Natrinema salsiterrestre sp. nov., an extremely halophilic archaeon isolated from a hypersaline soil

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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; S-DGD-1, sulfated diglycosyl diether; NCBI, National Center for Biotechnology Information.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB'* gene sequences of *Natrinema salsiterrestre* S1CR25-10^T are ON653413 and ON668046, respectively, and that of its complete genome is JAMQOT000000000.

Abstract: An extremely halophilic archaeal strain, designated S1CR25-10^T, was isolated from a hypersaline soil located in Odiel Saltmarshes Natural Area in Southwestern Spain (Huelva) and subjected to a polyphasic taxonomic characterization. The cells were Gram-stain-negative, motile and their colonies were pink-pigmented. It was a strictly aerobic haloarchaeon that could grow at 25-55 °C (optimum, 37 °C), at pH 6.0-9.0 (optimum, pH 7.0-8.0), and in the presence of 12-30 % (w/v) total salts (optimum, 20-25 % [w/v]). The phylogenetic analysis based on the comparison of the 16S rRNA gene sequences revealed that strain S1CR25-10^T belongs to the genus *Natrinema*, with 98.9 % identity to *Natrinema salinisoli* SLN56^T. In addition, the values of Orthologous Average Nucleotide Identity (OrthoANI), digital DNA-DNA hybridization (dDDH), and Average Amino acid Identity (AAI) were below the threshold limits accepted for prokaryotic species delineation, with *Natrinema salinisoli* SLN56^T showing the highest relatedness values (92.6 % and 48.4 %, respectively). The major polar lipids were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and a glycolipid chromatographically identical to sulfated diglycosyl diether (S-DGD-1). The DNA G+C content of the isolate was 63.8 mol%. Based on the phylogenetic, phenotypic, and chemotaxonomic characterization and the whole genome analysis, strain S1CR25-10^T represents a new species within the genus *Natrinema*, for which the name *Natrinema salsiterrestre* sp. nov., with type strain S1CR25-10^T (= CECT 30623^T = CCM 9251^T), is proposed.

INTRODUCTION

The genus *Natrinema* was proposed by McGenity et al. in 1998 by reassigning two strains, previously described as *Halobacterium salinarum* NCIMB 786^T and *Halobacterium halobium* NCIMB 777^T, to the new genus *Natrinema*, as *Natrinema pellirubrum* and *Natrinema pallidum*, respectively [1]. Following the description of the genus *Haloterrigena* in 1999 [2] there have been differing opinions regarding the taxonomic position of species classified within this genus and the genus *Natrinema*. The studies based on the analysis of molecular markers (such as 16S rRNA, *atpB*, *EF-2*, *radA*, *rpoB'*, and *secY* gene sequences) and DNA-DNA hybridization data resulted in overlap among members in phylogenetic tree reconstructions, confusing the border between two genera [3-8]. The issue was finally clarified by a detailed phylogenomic and comparative genomic study based on whole genome sequences, causing a transfer of several *Haloterrigena* species into the genus *Natrinema* [8]. Currently, the genus *Natrinema* comprises 17 species that were isolated from various habitats including salt lakes, salterns, saline soils, and salted food [8, 9]. Cells of species of this genus are rod-shaped, but pleomorphic in unfavourable conditions, aerobic, and stain Gram-negative [1]. The polar lipids found in all members of this genus are phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (PGP-Me). Some species, in addition, possess phosphatidylglycerol sulfate (PGS), sulfated diglycosyl diether (S-DGD-1), and mannose-2,6-disulfate (1→2)-glucose glycerol diether (S_2-DGD-1) [8].

The aquatic hypersaline habitats, such as saline lakes and salterns, have been thoroughly investigated, while more recent studies have focused on the microbiology of hypersaline soils. Strain S1CR25-10^T was isolated from a hypersaline soil located in Odiel Saltmarshes, a natural area of tidal wetlands located at the estuary of the Tinto and Odiel rivers in the province of Huelva (Southwestern Spain). In this study, we describe the isolation, characterization, and complete taxogenomic analysis of this halophilic archaeon from a hypersaline soil and we propose it to be considered as a new species of the genus *Natrinema*.

