *Halobacteria* DSMZ medium 372 after 1-2 weeks of incubation at 37°C. Growth occurs with 1.7-5.1 M NaCl (optimum 4.3-5.1 M), at pH 5.0-9.0 (optimum pH 7.0-8.0) and at 25-55°C (optimum 35-45°C). Mg^{2+} is not required, but optimum growth occurs with 0.05-0.25 M. Anaerobic growth was observed in the presence of nitrate and DMSO, but not in the presence of L-arginine. Nitrate is reduced to nitrite, and gas production is observed from nitrate. The major polar lipids are C20C20 glycerol diether derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether, diglycosyl diether. The major respiratory lipoquinone is MK-8 along with two minor quinones: MK-7 and MK-8(H_2). Indole formation is negative. Gelatin, starch, and Tween 80 are hydrolysed, but not casein. Catalase- and oxidase-positive. D-glucose, D-galactose, D-xylose, D-ribose, D-fructose, sucrose, D-trehalose, cellobiose, glycerol, sorbitol, and L-lysine are used as single carbon and energy source for growth. No growth occurs on D-mannose, D-lactose, L-aspartic acid, or D-raffinose as single carbon sources. Acid is produced from D-glucose, D-galactose, D-xylose, D-ribose, glycerol and sorbitol, but not from D-fructose, sucrose and D-trehalose. The organisms are susceptible to anisomycin and novobiocin, and resistant to aphidicoline, bacitracin, erythromycin, rifampicin, neomycin, ampicillin, and chloramphenicol. The cells are highly tolerant to the presence of decane and isooctane in a growth medium containing 3.4 or 5.1 M NaCl, but do not grow in the presence of cyclooctane. The DNA of strain SWO25^T has a G+C content of 61.1 mol%.

The type strain SWO25^T (=CIP 110583^T=JCM 19018^T) was isolated from salted water sampled in the sebkha of Ouargla in the Sahara (Algeria).

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Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

[1] **Cui H-L, Mou Y-Z, Yang X, Zhou Y-G, Liu H-C *et al.*** *Halorubellus salinus* gen. nov., sp. nov.and *Halorubellus litoreus* sp. nov., novel halophilic archaea isolated from a marine solar saltern. *Syst Appl Microbiol* 2012;35:30-34.

[2] **Echigo A, Minegishi H, Shimane Y, Kamekura M, Itoh T *et al.*** *Halomicroarcula pellucida* gen. nov., sp. nov., a non-pigmented, transparent-colony-forming, halophilic archaeon isolated from solar salt. *Int J Syst Evol Microbiol* 2013;63:3556-3562.

[3] **Makhdoumi-Kakhki A, Amoozegar MA, Bagheri M, Ramezani,M, Ventosa A.** *Haloarcheobius iranensi*s gen. nov., sp. nov., an extremely halophilic archaeon isolated from a saline lake. *Int J Syst Evol Microbiol* 2011;62:1021-1026.

[4] **Minegishi H, Kamekura M, Kitajima-Ihara T, Nakasone K, Echigo A *et al.*** Gene orders in the upstream of 16SrRNA genes divide genera of the family *Halobacteriaceae* into two groups. *Int J Syst Evol Microbiol* 2012;62:188-195.

[5] **Song H.S, Cha I-T, Yim KY, Lee H-W, Hyun D-W *et al.*** *Halapricum salinum* gen. nov., sp. nov., an extremely halophilic archaeon isolated from non-purified solar salt. *Ant van Leeuw JG* 2014;105:976-986.

[6] S**ong HS, Cha I-T, Rhee J-K, Yim K-J, Kim AY *et al.*** *Halostella salina* gen. nov, sp. nov., an extremely halophilic archaeon isolated from solar salt. *Int J System Evol Microbiol* 2016;66:2740-2746.

[7] **Xu W-D, Zhang W-J, Han D, Cui H-L, Yang K.** *Halorussus ruber* sp nov., isolated from an inland salt lake of China. *Arch Microbiol* 2015;197:91-95.

