Characterization of *Haloarcula terrestris* sp. nov., and reclassification of a *Haloarcula* species based on a taxogenomic approach

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Abbreviations: OrthoANI, Orthologous Average Nucleotide Identity; dDDH, digital DNA–DNA Hybridization; AAI, Average Amino acid Identity; DMSO, dimethyl sulfoxide; GGDC, Genome-to-Genome Distance Calculator; OC, orthologous cluster; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; NCBI, National Center for Biotechnology Information.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB'* gene sequences of *Haloarcula terrestris* S1AR25-5A^T are ON653024 (*rrnA*), ON653023 (*rrnB*), and ON668040, respectively, and that of its complete genome is JAMQOM000000000. The GenBank/EMBL/DDBJ accession number of the whole genome sequence of *Haloarcula tradensis* JCM 15760^T is JAMQCP000000000.

Abstract: An extremely halophilic archaeon, strain S1AR25-5A^T, was isolated from a hypersaline soil located in Odiel Saltmarshes Natural Area (Huelva, Spain). The cells were Gram-stain-negative, motile, pleomorphic rods. Cells growth was observed in the presence of 15-30 % (w/v) NaCl (optimum, 25 % [w/v] NaCl), at pH 6.0-9.0 (optimum, pH 6.5-7.5), and at a temperature range of 25-50 °C (optimum, 37 °C). Based on the 16S rRNA and *rpoB'* gene sequences comparison, strain S1AR25-5A^T was affiliated to the genus *Haloarcula*. Taxogenomic analysis, including comparison of the genomes and the phylogenomic tree based on the core-orthologous proteins, together with the genomic indices, i.e., Orthologous Average Nucleotide Identity (OrthoANI), digital DNA-DNA hybridization (dDDH), and Average Amino acid Identity (AAI), confirmed that strain S1AR25-5A^T (= CCM 9249^T = CECT 30619^T) represents a new species of the genus *Haloarcula*, for which we propose the name *Haloarcula terrestris* sp. nov. The major polar lipids were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, and an unidentified glycolipid that correlated with the lipid profile of species of the genus *Haloarcula*. In addition, based on the modern approach in description of species in taxonomy of prokaryotes, the above mentioned genomic indexes indicated that the species *Haloarcula tradensis* should be considered as a heterotypic synonym of *Haloarcula argentinensis.*

INTRODUCTION

The genus *Haloarcula* belongs to the family *Haloarculaceae*, within the order *Halobacteriales*, and class *Halobacteria*. It was proposed by Torreblanca et al. in 1986 [1] by reassigning a species, previously described as *Halobacterium vallismortis*, to the new genus *Haloarcula*, as *Haloarcula vallismortis*. Currently, this genus includes 12 species according to the List of Prokaryotic names with Standing in Nomenclature [2], although very recently, the species *Haloarcula salaria* and *Haloarcula quadrata* have been proposed as later heterotypic synonyms of *Haloarcula argentinensis* and *Haloarcula marismortui*, respectively [3]. The species of the genus *Haloarcula* were isolated from hypersaline habitats, including solar salterns, salt lakes, the Dead Sea, salt flats, and salted food [1, 4-12]. Cells of the species of this genus are short pleomorphic rods, with shapes ranging from almost regular rods to triangular and irregular shapes. Colonies have red, pink, or red to orange pigmentation [1, 8]. The most common feature of species of the genus *Haloarcula* is the presence of heterogeneous multiple copies of the 16S rRNA gene [4, 8, 10-12], which were originally detected in *Haloarcula marismortui* [13].

As part of recent studies on the characterization of the microbiota of hypersaline soils located in Odiel Saltmarshes, a natural area in the province of Huelva (Southwestern Spain), strain S1AR25-5A^T was isolated. The initial analysis based on 16S rRNA gene sequences revealed that the isolate was related to the genus *Haloarcula*, that was confirmed by genome comparison. The post genomic era introduced genomic indexes, i.e., Orthologous Average Nucleotide Identity (OrthoANI), digital DNA-DNA hybridization (dDDH), and Average Amino acid Identity (AAI) [14-17], which facilitate and clarify the delineation of prokaryotic taxa. Currently, the Overall Genome Relatedness Indexes (OGRI), represent a modern approach in prokaryotic taxonomy and their use should not be omitted in describing new taxa. In this study, we determined the whole genome sequence of *Haloarcula tradensis* JCM 15760^T, which was the only one missing in genome databases of type species of *Haloarcula*, in order to carry out a complete comparative taxogenomic analysis of the genus *Haloarcula*. Our data suggest that strain S1AR25-5A^T constitutes a novel *Haloarcula* species, for which we propose the name of *Haloarcula terrestris* sp. nov., and that the species *Haloarcula tradensis* should be considered as a heterotypic synonym of *Haloarcula argentinensis*. In addition, our data support the recent study proposing the reclassification of *Haloarcula salaria* as a heterotypic synonym of *Haloarcula argentinensis* and *Haloarcula quadrata* as a heterotypic synonym of *Haloarcula marismortui* [3].

MATERIALS AND METHODS

Analysis of Soil Sample and Isolation and Cultivation Conditions of the New Haloarchaeal Strain

Strain S1AR25-5A^T was isolated from a hypersaline soil located in Odiel Saltmarhes Natural Area, Southwestern Spain (37°12'26.6"N 6°57'52.5"W) in July 2020. We analyzed the physico-chemical characteristics of the sampled soil, which included the determination of the pH and the electrical conductivity with a pH meter (CRISON BASIC 20) and a conductometer (CRISON 35+), respectively, after a 1:5 dilution. The presence of heavy metals in the soil sample of the isolation place was detected after an analysis carried out by Innoagral Laboratories in Mairena del Aljarafe (Spain).