ISOLATION AND CULTIVATION FROM A TERRESTRIAL HABITAT

Strain S1CR25-10^T was isolated from a hypersaline soil sample of a natural area located in Odiel Saltmarshes, Southwestern Spain (37°12'26.6"N 6°57'52.5"W). The sampling was performed in July 2020. The physico-chemical characteristics of the sampled soil included the determination of the pH values and the electrical conductivity with a pH meter (CRISON BASIC 20) and a conductometer (CRISON 35+), respectively, after a 1:2.5 dilution. The measured pH was 8.3 and the electrical conductivity was 69.9 mS/cm. To investigate a potential increased level of heavy metals due to past metallurgic operations in this area, the soil sample was analyzed by Innoagral Laboratories in Mairena del Aljarafe (Spain). Several most prevalent heavy metals (copper, lead, and cadmium) fulfilled the standards of uncontaminated soils designated by the Environment Department of the regional Government of Andalusia [10], with concentrations of 85.7, 28.8, and 0.5 mg/kg, respectively. Nevertheless, arsenic and zinc concentrations (11.1 and 84.4 mg/kg, respectively) exceeded the reference intervals, suggesting a certain heavy metal tolerance or resistance of the isolated strain. Further investigations should be considered as haloarchaeal strains have demonstrated significant capacity in resistance to toxic heavy metals [11-13], and thus, they could possibly be applied in bioremediation.

For the isolation of this new strain, the sample was serially diluted and plated under sterile conditions and incubated at 37 °C for up to 3 months. R2A medium (Difco), a low nutrient medium consisting 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 sulfate, 0.05, was used as an isolation and cultivation medium, containing 25 % (w/v) of total salts (designated as R2A 25 % medium), prepared from a 30 % (w/v) stock salt solution [14] which contained (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). After succeeding cultivation, strain S1CR25-10^T was

obtained in pure culture by the streak plate method. For long-term preservation, cultures were maintained at -80 °C in R2A 25 % (w/v) liquid medium containing 40 % (v/v) glycerol. As reference strains for comparative phenotypic and chemotaxonomic analysis, we used *Natrinema pellirubrum* DSM 15624^T, *Natrinema versiforme* DSM 16034^T and *Natrinema salaciae* CECT 8172^T. These reference strains were grown on R2A 25 % medium under the same conditions than strain S1CR25-10^T.

PHYLOGENETIC STUDIES

The genomic DNA of the strain S1CR25-10^T was extracted using the method described by Marmur [15] adjusted for small volumes. The 16S rRNA and *rpoB'* genes were amplified by PCR [16] using a Bio-Rad T100 Thermal Cycler. We selected the universal archaeal primers ArchF and ArchR [17, 18] for 16S rRNA gene amplification and the primers used for PCR of *rpoB'* gene described by Fullmer et al [19]. The integrity of both genomic DNA and PCR amplicons was checked by 1 % agarose gel electrophoresis. To purify the DNA and PCR products, we used MEGAquick-spinTM Plus Fragment DNA Purification Kit (iNtRON Biotechnology), following the manufacturer's instructions.