[8] **Sun D-L, Jiang X, Wu QL, Zhou N-Y.** Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. *Appl Env Microbiol* 2013;79:5962-5969.

[9] **Stackebrandt E, Ebers J.** Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* 2006;33:152-155.

[10] **Amoozeagar MA, Siroosi M, Alashgahi S, Smidt, Ventosa A.** Systematics of haloarchaea and biotechnological potential for their hydrolytic enzymes. *Microbiology* 2017;63:623-645.

[11] **Gupta RS, Naushad S, Fabros R, Adeolu M.** A phylogenomic reappraisal of family-level divisions within the class *Halobacteria*: proposal for divide the order *Halobacteriales* into the families *Halobacteriaceae*, *Haloarculaceae* fam. nov., and *Halococcaceae* fam. nov., and the order *Haloferacales* into the families, *Haloferacaceae* and *Halorubraceae* fam. nov. *A Van Leew JG* 2016;109:565-587.

[12] **Cui H-L, Zhou P-J, Oren A, Liu S-J.** Intraspecific polymorphism of 16S rRNA genes in two halophilic archaeal genera, *Haloarcula* and *Halomicrobium*. *Extremophiles* 2009;13:31-37.

[13] **Vreeland RH, Straight S, Krammes J, Dougherty K, Rosenweigh WD *et al.*** *Halosimplex carlsbadense* gen. nov., sp., nov., a unique halophilic archaeon with three 16S rRNA genes, that grows only on defined medium with glycerol and acetate or pyruvate. *Extremophiles* 2002;6:445-452.

[14] **Zhang W-J, Cui H-L.** *Halomicroarcula salina* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* 2015;65:1628-1633.

[15] **Amann G, Stette, KO, Llobet-Brosa E, Amann R, Antón J.** Direct proof for the presence and expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui*. *Extremophiles* 2000;4:373-376.

[16] **Mylvaganam S, Dennis PP.** Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* 1992;130:399-410.

[17] **Gemmell RT, McGenity TJ, Grant WD.** Use of molecular techniques to investigate possible long-term dormancy of halobacteria in ancient halite deposits. *Ancient Biomol* 1998;2:125-133.

[18] **Baliga NS, Bonneau R, Facciotti MT, Pan, M, Glusman G *et al.*** Genome sequence of *Haloarcula marismortui*: a halophilic archaeom from the Dead Sea. *Genome Res* 2004;14:2221-2234.

[19] **Imadalou-Idres N, Carré-Mlouka A, Vandervennet M, Yahiaoui H, Peduzzi J *et al.*** Diversity and antimicrobial activity of cultivable halophilic Achaea from three Algerian sites. *J. Life Sci* 2013;10:1057-1069.

[20] **Usami R, Fukushima T, Mizuki T, Inoue A, Yoshida Y *et al.*** Organic tolerance of halophilic Archaea. *Biosci Biotechnol Biochem* 2003;67:1809-1812.

[21] **Usami R, Fukushima T, Mizuki T, Yoshida Y, Inoue A *et al.*** Oranic solvent tolerance of halophilic archaea, *Haloarcula* strains: effect of NaCl concentration on the tolerance and polar lipid composition. *J Biosci Bioeng* 2005;99:169-174.

[22] **Oren A, Ventosa A, Grant WD.** Proposed minimal standards for description of new taxa in the order *Halobacteriales*. *Int J Syst Bacteriol* 1997;47:233-238.

[23] **Dussault HP.** An improved technique for staining red halophilic bacteria. *J Bacteriol* 1995;70:484-485.

[24] **Ihara K, Watanabe S, Tamura T.** *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two extremely halophilic Archaea collected in Argentina. Int. *J Syst Bacteriol* 1997;47:73-77.

[25] **Oren A, Elevi R, Watanabe S, Ihara K, Corcelli A.** *Halomicrobium mukohataei* gen. nov., comb. nov., and emended description of *Halomicrobium mukohataei*. *Int J Syst Evol Microbiol* 2002;52:1831-1835.