The soil sample was serially diluted and plated under sterile conditions and incubated at 37 °C for up to 3 months. R2A 25 % medium was used as an isolation and cultivation medium. It consists of (g/l): yeast extract, 0.5; proteose peptone no. 3, 0.5; casamino acids, 0.5; dextrose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; dipotassium phosphate, 0.3; magnesium sulphate, 0.05; and it was supplemented with 25 % (w/v) seawater salt solution (designated as R2A 25 % medium), prepared by dilution of 30 % stock solution, which is composed of (g/l): NaCl, 195; $MgCl_2{\cdot}6H_2O$, 32.5; $MgSO_4{\cdot}7H_2O$, 50.8; $CaCl_2$, 0.83; KCl, 5.0; $NaHCO_3$, 0.17; NaBr, 0.58. The pH of the medium was adjusted to 7.5 with 1 M KOH and, if needed, solidified with purified agar to a final concentration of 2 % (w/v). Strain S1AR25-5A^T was isolated in pure culture after successive cultivations. *Haloarcula tradensis* JCM 15760^T was obtained from a freeze-dried culture of the Japan Collection of Microorganisms and cultured under the same growth conditions as strain S1AR25-5A^T. Cultures were maintained at -80 °C in R2A 25 % liquid medium containing 40 % (v/v) glycerol for long-term preservation.

DNA Extraction, Amplification and Sequencing

The genomic DNA of strain S1AR25-5A^T was extracted by using the methodology of Marmur [18] adjusted for small volumes. Bio-Rad T100 Thermal Cycler was used for the PCR reactions to amplify the 16S rRNA and *rpoB'* genes. The universal archaeal primers ArchF and ArchR [19, 20] and rpoBF and rpoBR [21] were used, respectively, for that purpose. The integrity of genomic DNA and PCR amplicons was checked by 1 % agarose gel electrophoresis and for purification of both, the genomic DNA and the PCR products, MEGAquick-spin^{TM} Plus Fragment DNA Purification Kit (iNtRON Biotechnology) was used, following the manufacturer's instructions. DNA concentration was determined by Qubit 4 Fluorometer (Thermo Fisher Scientific), and the quality of the extracted DNA was checked spectrophotometrically with NanoDrop One (Thermo Fisher Scientific). The PCR amplicons were sequenced by Stab Vida (Caparica, Portugal) using the Sanger chain-termination method. The whole genome sequencing of strain S1AR25-5A^T was determined by Novogene Europe (Cambridge, United Kingdom) at the Illumina NovaSeq 6000 platform following a 2 × 150 paired-ends approach. Additionally, we obtained the whole genome sequence of *Haloarcula tradensis* JCM 15760^T , which could not be found in genome databases, by following the same methodology.

Phylogenetic Studies

The 16S rRNA and *rpoB'* gene sequences of the isolated strain were assembled with ChromasPro v.1.5 software (Technelysium Pty Ltd.) and taxonomically associated with phylogenetic neighbors by comparison with the sequences available in the EzBioCloud database using its own pairwise alignment tool [22] and in NCBI GenBank database by BLASTN search [23]. Multiple sequence alignments of 16S rRNA and *rpoB'* gene sequences from strain S1AR25-5A^T and the closest relatives were carried out using, respectively, Fast Aligner tool of ARB suite [24] and ClustalW v.1.4 [25] as implemented in BioEdit program v.7.2.5 [26]. Subsequently, the alignments were visually inspected and corrected. Phylogenetic tree reconstructions based on the 16S rRNA and *rpoB'* gene sequences were conducted using the maximum-likelihood [27], neighbor-joining [28], and maximum-parsimony algorithms [29] implemented in the ARB software [24]. The "gitana" script was used for formatting and the tree visualization [30]. The 16S rRNA and *rpoB'* gene sequences of strain S1AR25-5A^T were deposited in GenBank/EMBL/DDBJ, under the accession numbers ON653024 (*rrnA*), ON653023 (*rrnB*), and ON668040, respectively.

Phylogenomic Analyses and Genomic Indices

For the taxogenomic analyses, publicly available genome sequences of the closely related species were obtained from GenBank database and the minimal standards for the use of genome data for taxonomic purposes were followed [31]. The reads of the genomes of strain S1AR25-5A^T and *Haloarcula tradensis* JCM 15760^T were assembled by k-mer strategy using Spades v.3.13.0 [32], and completeness and contamination of the assemblies were checked by CheckM v.1.0.5 [33]. The standard genome annotation was conducted by Prokka v.1.12 [34]. The curated whole-genome sequences of strain S1AR25-5A^T and *Haloarcula tradensis* JCM 15760^T were deposited in GenBank/EMBL/DDBJ, under the accession numbers JAMQOM000000000 and JAMQCP000000000, respectively. The Enveomics toolbox [35] was used to identify the clusters of orthologous proteins shared by all analyzed strains, as previously described [36]. The single-copy core-orthologous proteins were individually aligned using MUSCLE v.5.1 [37] and further concatenated to create a supermatrix. In order to determine the phylogenomic position of the studied strain with respect to species of *Haloarcula* and other related haloarchaea, an approximately maximum-likelihood phylogenomic tree was constructed using FastTreeMP v.2.1.8 [38].

OGRI were calculated among strain S1AR25-5A^T and the species of the genus *Haloarcula* and other related species of the family *Haloarculaceae*. OrthoANI values were determined by OrthoANIu tool v.1.2 [39], dDDH values were obtained using the Genome-to-Genome Distance Calculator (GGDC v.3.0) from the Leibniz Institute DSMZ (Germany) [40], and AAI values were calculated using the 'aai.rb' script from the Enveomics collection

[35]. The OrthoVenn2 online tool [41] was used to compare the orthologous clusters (OCs) among strain S1AR25-5A^T and its phylogenomically closest related species: *Haloarcula mannanilytica* MD130-1^T, *Haloarcula amylolytica* JCM 13557^T, *Haloarcula hispanica* ATCC 33960^T, and *Haloarcula japonica* DSM 6131^T. Additionally, isoelectric point of predicted proteins was calculated by the 'iep' program of the EMBOSS package [42].

Phenotypic Characterization

A complete phenotypic characterization was performed according to the minimal standards for the description of new taxa in the class *Halobacteria* [43]. Morphology and motility of the cells were observed by a phase-contrast microscope (Zeiss Axioscope 5). Colonial morphology, pigmentation, and size were examined on R2A 25 % solid medium after 7 days of incubation at 37 °C. Gram staining was performed as described by Dussault [44]. The growth of the studied strain was determined in R2A medium using a range of gradually increasing concentration of salts: 5, 7.5, 10, 12, 15, 17, 20, 22, 25, and 30 % (w/v). The pH range and optimum of growth were determined in R2A 25 % buffered medium with pH adjusted to 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5 and the temperature range and optimum that enables cell growth was examined by incubation at 20 °C to 55 °C (at intervals of 5 °C). To determine the ability of anaerobic growth, cultures on R2A 25 % medium plates supplemented with three alternative electron acceptors (L-arginine, DMSO, and KNO_3) were incubated at 37 °C during 14 days in a gas-pak system using AnaeroGen^{TM} (Oxoid).