The PCR products were sequenced by StabVida (Caparica, Portugal) using the Sanger chain-termination method. In addition to the aforementioned PCR primers, two more 16S rRNA locus-targeted reverse primers, i.e., B36 (GGA CTA CCA GGG TAT CTA) and D34 (GGT CTC GCT CGT TGC CTG), were used for sequencing. The overlapping 16S rRNA and *rpoB'* gene sequences obtained from the studied strain were individually assembled with ChromasPro v.1.5 program (Technelysium Pty Ltd) and taxonomically identified by BLASTN search [20] against NCBI GenBank database and by EzBioCloud identification service [21]. Strain S1CR25-10^T was most closely related to *Natrinema salinisoli* SLN56^T, *Natrinema altunense* JCM 12890^T, and *Natrinema pallidum* JCM 8980^T with 98.9 %, 98.5 %, and 98.4 % 16S rRNA gene sequence identity, respectively. Based on *rpoB'* gene sequence identity the most closely related species were *Natrinema salinisoli* SLN56^T (97.2 %), *Natrinema longum* ABH32^T (94.9 %), and *Natrinema soli* 5-3^T (94.4 %). Phylogenetic tree reconstructions based on the 16S rRNA sequences were conducted using the maximum-parsimony [22], neighbor-joining [23] and maximum-likelihood [24] algorithms as implemented in the ARB software [25] and they confirmed the placement of the new isolate into a separate branch along with *Natrinema salinisoli* SLN56^T (**Figure 1**). In addition, *rpoB'* gene-based phylogenetic tree reconstructed by the ARB software, clustered strain S1CR25-10^T with the already described species of the genus *Natrinema* (**Figure 2**), but distant enough from them as to be suggested to represent a new species within this genus. The 16S rRNA and *rpoB'* gene sequences of strain S1CR25-10^T were deposited in GenBank/EMBL/DDBJ, under the accession numbers ON653413 and ON668046, respectively.

PHYLOGENOMIC ANALYSIS

The concentration of the extracted DNA was determined by Qubit 4 Fluorometer (Thermo Fisher Scientific), and its quality was checked spectrophotometrically with NanoDrop One (Thermo Fisher Scientific). The whole genome sequencing of strain S1CR25-10^T was carried out by Novogene Europe (Cambridge, United Kingdom) using an Illumina HiSeq 4000 platform. The guidelines for the use of genome data for taxonomic purposes were followed [26]. The draft genome of the studied strain was assembled into 27 contigs (N50, 475,096; coverage, 346X) using Spades v.3.13.0 [27], with a total size of 4,787,139 bp and a G+C content of 63.8 mol%. The standard genome annotation was conducted by Prokka v.1.12 [28]. The PCR amplified genes (16S rRNA and *rpoB'*) matched the sequences determined by genomic sequencing. Further general genomic features of strain S1CR25-10^T and other type strains of species of the genus *Natrinema* are shown in **Table S1**. Completeness and contamination of the new assembly were also verified by CheckM v1.0.5 [29]. The curated whole-genome sequence of strain S1CR25-10^T was deposited in GenBank/EMBL/DDBJ, under the accession number JAMQOT000000000. The publicly available genome sequences of the type strains of species of the genus

Natrinema and those of the closely related taxa of the family *Natrialbaceae* were downloaded from NCBI GenBank database and further used to carry out a phylogenomic comparative analysis. The Enveomics toolbox [30] was employed to identify the orthologous protein set shared by all analyzed strains, as previously described elsewhere [31]. An approximately maximum-likelihood phylogenomic tree (**Figure 3**) was reconstructed based on the sequence of the 1,524 core-orthologous proteins using FastTreeMP v.2.1.8 [32]. The tree topology revealed that the studied strain grouped again with *Natrinema salinisoli* SLN56^T but far enough as to constitute a new species within the genus *Natrinema*.

To verify that hypothesis, we calculated the overall genome relatedness indexes, specifically the Orthologous Average Nucleotide Identity (OrthoANI), the digital DNA-DNA hybridization (dDDH), and the Average Amino acid Identity (AAI), which were estimated for each genome pair using OrthoANIu tool v.1.2 [33], the Genome-to-Genome Distance Calculator (GGDC 3.0) from the Leibniz Institute DSMZ (Germany) [34], and the 'aai.rb' script from the Enveomics collection [30], respectively. The relatedness values determined between strain S1CR25-10^T and the type strains of the species of the genus *Natrinema* were 81.3-92.6 % for orthoANI, 23.8-48.4 % for dDDH, and 76.4-92.6 % for AAI. Therefore, all genomic indexes displayed values lower than the accepted cutoff limits for prokaryotic species delineation [35, 36, 37, 38], confirming that the strain S1CR25-10^T constitutes a novel species. Besides, AAI values were above the 65 % threshold for genus demarcation, proving that this strain must be placed within the genus *Natrinema* (**Figures 4** and **5**).