[26] **Takashina T, Hamamoto T, Otozai K, Grant WD, Horikoshi K.** *Haloarcula japonica* sp. nov., a new triangular halophilic Archaebacterium. *System Appl Microbiol* 1990;17:177-181.

[27] **Namwong S, Tanasupawat S, Kudo T, Itoh T.** *Haloarcula salaria* sp. nov. and *Haloarcula tradensis* sp. nov. , isolated from salt in Thai fish sauce. *Int J System Evol Microbiol* 2011;61:231-236.

[28] **Bligh EG, Dyer WJ.** A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; 37:911-917.

[29] **Oren A.** Characterization of the halophilic archaeal community in saltern crystallize ponds by means of polar lipid analysis. *Int J Salt Lake Res* 1994;3:15-29.

[30] **Chiaradia L, Lefebvre C, Parra J, Marcoux J, Burlet-Schiltz O, Etienne G, Tropis M, Daffé M.** Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Sci Rep* 2017;7:12807.

[31] **Tindall BJ.** A comparative study of the lipd composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* 1990;13:128-130.

[32] **Oren A, Arahal DR, Ventosa A.** Emended description of genera of the familly *Halobacteriaceae*. *Int J System Evol Microbiol* 2009;59:637-642.

[33] **Cashion P, Holder-Franklin MA, Mc Cully J, Franklin M.** A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* 1977;81:461-466.

[34] **Mesbah M, Premachandran U, Whitman WB.** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* 1989;39:159-167.

[35] **De Ley J, Cattoir H, Reynaerts A.** The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 1970;12:133-142.

[36] **Huss VAR, Festl H, Schleifer KH.** Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* 1983;4:184-192.

[37] **Minegishi M, Kamekura M, Itoh T, Echigo A, Usami R *et al.*** Further refinement of the phylogeny of the *Halobacteriaceae* based on the full-length RNA polymersae sububit B' (*rpoB'*) gene. *Int J System Evol Microb* 2010;60:2398-2408.

[38] **Dereeper A, Gyugnon V, Blanc G, Audic S, Buffet S *et al.*** Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 2008;36:W465-W469.

Figure legends :

Figure 1:

Maximum Likelihood tree based on the 16S rRNA genes showing the relationships between strain SWO25^T and other members of the genus *Haloarcula*. All the sequences used to construct the phylogenetic tree were designated *rrnA* for type I sequences, and *rrnB* for type II sequences, as defined in [19]. The sequences of *Halobacterium salinarum* corrig. (Harrison and Kennedy 1922) Elazari-Volcani 1957 (Approved Lists 1980) (JCM 8978^T) were used as an outgroup. GenBank accession numbers are indicated in parentheses. Boostrap values (expressed as percentages of 1000 replications) higher than 80% are indicated. Bars represent expected numbers of substitutions per nucleotide position.

Figure 2:

Maximum Likelihood tree based on *rpoB'* genes showing the relationships between strain SWO25^T and other members of the genus *Haloarcula*. The sequences of *Hbt. salinarum* were used as an outgroup. GenBank accession numbers are indicated in parentheses. Boostrap values (expressed as percentages of 1000 replications) higher than 80% are indicated. Bars represent expected numbers of substitutions per nucleotide position.

Supplementary Figure S1:

One-dimensional thin-layer chromatogram on silica gel were performed for total polar lipids extracted from strain SWO25^T (a), *Har. tradensis* (b), *Har. salaria* (c), *Har. argentinensis* (d). The plates were migrated using a solvent system composed of chloroform: methanol: water (65:25:4, v/v). Total polar lipids were detected by spraying the plate with 5% phosphomolybdic acid in ethanol, followed by heating the chromatogram at 120°C (A). Phosphate-containing lipids appeared as blue spots after spraying a second plate with

molybdenum blue solution (B). A third plate was sprayed with 0.2% anthrone in sulfuric acid, then heated at 120°C, to reveal glycolipids and glycerol containing lipids (C).PG: phosphatidylglycerol; PGP: phosphatidylglycerol phosphate; PGS: phosphatidylglycerol sulfate; TGD-2: triglycosyl diether; DGD: diglycosyl diether.