The catalase test was carried out by adding a few drops of 3 % H_2O_2 (v/v) to a young culture of the microorganism [45]. Oxidase activity was determined by using 1 % (v/v) tetramethyl-p-phenylenediamine [46]. Hydrolysis of casein, gelatin, and starch were examined as described by Mata et al. [47]. Aesculin hydrolysis was detected according to Barrow and Feltham [48]. Hydrolysis of Tween 80 was carried out as previously described by Gutiérrez and González [49]. Indole production test conducted on the medium containing tryptone and yeast extract, and the reduction of nitrate and nitrite were carried out following the methodology of Gerhardt et al. [50]. Methyl red, Voges–Proskauer, and Simmons' citrate tests were determined according to Oren et al. [43]. The production of H_2S was detected by a strip impregnated with lead acetate [51]. The urease test was conducted as previously described [52] to determine the ability of the studied strain to split urea. Acid production from carbohydrates and sugar alcohols was determined using the following substrates: D-arabinose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-trehalose D-xylose, glycerol, lactose, and sucrose, which were added to the media to a final concentration of 1 % (w/v), containing phenol red as indicator [53]. The nutritional tests were carried out to determine the utilization of carbohydrates, alcohols, organic acids, and amino acids as sole carbon, nitrogen (in case of amino acids), and energy sources following the methodology described by Ventosa et al. [53]. *Haloarcula vallismortis* ATCC 29715^T, *Haloarcula hispanica* ATCC 33960^T, and *Haloarcula japonica* DSM 6131^T were used as reference strains for phenotypic feature comparison.

Chemotaxonomic Analysis

The identification of polar lipids was carried out by High Performance Thin Layer Chromatography (HPTLC). The polar lipids were obtained from a cell biomass of strains S1AR25-5A^T, *Haloarcula vallismortis* ATCC 29715^T, *Haloarcula hispanica* ATCC 33960^T, and *Haloarcula japonica* DSM 6131^T. The HPTLC silica gel glass plates (10 × 20 cm, Merck) were washed in chloroform/methanol 1:1 (v/v) and developed with chloroform/methanol/90 % acetic acid (39.4/2.42/18.18 [ml]) solvent system [54, 55]. The polar lipids were detected by 5 % (v/v) sulfuric acid followed by heating at 160 °C. Glycolipids appear as purple spots, and the remaining polar lipids as brown spots after prolonged heating. Finally, phospholipids were revealed by molybdenum blue spray reagent. The polar lipids extracted from *Halorubrum saccharovorum* DSM 1137^T and *Halobacterium salinarum* DSM 3754^T were used as reference for identifying the polar lipid pattern of the isolate.

RESULTS AND DISCUSSION

Soil Sample Composition of the Sampling Area

The soil sample obtained from the hypersaline soil located in Odiel Saltmarshes Natural Area in Huelva (Spain) had pH value of 8.3 and its electrical conductivity was 46.5 mS/cm. The soil sample was tested for the presence of heavy metals due to past mining practices in this region. Some of the most prevalent heavy metals (cadmium, copper, and lead) were in accordance with the standards of uncontaminated soils designated by the Environment Department of the Regional Government of Andalusia [56], with concentrations (mg/kg) of 0.4, 89.2, and 15.1, respectively. However, zinc and arsenic reached higher concentrations (71.4 and 8.0 mg/kg, respectively) than the given limits (10-70 and 2-5 mg/kg, respectively), which imply a possible heavy metal tolerance or/and resistance of the studied strain as haloarchaeal strains have demonstrated a significant capacity of resistance to toxic heavy metals [57-59], yet further investigation should be performed.

Phylogenetic Studies

Strain S1AR25-5A^T was isolated as part of the studies focused on the characterization of prokaryotes of hypersaline soils located in Odiel Saltmarshes Natural Area (Huelva). The analysis of partial 16S rRNA sequence enabled us to determine the preliminary phylogenetic position of the isolate S1AR25-5A^T, which was related to the genus *Haloarcula*. The species of the genus *Haloarcula* possess at least two heterogeneous copies of the 16S rRNA gene. Two 16S rRNA (*rrnA*, 1,465 bp, and *rrnB*, 931 bp) gene sequences were determined for the studied strain and compared with those of the type strains of species of the genus *Haloarcula*. Strain S1AR25-5A^T was most closely related to *Haloarcula hispanica* ATCC 33960^T, *Haloarcula marismortui* ATCC 43049^T, and *Haloarcula mannanilytica* MD130-1^T with 99.7 %, 99.5 %, and 99.5 % (*rrnA*) and *Haloarcula marismortui* ATCC 43049^T, *Haloarcula hispanica* ATCC 33960^T and *Haloarcula argentinensis* JCM 9737^T with 99.8 %, 99.7 % and 99.5 % (*rrnB*) 16S rRNA gene sequence identity, respectively. The presence of multiple copies with sequence variations may pose an obstacle in classification and cause an overestimation of prokaryotic diversity [60, 61]. It has been proven that the 16S rRNA gene has many disadvantages for being utilized as a taxonomic marker for the class *Halobacteria* [62], and that full-length *rpoB'* gene constitutes an useful supplementary biomarker in determining the phylogenetic position of new strains [63]. Analysis of the complete *rpoB'* gene sequence (1,827 bp) of the studied strain in comparison with *rpoB'* gene sequences of species of the family *Haloarculaceae* showed the closest relatedness to *Haloarcula amylolytica* JCM 13557^T (95.3 %), *Haloarcula vallismortis* ATCC 29715^T (95.0 %), and *Haloarcula japonica* DSM 6131^T (95.0 %). The phylogenetic reconstruction based on the 16S rRNA (**Figure 1**) and *rpoB'* (**Figure 2**) gene sequences confirmed the affiliation of strain S1AR25-5A^T to the genus *Haloarcula*.