With the aim to compare the orthologous clusters (OCs) detected for strains S1CR25-10^T, *Nnm. salinisoli* SLN56^T, *Nnm. longum* ABH32^T, *Nnm. soli* DC36^T, and *Nnm. amylolyticum* LT61^T, the online OrthoVenn2 server [39] was used. The analyzed species of the genus *Natrinema* shared a total of 2,456 OCs as shown in **Figure S1**. Aditionally, calculation of the isoelectric point of the predicted proteins, generated by Prodigal v.2.60 [40], from all the species of the genus *Natrinema* and other reference taxa for comparative purposes was computed using the 'iep' program of the EMBOSS package [41]. The strain S1CR25-10^T and the species of *Natrinema* shared a compliant isoelectric profile with a peak at around 4 (**Figure S2**), showing an acidic proteome and, thus, a "salt-in" osmoregulation strategy [42].

PHENOTYPIC FEATURES

A complete phenotypic characterization, which involved morphological, physiological, biochemical, and nutritional features, was performed according to the minimal standards for the description of novel taxa of the class *Halobacteria* [34]. Cell morphology and motility were examined by a phase-contrast microscope (Zeiss Axioscope 5). Gram staining was performed according to Oren et al. [43]. The range and optimal growth of strain S1CR25-10^T was determined in R2A medium using a range of gradually increasing salt concentrations (0.5, 5, 10, 15, 20, 25, and 30 % [w/v]), prepared from the 30 % (w/v) stock solution. Similarly, the pH (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5) and temperature (from 20 °C to 55 °C, at 5 °C intervals) ranges and optimal values supporting growth were determined in R2A 25 % (w/v) buffered medium. Colonial morphology, size, and pigmentation were observed on R2A 25% medium after 10 days of incubation at 37 °C. Cells of strain S1CR25-10^T were motile, Gram-stain-negative, 0.5 × 2-6 μm in size, and rod-shaped, but became pleomorphic under unfavorable conditions (**Figure S3**). Colonies were small, circular, 0.2-0.3 mm in diameter, pink-pigmented, and rough at the edges on older cultures. Growth was detected in the presence of 12-30 % (w/v) total salts (optimum, 20-25 % [w/v]), at pH 6.0-9.0 (optimum, pH 7.0-8.0), and a temperature range of 25-55 °C (optimum, 37 °C).

The catalase test was conducted by adding a few drops of 3 % H_2O_2 (v/v) to a young culture of the microorganism [44]. Oxidase activity was determined by using 1 % (v/v) tetramethyl-p-phenylenediamine [45]. To examine whether the strain S1CR25-10^T was able to grow anaerobically, R2A medium plates supplemented with alternative electron acceptors (L-arginine, DMSO, and KNO_3) were incubated at 37 °C during 14 days in a gas-

pak system using AnaeroGen (Oxoid) and an anaerobic indicator (Oxoid) in order to provide and control, respectively, an anaerobic environment. Starch, aesculin, gelatin, and Tween 80 hydrolysis were assessed as previously described by Durán-Viseras et al. [46]. Other biochemical tests were carried out following the methodology described by Oren et al. [33] (methyl red, Voges-Proskauer, and Simmons' citrate tests), Smibert et al. [47] (nitrate and nitrite reduction), Gerhardt et al. [48] (indole production), Christensen et al. [49] (urease test), and Ventosa et al. [50] (H_2S formation and acid production from carbohydrates). Strain S1CR25-10^T possesed the enzyme catalase but did not show oxidase activity. The isolate did not grow anaerobically. It hydrolysed aesculin, but not starch, gelatin, and Tween 80. Methyl red test and nitrate reduction were positive, but nitrite reduction and the remaining biochemical tests were negative. Susceptibility to antibiotics was determined by the disc diffusion method after spreading strain S1CR25-10^T on R2A 25% solid medium [43]. Strain S1CR25-10^T was resistant to ampicillin (10 μg), chloramphenicol (30 μg), neomycin (30 μg), gentamicin (10 μg), penicillin G (10 IU) and nalidixic acid (30 μg) but was susceptible to rifampin (5 μg). Acid was produced from D-arabinose, D-fructose, D-galactose, D-glucose, sucrose, and D-xylose. In order to determine nutritional characteristics, we followed the methodology described by Ventosa et al. [50]. The following substrates were used as sole carbon and energy sources: D-arabinose, D-cellobiose, citrate, ethanol, formic acid, D-fructose, fumarate, D-galactose, D-glucose, melezitose, melibiose, pyruvate, D-ribose, salicin, D-sorbitol, sucrose, xylitol, and D-xylose. L-alanine, L-arginine, and L-glutamine were used as sole carbon, nitrogen, and energy sources. *Natrinema pellirubrum* DSM 15624^T, *Natrinema versiforme* DSM 16034^T, *Natrinema salaciae* CECT 8172^T, and *Natrinema salinisoli* SLN56^T were chosen as reference strains for phenotypic feature comparison (**Table 1**).