Supplementary Figure S2:

Two-dimensional thin-layer chromatogram on silica gel of total polar lipids extracted from strain SWO25^{T} (a), *Har. tradensis* (b), *Har. salaria* (c), *Har. argentinensis* (d). The plates were migrated using a solvent system composed of chloroform: methanol: water (65:25:4, v/v) in the first dimension and (chloroform: methanol: acetic acid: water (80:12:15:4, v/v) in the second dimension. Total polar lipids were detected by spraying the plate with 5% phosphomolybdic acid in ethanol, followed by heating the chromatogram at 120°C (A). Phosphate-containing lipids appeared as blue spots after spraying a second plate with molybdenum blue solution (B). A third plate was sprayed with 0.2% anthrone in sulfuric acid, then heated at 120°C, to reveal glycolipids and glycerol containing lipids (C). PG: phosphatidylglycerol; PGP: phosphatidylglycerol phosphate; PGS: phosphatidylglycerol sulfate.

Figure 1

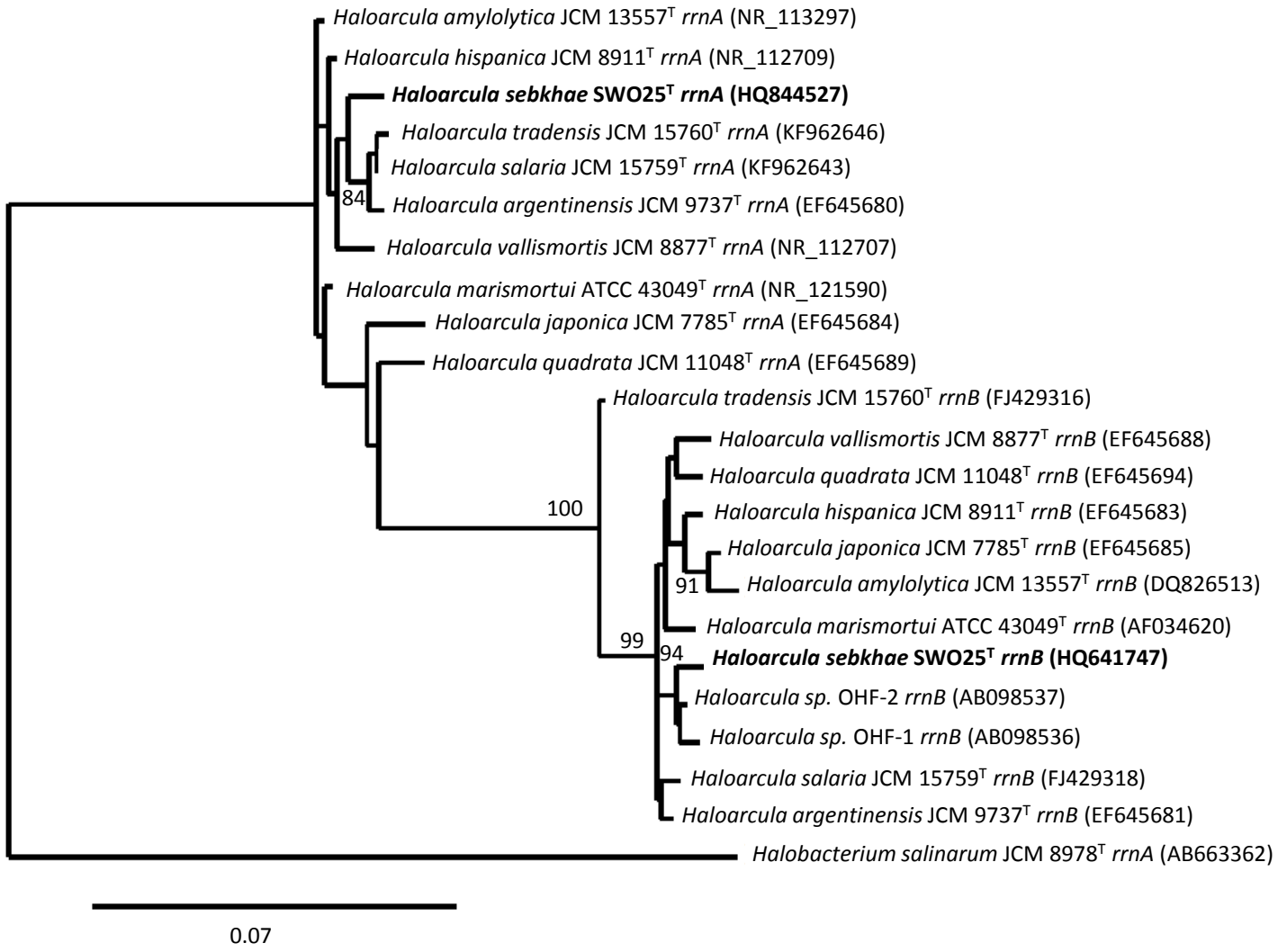


Figure 2

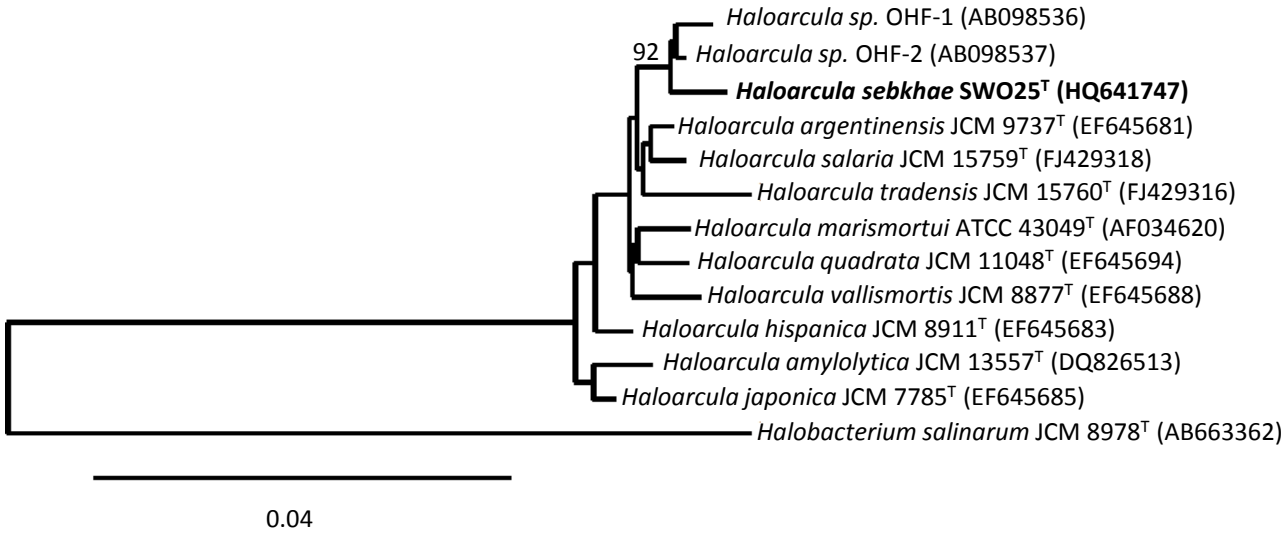
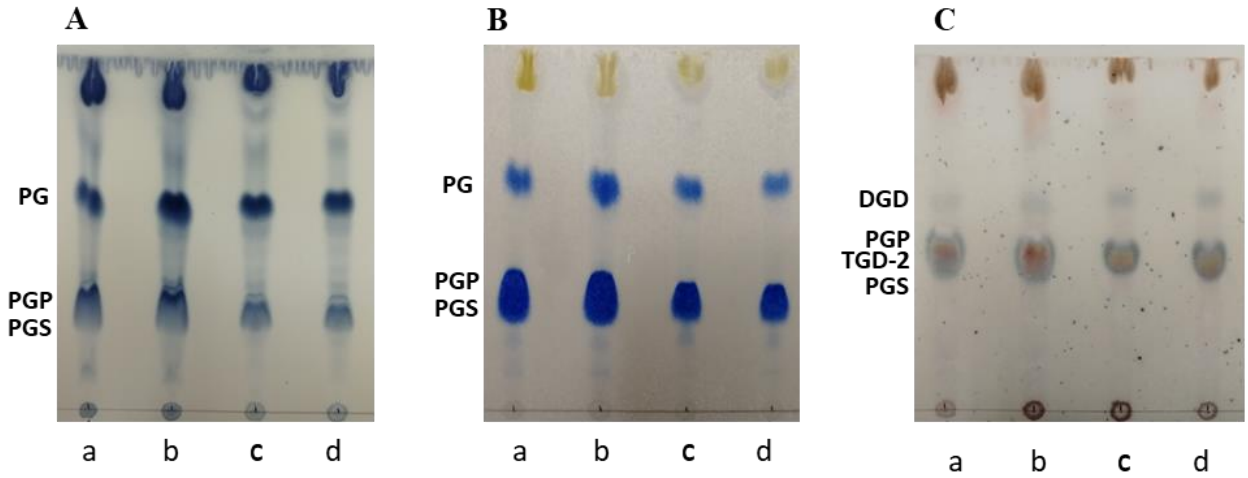