Phylogenomic Analyses

The draft genome of strain S1AR25-5A^T was assembled into 41 contigs (N50, 546,503 bp; coverage, 539X, completeness, 99.5 %; contamination, 0.8 %). The whole genome sequence of *Haloarcula tradensis* JCM 15760^T, which was not previously available, was obtained in order to carry out a complete taxogenomic analysis, including all current species of the genus *Haloarcula*. The general genomic features of the studied strain and other type strains of species of the genus *Haloarcula* are shown in **Supplementary Table S1**. The PCR amplified genes (16S rRNA and *rpoB'*) of strain S1AR25-5A^T matched the sequences determined by whole genome sequencing. An approximately maximum-likelihood phylogenomic tree (**Figure 3**) was constructed based on 1,061 single-copy core-orthologous protein sequences and showed that strain S1AR25-5A^T clustered with *Haloarcula mannanilytica* MD130-1^T, but far enough as to constitute a new species within the genus *Haloarcula*. In addition, the tree topology revealed that *Haloarcula argentinensis* DSM 12282^T, *Haloarcula tradensis* JCM 15760^T, and *Haloarcula salaria* JCM 15759^T grouped tightly together, as well as *Haloarcula marismortui* ATCC 43049^T and *Haloarcula quadrata* DSM 11927^T, which confirms the latest results of Ma et al. [3] proposing *Haloarcula salaria* and *Haloarcula quadrata* as later heterotypic synonyms of *Haloarcula argentinensis* and *Haloarcula marismortui*, respectively. Moreover, the phylogenomic position of *Haloarcula tradensis* JCM 15760^T suggests that it could represent another heterotypic synonym of *Haloarcula argentinensis*.

To verify that strain S1AR25-5A^T constitutes a new species and to elucidate the taxonomic position of *Haloarcula tradensis*, we calculated OrthoANI and dDDH values, which are used for the species delineation [35, 36]. The percentages of identity obtained between strain S1AR25-5A^T and the species of the genus *Haloarcula* were 89.2-81.9 % (OrthoANI) and 38.0-25.0 % (dDDH), confirming unequivocally that strain S1AR25-5A^T represents a novel species according to the accepted thresholds for the prokaryotic species delineation: ~95-96 % for OrthoANI and 70 % for dDDH [64, 65, 66]. On the other hand, OrthoANI and dDDH values among strains *Haloarcula argentinensis* DSM 12282^T, *Haloarcula tradensis* JCM 15760^T, and *Haloarcula salaria* JCM 15759^T were: 99.9 %, 99.1 %, 98.9 % and 99.4 %, 91.0 % and 89.7 %, respectively (**Figure 4**), which unquestionably indicate that they constitute the same species. OrthoANI and dDDH values between the species *Haloarcula marismortui* ATCC 43049^T and *Haloarcula quadrata* DSM 11927^T were 98.4 % and 85.6 %, that confirm that these two strains represent the same species, as it was previously reported [3]. AAI cutoff value for delineation of prokaryotes at species level have also been established at ~95 to 96% [67], but the threshold at genus level is not definite, although an approximate value of 65 % have been proposed [68], which yet is not applicable for all archaeal and bacterial families [69]. AAI values between strain S1AR25-5A^T and the species of the genus *Haloarcula* were 91.5-80.3 %, below the AAI limit for species delineation, whereas AAI values among strains *Haloarcula argentinensis* DSM 12282^T, *Haloarcula tradensis* JCM 15760^T, and *Haloarcula salaria* JCM 15759^T were 99.7 %, 98.7 % and 98.6 %, and AAI value between *Haloarcula marismortui* ATCC 43049^T and *Haloarcula quadrata* DSM 11927^T was 98.0 %, which confirm the previous findings that these strains are members of the same species (**Figure 5**).

The studied haloarchaeal strain S1AR25-5A^T and the phylogenomically most closely related species: *Haloarcula mannanilytica* MD130-1^T, *Haloarcula amylolytica* JCM 13557^T, *Haloarcula hispanica* ATCC 33960^T, and *Haloarcula japonica* DSM 6131^T, shared a total of 2,852 orthologous protein clusters (OCs) (**Supplementary Figure S1A**). *Haloarcula tradensis* JCM 15760^T contained a total of 4,088 OCs, of which 3,488 OCs were shared with both, *Haloarcula argentinensis* DSM 12282^T and *Haloarcula salaria* JCM 15759^T, 583 OCs were only shared with *Haloarcula argentinensis* DSM 12282^T and 17 OCs only with *Haloarcula salaria* JCM 15759^T. No singleton OCs were detected in *Haloarcula tradensis* JCM 15760^T (**Supplementary Figure S1B**). In addition, the isoelectric point of predicted proteins was calculated for species of the genus *Haloarcula* and other reference taxa for comparative purposes. Strain S1AR25-5A^T and *Haloarcula* species shared a compliant isoelectric profile with a peak at around 4 (**Supplementary Figure S2**), showing an acidic proteome and, thus, the "salt-in" osmoregulation strategy typical of members of the class *Halobacteria* [70].

Phenotypic Features

A complete phenotypic analysis was performed, including morphological, physiological, biochemical, and nutritional characterization of the new isolate. The optimal cell growth of the isolate S1AR25-5A^T was determined in the presence of 25 % (w/v) NaCl, at pH 6.5-7.5, and at 37 °C. However, the strain could grow in the range of 15-30 % (w/v) NaCl, at pH 6.0-9.0, and at temperatures between 25 °C and 50 °C. Cells were Gram-stain-negative, 0.5 × 2-5 μm in size, motile, predominantly pleomorphic rods, although irregular-shaped cells were also present. Colonies were round, 0.8-1.3 mm in diameter, convex, showed pinkish pigmentation, and were mucoid on older cultures. The isolate was a strictly aerobic extremely halophilic archaeon. It possesed the enzyme catalase but did not show oxidase activity. Aesculin was hydrolized, but casein, gelatin, starch, and Tween 80 were not. Methyl red test was positive. Nitrate was reduced by the studied strain, but nitrite was not. Voges–Proskauer, urease, indole, and Simmons' citrate tests were negative. The production of H_2S was not detected. Further phenotypic characteristics of strain S1AR25-5A^T and other strains of the genus *Haloarcula* are detailed in **Supplementary Table S2** and in the new species description.