CHEMOTAXONOMY

Polar lipids composition has been found to be very useful in the characterization and differentiation of haloarchaea [43, 51]. We extracted polar lipids from a cell biomass of strain S1CR25-10^T cultivated in R2A 25 % (w/v) liquid medium. The polar lipids of the reference strains *Halobacterium salinarum* DSM 3754^T, *Halorubrum saccharovorum* DSM 1137^T, *Natrinema pellirubrum* DSM 15624^T, *Natrinema versiforme* DSM 16034^T, and *Natrinema salaciae* CECT 8172^T were also extracted in order to compare their polar lipids profiles with that of the new strain and, therefore, to confirm its assignment to the genus *Natrinema*. The chemotaxonomic characterization was carried out by High Performance Thin Layer Chromatography (HPTLC) on silica gel glass plates (10 × 20 cm, Merck) washed in methanol/chloroform 50:50 (v/v) and developed in chloroform (39.4 ml)/ methanol (2.42 ml)/90 % acetic acid (18.18 ml) solvent system [52, 53]. To detect all polar lipids, 5 % (v/v) sulfuric acid was used followed by heating at 160 °C. The chromatography plate showed that strain S1CR25-10^T harbored the following polar lipids: phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and a glycolipid chromatographically identical to sulfated diglycosyl diether (S-DGD-1), which is in concordance with the lipid profile of species of the genus *Natrinema* (**Figure S4A**). The phospholipids (**Figure S4B**) were revealed by molybdenum blue spray reagent to complete the chemotaxonomic study and double check the affiliation of the sudied strain to the genus *Natrinema*.

The phylogenetic and phenotypic studies, polar lipids determination, and comparative genomic analysis demonstrated that strain S1CR25-10^T represents a novel species within the genus *Natrinema*, family *Natrialbaceae*, order *Natrialbales*, for which the name *Natrinema salsiterrestre* sp. nov. is proposed.

DESCRIPTION OF *NATRINEMA SALSITERRESTRE* SP. NOV.

Natrinema salsiterrestre (sal.si.ter.res'tre. L. masc. perf. part. *salsus*, salted, salty; L. masc. adj. *terrestris*, of or belonging to the earth, terrestrial; N.L. neut. adj., *salsiterrestre*, of a salted soil).

Cells are Gram-stain-negative, motile, 0.5 × 2-6 μm rods, that become pleomorphic in old cultures under unfavorable conditions. Colonies are small, circular, 0.2-0.3 mm in diameter and pink-pigmented after 10 days of