Table S1. DNA-DNA relatedness of strain SWO25^T with other species of the genus *Haloarcula*

	% DNA-DNA similarity with strain SWO25^T
Har. japonica DSM6131^T	68.8-67.2
Har. tradensis JCM15760^T	68.8-66.0
Har. salaria JCM15759^T	68.0-66.0
Har. argentinensis DSM12282^T	48.2-54.7
Har. marismortui DSM3752^T	51.2-49.8
Har. amylolytica JCM 13557^T	50.2-48.5
Har. hispanica DSM4426^T	47.5-46.1
Har. vallismortis DSM3756^T	43.4-47.3
Har. quadrata DSM11927^T	16.5-15.2

The given values are results of measurements in duplicate.

Supplementary Figure S1

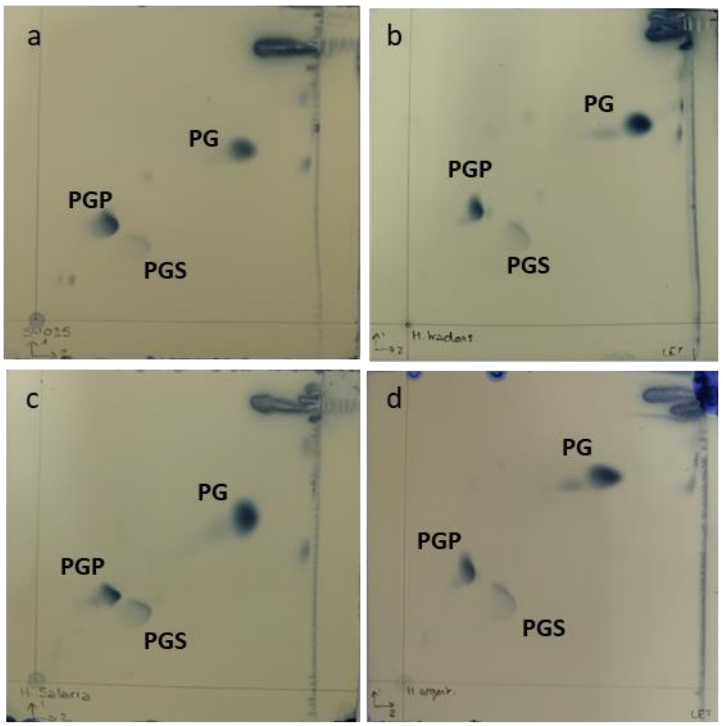


Supplementary Figure S1:

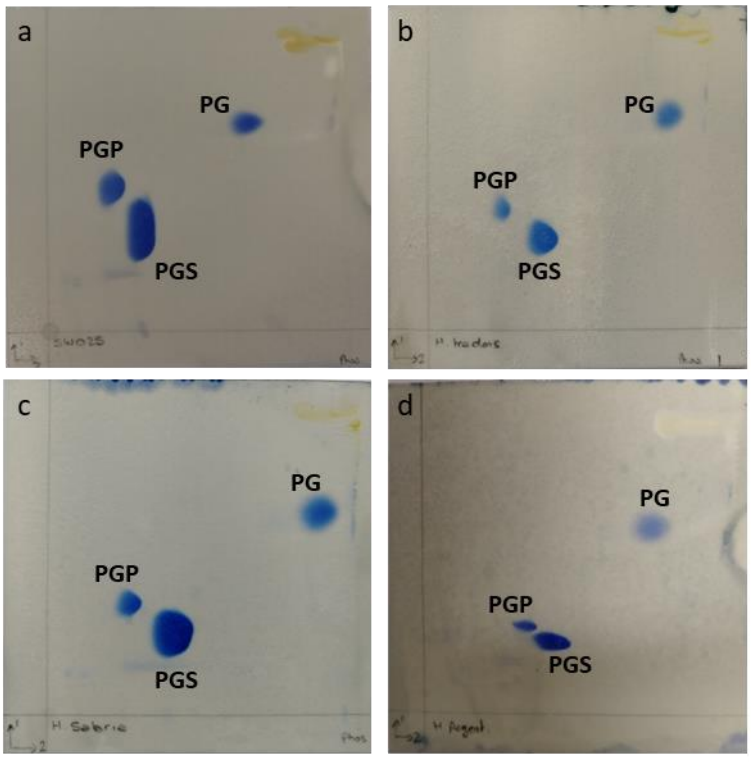
One-dimensional thin-layer chromatogram on silica gel were performed for total polar lipids extracted from strain SWO25^T (a), *Har. tradensis* (b), *Har. salaria* (c), *Har. argentinensis* (d).. The plates were migrated using a solvent system composed of chloroform: methanol: water (65:25:4, v/v). Total polar lipids were detected by spraying the plate with 5% phosphomolybdic acid in ethanol, followed by heating the chromatogram at 120°C (A). Phosphate-containing lipids appeared as blue spots after spraying a second plate with molybdenum blue solution (B). A third plate was sprayed with 0.2% anthrone in sulfuric acid, then heated at 120°C, to reveal glycolipids and glycerol containing lipids (C).PG: phosphatidylglycerol; PGP: phosphatidylglycerol phosphate; PGS: phosphatidylglycerol sulfate; TGD-2: triglycosyl diether; DGD: diglycosyl diether.

Supplementary Figure S2

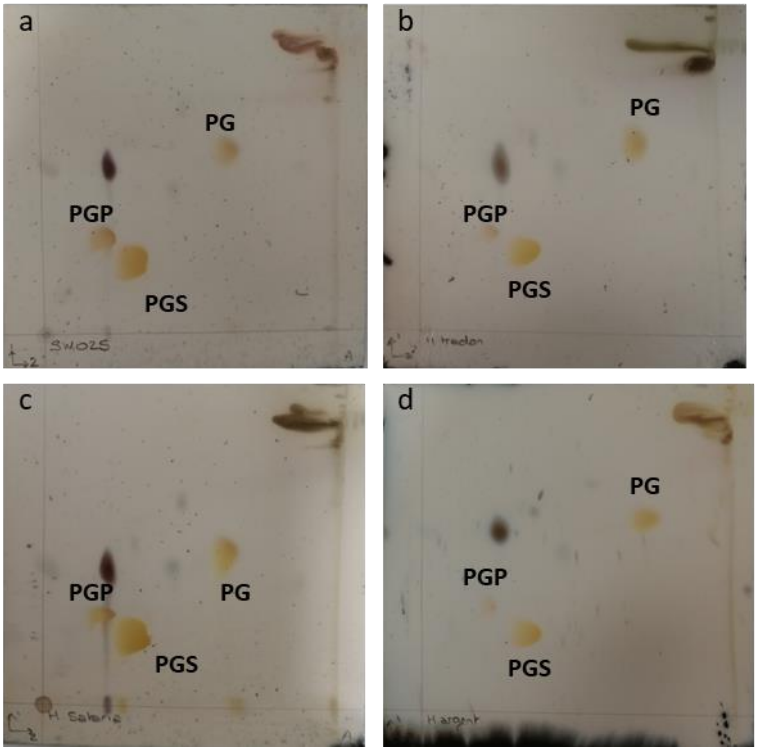
A



B



C



Supplementary Figure S2:

Two-dimensional thin-layer chromatogram on silica gel of total polar lipids extracted from strain SWO25^{T} (a), *Har. tradensis* (b), *Har. salaria* (c), *Har. argentinensis* (d). The plates were migrated using a solvent system composed of chloroform: methanol: water (65:25:4, v/v) in the first dimension and (chloroform: methanol: acetic acid: water (80:12:15:4, v/v) in the second dimension. Total polar lipids were detected by spraying the plate with 5% phosphomolybdic acid in ethanol, followed by heating the chromatogram at 120°C (A). Phosphate-containing lipids appeared as blue spots after spraying a second plate with molybdenum blue solution (B). A third plate was sprayed with 0.2% anthrone in sulfuric acid, then heated at 120°C, to reveal glycolipids and glycerol containing lipids (C). PG: phosphatidylglycerol; PGP: phosphatidylglycerol phosphate; PGS: phosphatidylglycerol sulfate.

Table 1

Differential characteristics between strains SWO25^T and the type strains of closely related species of the genus *Haloarcula*.

Strains: 1, SWO25^T (this study); 2, *Har. japonica* TR-1^T [26]; 3, *Har. salaria* HST03^T [27]; 4, *Har. tradensis* HST01-2R^T [27]; 5, *Har. argentinensis* arg-1^T [24].

+, positive; –, negative; R, resistant; S, sensitive. ND, No data available.

Characteristic	**1**	**2**	**3**	**4**	**5**
Cell morphology	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic
Pigmentation	Red	Red-orange	Red	Red	Orange-red
Range (optimum) growth:					
NaCl (M)	1.7-5.1 (4.3-5.1)	2.5-5.1 (3.4)	2.6-5.1 (3.4-4.3)	2.6-5.1 (3.4-4.3)	2-4.5 (3-3.5)
Mg^{2+} (M)	0-0.3 (0.05-0.25)	0.04-0.3 (0.08)	0.08-0.4 (0.16-0.24)	0.08-0.4 (0.16-0.24)	0.03-0.15 (0.1)
Temperature (°C)	25-55 (35-45)	24-45 (42-45)	15-45 (37)	15-45 (37)	25-55* (35-40)*
pH	5-9 (6-8)	6-8 (7-7.5)	6-8 (7)	6-8 (7)	5-9* (6-7)*
Sensitivity to:					
Bacitracin	R	S	S	S	R*
Rifampicin	R	R	R	R	R*
Ampicillin	R	R	R	R	R*
Chloramphenicol	R	R	R	R	R
Novobiocin	S	S	R	S	S
Hydrolysis of:					
Gelatin	+	-	-	-	+*
Starch	+	-	+	+	+*
Casein	-	-	-	-	-*
Tween 80	+	ND	+	+	+*
Nitrate reduction	+	+	ND	ND	+*
Gas production from $NaNO_3$	+	+	-	-	+*
Indole formation	-	+	-	-	-*
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Utilization of:					
D-Glucose	+	+	+	+	+
D-Galactose	+	+	-	-	+
Glycerol	+	+	+	+	+
Sorbitol	+	+	-	-	+*
L-aspartic acid	-	ND	+	+	-*
L-Lysine	+	ND	-	+	-*
Acid production from:					
D-Glucose	+	+	-	-	+
D-Galactose	+	+	-	-	+
DNA G+C content (mol%)	61.1	63.3	61.6	62.2	62.0

* Determined in this study