Chemotaxonomic Analysis

The HPTLC chromatograms (**Supplementary Figures S3A** and **S3B**) showed that the major polar lipids of strain S1AR25-5A^T were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and an unidentified glycolipid that matched the lipid profile of *Haloarcula* species. Polar lipid composition has been found to be useful in characterization and differentiation of haloarchaea

[1, 43]. The determined polar lipids of the studied strain are in concordance with the lipid profiles of species of the genus *Haloarcula* [1], which confirms the affiliation of strain S1AR25-5A^T to this genus.

TAXONOMIC CONCLUSIONS

As part of the studies focused on the characterization of prokaryotes that inhabit hypersaline soils of the Odiel Saltmarshes Natural Area in Huelva (Spain), we isolated strain S1AR25-5A^T. The initial 16S rRNA gene analysis affiliated the isolate to the genus *Haloarcula*. The multiple copies of the 16S rRNA gene, that are typically found for the genus *Haloarcula*, may exhibit remarkable levels of divergence. A more recent approach in the taxonomy of prokaryotes involves using the whole genome sequence for calculation of the Overall Genome Related Indexes, as well as the determination of the phylogenomic position. The phylogenetic studies, polar lipid profile, phenotypic features, and comparative taxogenomic analysis demonstrated that strain S1AR25-5A^T constitutes a new species within the genus *Haloarcula*, for which we propose the name *Haloarcula terrestris* sp. nov. A detailed species description is included below.

Additionally, in conformity with the International Code of Nomenclature of Prokaryotes (Section 5, Rule 23a) [71], we propose *Haloarcula tradensis* Namwong *et al*. 2011 as a later heterotypic synonym of *Haloarcula argentinensis* Ihara *et al*. 1997 (type strain, arg-1^T = CIP 105173^T = ATCC 700875^T = DSM 12282^T = JCM 9737^T; reference strain, HST03 = BCC 40030 = JCM 15760 = PCU 314). Besides, our study confirms the synonym between *Haloarcula argentinensis* and *Haloarcula salaria*, as well as between *Haloarcula marismortui* and *Haloarcula quadrata* [3]. On the basis of these studies, and considering that *Haloarcula argentinensis*, *Haloarcula tradensis* and *Haloarcula salaria* have some relevant differences, we propose an emended description of *Haloarcula argentinensis*, including the features of the other two species.

Description of *Haloarcula terrestris* sp. nov.

Haloarcula terrestris (ter.res'tris. L. fem. adj. *terrestris*, of or belonging to the earth, terrestrial).

Cells are Gram-stain-negative, 0.5 × 2-5 μm in size, motile, predominantly pleomorphic rods with the minor presence of irregular-shaped cells. Colonies are round, 0.8-1.3 mm in diameter, convex, mucoid on older cultures, and show pink pigmentation after 7 days of incubation at 37 °C. Extremely halophilic, able to grow at 15-30 % (w/v) NaCl (optimally at 25 % [w/v] NaCl); pH 6.0-9.0 (with an optimum at pH 6.5-7.5); and 25-50 °C (optimum at 37 °C). No growth occurs anaerobically with potassium nitrate, L-arginine, or DMSO. Catalase positive, oxidase negative. Aesculin is hydrolyzed, but casein, gelatin, starch, and Tween 80 are not. Nitrate is reduced but not nitrite. H_2S and indole are not produced. Methyl red test is positive. Voges–Proskauer, urease, and Simmons´s citrate tests are negative. It requires at least 0.5 % (w/v) Mg^{2+} and optimum Mg^{2+} concentration is 0.5-2.0 % (w/v). Acid is produced from D-arabinose, D-glucose, D-maltose, and D-xylose and is not produced from D-trehalose, glycerol, and mannitol. The following substrates are used as sole carbon and energy sources: D-fructose, D-glucose, D-maltose, hippurate, pyruvate, and xylitol, while D-cellobiose, D-melezitose, D-raffinose, D-trehalose, dulcitol, glycerol, L-arabinose, mannitol, propionate, and starch are not. L-alanine, L-arginine, L-glycine, L-glutamine, L-isoleucine, L-methionine, L-phenylalanine, lysine, and ornithine are used as sole carbon, nitrogen and energy sources, while L-cysteine and L-serine are not. The major polar lipids are phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and an unidentified glycolipid that matched the lipid profile of *Haloarcula* species. The DNA G+C content is 62.3 mol% (genome).

The type strain is S1AR25-5A^T (= CCM 9249^T = CECT 30619^T), isolated from a hypersaline soil located in Odiel Saltmarshes Natural Area, Huelva, Spain. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB'* gene sequences of strain S1AR25-5A^T are ON653024 (*rrnA*), ON653023 (*rrnB*), and ON668040, respectively. The GenBank/EMBL/DDBJ accession number of the whole genome sequence of strain S1AR25-5A^T is JAMQOM000000000.

Emended description of Haloarcula argentinensis Ihara et al. 1997

Haloarcula argentinensis (ar.gen.tin.en'sis. N.L. fem. adj. *argentinensis*, pertaining to Argentina, Argentinian).

Cells are Gram-stain-negative, triangular and flat (0.3 × 1.0 µm) or pleomorphic rods (0.6-1.2 × 1.0-2.5 µm. Nonmotile or motile by polar archaella. Colonies are orange-red or red pigmented. Chemoorganotrophic. Strictly aerobic. Extremely halophilic. Growth occurs in media containing 12–30 % (w/v) NaCl. Optimum growth at 15–25 % (w/v) NaCl. Mg^{2+} concentration ranges from 0.2 to 6.0 % (w/v). Optimum Mg^{2+} concentration, 0.5 % (w/v). Growth temperature: 15-50 ºC (with an optimum at 37-40 °C). pH range for growth 6.0-8.0 (with an optimum at pH 7.0). Catalase and oxidase positive. Starch and Tween 80 are hydrolyzed. Different reactions may be observed for hydrolysis of gelatin. Indole is not produced. Nitrate is not reduced. Acid may be produced from glucose and other sugars (sucrose, maltose, galactose, mannose, ribose, glycerol, and fructose). The major polar lipids are phosphatidylglycerol (PG), phsophatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), triglycosyl diether (TGD-2), and diglycosyl diether (DGD-2). The DNA G+C content is 61.3 mol% (genome).

The type strain is arg-1 (= ATCC 700875^T = CIP 105173^T = DSM 12282^T = JCM 9737^T), isolated from soil of salterns in Argentina. The DNA G+C content of the type strain is 61.3 mol% (genome). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB'* gene sequences of strain JCM 9737^T are EF645680 (*rrnA*), EF645681 (*rrnB*), and AB477143, respectively. The GenBank/EMBL/DDBJ accession number of the whole genome sequence of strain DSM 12282^T is AOLX00000000.

Haloarcula salaria strain HST01-2R^T (= BCC 40029^T = JCM 15759^T = PCU 313^T), isolated from salt from a sample of fish sauce from Thailand, is an additional strain of *Haloarcula argentinensis*, and *Haloarcula salaria* Namwong *et al*. 2011 is a later heterotypic synonym of *Haloarcula argentinensis*.

Haloarcula tradensis strain HST03^T (= BCC 40030^T = JCM 15760^T = PCU 314^T), isolated from salt from a sample of fish sauce from Thailand, is an additional strain of *Haloarcula argentinensis*, and *Haloarcula tradensis* Namwong *et al*. 2011 is a later heterotypic synonym of *Haloarcula argentinensis.*

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References

1. **Torreblanca M, Rodriguez-Valera F, Juez G, Ventosa A, Kamekura M, *et al***. Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* 1986;8:89–99. Doi: 10.1016/S0723-2020(86)80155-2.

2. **Parte AC, Sardà Carbasse J , Meier-Kolthoff JP, Reimer LC, Göker M** . List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol* 2020;70:5607-5612. doi: 10.1099/ijsem.0.004332.

3. **Ma X, Hu Y, Li XX, Tan S, Cheng M, *et al***. *Halomicroarcula laminariae* sp. nov. and *Halomicroarcula marina* sp. nov., extremely halophilic archaea isolated from salted brown alga *Laminaria* and coastal saline-alkali lands. *Int J Syst Evol Microbiol* 2023;73:005889. Doi: 10.1099/ijsem.0.005889.

4. **Yang Y, Cui HL, Zhou PJ, Liu SJ**. *Haloarcula amylolytica* sp. nov., an extremely halophilic archaeon isolated from Aibi salt lake in Xin-Jiang, China. *Int J Syst Evol Microbiol* 2007;57:103–106. Doi: 10.1099/ijs.0.64647-0.

5. **Ihara K, Watanabe S, Tamura T**. *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. *Int J Syst Bacteriol* 1997;47:73–77. Doi: 10.1099/00207713-47-1-73.

6. **Juez G, Rodriguez-Valera F, Ventosa A, Kushner DJ**. *Haloarcula hispanica* spec. nov. and *Haloferax gibbonsii* spec. nov., two new species of extremely halophilic archaebacteria. *Syst Appl Microbiol* 1986; 8:75–79. Doi: 10.1099/00207713-36-4-57.

7. **Takashina T, Hamamoto T, Otozai K, Grant WD, Horikoshi K**. *Haloarcula japonica* sp. nov., a new triangular halophilic archaebacterium. *Syst Appl Microbiol* 1990;13:177–181. Doi: 10.1016/S0723-2020(11)80165-7.

8. **Enomoto S, Shimane Y, Ihara K, Kamekura M, Itoh T, *et al***. *Haloarcula mannanilytica* sp. nov., a galactomannan-degrading haloarchaeon isolated from commercial salt. *Int J Syst Evol Microbiol* 2020; 70: 6331–6337. Doi: 10.1099/ijsem.0.004535.

9. **Oren A, Ginzburg M, Ginzburg BZ, Hochstein LI, Volcani BE**. *Haloarcula marismortui* (Volcani) sp. nov., nom. rev., an extremely halophilic bacterium from the Dead Sea. *Int J Syst Bacteriol* 1990; 40: 209–210. Doi: 10.1099/00207713-40-2-209.

10. **Oren A, Ventosa A, Gutiérrez MC, Kamekura M**. *Haloarcula quadrata* sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). *Int J Syst Bacteriol* 1999; 49:1149–1155. Doi: 10.1099/00207713-49-3-1149.

11. **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 Syst Evol Microbiol* 2011;61:231–236. Doi: 10.1099/ijs.0.021790-0.

12. **Barreteau H, Vandervennet M, Guedon L, Point V, Canaan S, *et al***. *Haloarcula sebkhae* sp. nov., an extremely halophilic archaeon from Algerian hypersaline environment. *Int J Syst Evol Microbiol* 2019;69:732–738. Doi: 10.1099/ijsem.0.003211.

13. **Mylvaganam S, Dennis PP**. Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* 1992;130:399–410. Doi: 10.1093/genetics/130.3.399.

14. **Konstantinidis KT, Tiedje JM**. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Curr Opin Microbiol* 2007;10:504–509. Doi: 10.1016/j.mib.2007.08.006.

15. **Borriss R, Rueckert C, Blom J, Bezuidt O, Reva O, *et al***. Whole genome sequence comparisons in taxonomy. In *Methods in Microbiology*. Taxonomy of Prokaryotes, eds Rainey, F.; Oren, A.; London: Academic Press, 2011; 38, pp. 409–436. Doi: 10.1016/B978-0-12-387730-7.00018-8.

16. **Chun J, Rainey FA**. Integrating genomics into the taxonomy and systematics of the *Bacteria* and *Archaea*. *Int J Syst Evol Microbiol* 2014;64:316–324. Doi: 10.1099/ijs.0.054171-0.

17. **Ramírez-Durán N, de la Haba RR, Vera-Gargallo B, Sánchez-Porro C, Alonso-Carmona S, *et al***. Taxogenomic and comparative genomic analysis of the genus *Saccharomonospora* focused on the identification of biosynthetic clusters PKS and NRPS. *Front Microbiol* 2021;12:603791. Doi: 10.3389/fmicb.2021.603791.

18. **Marmur J**. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* 1961;3:208–218. doi: 10.1016/S0022-2836(61)80047-8.

19. **Delong EF**. Archaea in coastal marine environments. *Proc Natl Acad Sci USA* 1992;89:5685–5689. Doi: 10.1073/pnas.89.12.5685.

20. **Arahal DR, Dewhirst FE, Paster BJ, Volcani BE, Ventosa A**. Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl Environ Microbiol* 1996;62:3779–3786. Doi: 10.1128/aem.62.10.3779-3786.1996.

21. **Fullmer M, Soucy SM, Swithers KS, Makkay AM, Wheeler, R, *et al***. Population and genomic analysis of the genus *Halorubrum*. *Front Microbiol* 2014;5:140. Doi: 10.3389/fmicb.2014.00140.

22. **Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, *et al***. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67: 1613–1617. Doi: 10.1099/ ijsem.0.001755.

23. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ**. Basic local alignment search tool. *J Mol Biol* 1990;215:403–410. Doi: 10.1016/S0022- 2836(05)80360-2.

24. **Ludwig W, Strunk O, Westram R, Richter L, Meier H, *et al***. ARB: a software environment for sequence data. *Nucleic Acids Res* 2004; 32:1363–1371. Doi: 10.1093/nar/gkh293.

25. **Thompson JD, Higgins DG, Gibson TJ**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680. Doi: 10.1093/nar/22.22.4673.

26. **Hall T**. BioEdit: an important software for molecular biology. *GERF Bull Biosci* 2011;2:60-61.

27. **Felsenstein J**. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981;17:368–376. Doi: 10.1007/BF01734359.

28. **Saitou N, Nei M**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425. Doi: 10.1093/oxfordjournals.molbev.a040454.

29. **Felsenstein J**. Parsimony in systematics: biological and statistical issues. *Annu Rev Ecol Syst* 1983;14:313–333. Doi: 10.1146/annurev.es.14.110183.001525.

30. **Galisteo C**. Gitana: phyloGenetic Imaging Tool for Adjusting Nodes and other Arrangements. 2022. Available online: https://github.com/cristinagalisteo/gitana (accessed on 3 June 2022).

31. **Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, *et al***. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466. Doi: 10.1099/ijsem.0.002516.

32. **Prjibelski A, Antipov D, Meleshko D, Lapidus A, Korobeynikov A**. Using SPAdes de novo assembler. *Curr Protoc Bioinformatics* 2020;70:e102. Doi: 10.1002/cpbi.102.

33. **Parks DH, Imelfort M, Skennerton C, Hugenholtz P, Tyson GW**. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;25:1043–1055. Doi: 10.1101/gr.186072.114.

34. **Seemann T**. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069. Doi: 10.1093/bioinformatics/btu153.

35. **Rodríguez-R LM, Konstantinidis KT**. The Enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ* 2016;4:e1900v1. Doi: 10.7287/peerj.preprints.1900v1.

36. **de la Haba RR, López-Hermoso C, Sánchez-Porro C, Ventosa A**. Comparative genomics and phylogenomic analysis of the genus *Salinivibrio*. *Front Microbiol* 2019;10:2104. Doi: 10.3389/fmicb.2019.02104.

37. **Edgar RC**. Muscle5: high-accuracy alignment ensembles enable unbiased assessments of sequence homology and phylogeny. *Nat Commun* 2022;13:6968. Doi: 10.1101/2021.06.20.449169.

38. **Price MN, Dehal PS, Arkin AP**. FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 2010;5:e9490. Doi: 10.1371/journal.pone.0009490.

39. **Lee I, Kim YO, Park SC, Chun J**. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 2016;66:1100–1103. Doi: 10.1099/ijsem.0.000760.

40. **Meier-Kolthoff JP, Sardà Carbasse J, Peinado-Olarte RL, Göker M**. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Res* 2020;50:801–807. Doi: 10.1093/nar/gkab902.

41. **Xu L, Dong Z, Fang L, Luo Y, Wei Z, *et al***. OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res* 2019;47:52-58. Doi: 10.1093/nar/gkz333.

42. **Rice P, Longden L, Bleasby A**. EMBOSS: the European molecular biology open software suite. *Trends Genet* 2000;16:276–277. Doi: 10.1016/S0168- 9525(00)02024-2.

43. **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. Doi: 10.1099/00207713-47-1-233.

44. **Dussault HP**. An improved technique for staining red halophilic bacteria *J Bacteriol* 1955;70:484–485. Doi: 10.1128/jb.70.4.484-485.1955.

45. **Cowan ST, Steel KJ**. *Manual for the Identification of Medical Bacteria*, 3rd ed.; Cambridge University Press: London, UK, 1965.

46. **Kovacs N**. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* 1956;178:703. Doi: 10.1038/178703a0.

47. **Mata JA, Martínez-Cánovas J, Quesada E, Béjar V**. A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* 2002;25:360–375. Doi: 10.1078/0723-2020-00122.

48. **Barrow GI., Feltham RKA**. *Cowan and Steel's Manual for the Identification of Medical Bacteria*; Cambridge University Press: Cambridge, UK, 2003; ISBN 9780521543.

49. **Gutiérrez C, González C**. Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. *Appl Microbiol* 1972;24:516–517. Doi: 10.1128/am.24.3.516-517.1972.

50. **Gerhardt P, Murray RG, Wood WA, Krieg N**. *Methods for General and Molecular Bacteriology*; American Society for Microbiology: Washington, DC, USA, 1994.

51. **Clarke PH**. Hydrogen sulphide production by bacteria. *J Gen Microbiol* 1953;8:397–407. Doi: 10.1099/00221287-8-3-397.

52. **Christensen WB**. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J Bacteriol* 1946;52:461–466. Doi: 10.1128/jb.52.4.461-466.1946.

53. **Ventosa A, Quesada E, Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A**. Numerical taxonomy of moderately halophilic Gram-negative rods. *Microbiology* 1982;128:1959–1968. Doi: 10.1099/00221287-128-9-1959.

54. **Angelini R, Corral P, Lopalco P, Ventosa A, Corcelli A**. Novel ether lipid cardiolipins in archaeal membranes of extreme haloalkaliphiles. *Biochim Biophys Acta* 2012;1818:1365–1373. Doi: 10.1016/j.bbamem.2012.02.014.

55. **Corral P, Gutiérrez MC, Castillo AM, Domínguez M, Lopalco P, *et al***. *Natronococcus roseus* sp. nov., a haloalkaliphilic archaeon from a hypersaline lake. *Int J Syst Evol Microbiol* 2013;63:104–108. Doi: 10.1099/ijs.0.036558-0.

56. **Consejería de Medio Ambiente de la Junta de Andalucía.** *Los criterios y estándares para declarar un suelo contaminado en Andalucía y la metodología y técnicas de toma de muestra y análisis para su investigación*. Sevilla: Junta de Andalucía, 1999.

57. **Krzmarzick MJ, Taylor DK, Fu X, McCutchan AL**. Diversity and niche of archaea in bioremediation. *Archaea* 2018;2018:1–17 Doi: 10.1155/2018/3194108.

58. **Vera-Bernal M, Martínez-Espinosa RM**. Insights on cadmium removal by bioremediation: the case of haloarchaea. *Microbiol Res* 2021;12:354–375. Doi: 10.3390/microbiolres12020024.

59. **Tavoosi N, Akhavan Sepahi A, Amoozegar MA, Kiarostami V**. Toxic heavy metal/oxyanion tolerance in haloarchaea from some saline and hypersaline ecosystems. *J Basic Microbiol* 2023;63:558–569. Doi: 10.1002/jobm.202200465.

60. **Sun DL, Jiang X, Wu QL, Zhou NY**. Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. *Appl Environ Microbiol* 2013;79:5962–5969. Doi: 10.1128/AEM.01282-13.

61. **Ibal JC, Pham HQ, Park CE, Shin JH**. Information about variations in multiple copies of bacterial 16S rRNA genes may aid in species identification. *PLoS One* 2019;14:e0212090. Doi: 10.1371/journal.pone.0212090.

62. **de la Haba RR, Corral P, Sánchez-Porro C, Infante-Domínguez C, Makkay AM, *et al***. Genotypic and lipid analyses of strains from the archaeal genus *Halorubrum* reveal insights into their taxonomy, divergence, and population structure. *Front Microbiol* 2018;9:512. Doi: 10.3389/fmicb.2018.00512.

63. **Minegishi H, Kamekura M, Itoh T, Echigo A, Usami R, *et al***. Further refinement of the phylogeny of the *Halobacteriaceae* based on the full-length RNA polymerase subunit B' (*rpoB'*) gene. *Int J Syst Evol Microbiol* 2010;60:2398–2408. Doi: 10.1099/ijs.0.017160-0.

64. **Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, *et al*.** DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81-91. Doi: 10.1099/ijs.0.64483-0.

65. **Richter M, Rossello-Mora R**. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U.S.A.* 2009;106:19126–19131. Doi: 10.1073/pnas.0906412106.

66. **Auch AF, von Jan M, Klenk H-P, Göker M**. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2010;2:117–134. Doi: 10.4056/sigs.531120.

67. **Konstantinidis KT, Tiedje JM**. (2005). Towards a genome-based taxonomy for prokaryotes. *J Bacteriol* 2005;187:6258-6264. Doi: 10.1128/JB.187.18.6258-6264.2005.

68. **Konstantinidis KT, Rosselló-Móra R, Amann R**. Uncultivated microbes in need of their own taxonomy. *ISME J* 2017;11:2399–2406. Doi: 10.1038/ismej.2017.113.

69. **de la Haba RR, Minegishi H, Kamekura M, Shimane Y, Ventosa A**. Phylogenomics of haloarchaea: the controversy of the genera *Natrinema-Haloterrigena*. *Front Microbiol* 2021;12:740909. Doi: 10.3389/fmicb.2021.740909.

70. **Becker EA, Seitzer PM, Tritt A, Larsen D, Krusor M, *et al***. Phylogenetically driven sequencing of extremely halophilic archaea reveals strategies for static and dynamic osmo-response. *PLoS Genet* 2014; 1:e1004784. Doi: 10.1371/journal.pgen.1004784.

71. **Oren A, Arahal DR, Göker M, Moore ER, Rossello-Mora R, Sutcliffe IC**. International Code of Nomenclature of Prokaryotes. Prokaryotic code (2022 revision). *Int J Syst Evol Microbiol* 2023;73:005585. Doi: 10.1099/ijsem.0.005585.
72. **Gonzalez C, Gutierrez C, Ramirez C**. *Halobacterium vallismortis* sp. nov. an amylolytic and carbohydrate-metabolizing, extremely halophilic bacterium. *Can J Microbiol* 1978;24:710715. Doi: 10.1139/m78-119.

Legends to Figures

Figure 1. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequence comparison of strain S1AR25-5A^T with species of *Haloarcula* and other related species within the family *Haloarculaceae*. The species *Haloferax volcanii* NCIMB 2012^T was used as an outgroup. Sequence accession numbers are shown in parentheses. Bootstrap values ≥70 % (based on 1,000 pseudo-replicates) are shown at branch points. Filled circles indicate branches that were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 expected substitutions per nucleotide position.

Figure 2. Neighbor-joining phylogenetic reconstruction based on *rpoB'* gene sequence comparison of strain S1AR25-5A^T with species of *Haloarcula* and related species of the family *Haloarculaceae*. The species *Haloferax volcanii* JCM 8879^T was used as an outgroup. Sequence accession numbers are shown in parentheses. Bootstrap values (%) higher than 70 % are indicated at branch points. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 expected substitutions per nucleotide position.

Figure 3. Approximately maximum-likelihood phylogenomic tree based on the comparison of 1,061 single-copy core-orthologous proteins showing the relationships of strain S1AR25-5A^T with species of *Haloarcula* and other related species within the family *Haloarculaceae*. Sequence accession numbers are shown in parentheses. Branch support values (%) are computed with the Shimodaira-Hasegawa test and are shown at branch points. Bar, 0.05 substitutions per amino acid position.

Figure 4. Heatmap displaying OrthoANI (upper right) and dDDH (lower left) percentages among strain S1AR25-5A^T, members of the genus *Haloarcula*, and other related species of the family *Haloarculaceae*.

Figure 5. Heatmap showing AAI percentages among *Haloarcula* species, including strain S1AR25-5A^T, and other related species of the family *Haloarculaeae*.