incubation at 37 °C. No growth occurs anaerobically with L-arginine, potassium nitrate, or DMSO. Extremely halophilic archaeon. Growth occurs in a wide range of salt concentrations between 12-30 % (w/v), with optimum at 20-25 % (w/v). Cells lyse in distilled water. Optimal growth occurs at pH values and temperature of 7.0-8.0 and 37 °C, respectively. The pH and temperature ranges permitting growth are 6.0-9.0 and 25-55 °C, respectively. Catalase-positive, oxidase-negative. Aesculin is hydrolyzed but gelatin, starch, and Tween 80 are not. Nitrate is reduced but nitrite is not. H_2S and indole are not produced. Methyl red test is positive whereas Voges-Proskauer and Simmons' citrate tests are negative. Acid was produced from D-arabinose, D-fructose, D-galactose, D-glucose, sucrose, and D-xylose. The following compounds are used as sole carbon and energy sources: D-arabinose, D-cellobiose, citrate, ethanol, formic acid, D-fructose, fumarate, D-galactose, D-glucose, melezitose, melibiose, pyruvate, D-ribose, salicin, D-sorbitol, sucrose, xylitol, and D-xylose. L-alanine, L-arginine, and L-glutamine were used as sole carbon, nitrogen, and energy sources The major polar lipids are phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and a glycolipid chromatographically identical to sulfated diglycosyl diether (S-DGD-1). The DNA G+C content is 63.8 mol%.

The type strain is S1CR25-10^T (= CECT 30623^T = CCM 9251^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 S1CR25-10^T are ON653413 and ON668046, respectively. The GenBank/EMBL/DDBJ accession number of the whole genome sequence of strain S1CR25-10^T is JAMQOT000000000.

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403 **Table 1.** Differential characteristics between strain S1CR25-10^T and related species of the genus *Natrinema*.

404 All data are from this study unless otherwise indicated. +, positive; -, negative; ND, not determined. [a]Data from McGenity et
405 al. [1]; [b]Data from Albuquerque et al. [54]; [c]Data from Xin et al. [55]; [d]Data from Bao et al. [56].

Characteristic	**Strain S1CR25-10^T**	***Nnm. pellirubrum* DSM 15624^T**	***Nnm. salaciae* CECT 8172^T**	***Nnm. versiforme* DSM 16034^T**	***Nnm. salinisoli* SLN56^T**
Morphology	Rods/ Pleomorphic	Rods[a]	Pleomorphic[b]	Pleomorphic[c]	Pleomorphic[d]
Colony pigmentation	Pink	Light red or orange[a]	Red[b]	Light red[c]	Red[d]
Salt requirement:					
Range (%, w/v)	12-30	10-25[a]	10-30[b]	≥9[c]	10-28[d]
Optimum (%, w/v)	20-25	20-25[a]	15-20[b]	20-25[c]	23[d]
Temperature requirement:					
Range (°C)	25-55	20-45[a]	30-52.5[b]	20-53[c]	35-55[d]
Optimum (°C)	37	ND	45[b]	37-46[c]	37[d]
pH requirement:					
Range	6.0-9.0	6.0-8.6[a]	6.5-9.0[b]	6.0-8.0[c]	7.0-9.5[d]
Optimum	7.0-8.0	7.2-7.8[a]	7.0-8.0[b]	6.5-7.0[c]	7.5[d]
Anaerobic growth with nitrate	-	-	+	+	+[d]
Hydrolysis of:					
Gelatin	-	-	+	-	-[d]
Tween 80	-	+	+	-	-[d]
Aesculin	+	-	+	+	ND
Acid production from glycerol	-	-	+	+	ND
Utilization as sole carbon and energy sources:					
Amygdalin	-	-	+	+	ND
D-Maltose	-	-	+	+	-[d]
D-Raffinose	-	-	+	+	ND
Mannose	-	+	+	+	+[d]
Starch	-	+	+	+	ND
Butanol	-	+	+	+	ND
Dulcitol	-	+	+	+	ND
Glycerol	-	+	+	+	+[d]
Mannitol	-	+	+	+	+[d]
Myo-inositol	-	-	+	+	ND
Benzoate	-	-	+	+	ND
Butyrate	-	+	+	+	ND
L-Glutamate	-	-	+	+	+[d]
L-Alanine	+	+	-	-	-[d]
L-Arginine	+	+	-	-	-[d]
L-Glutamine	+	+	-	-	ND

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Legends to Figures

Figure 1. Maximum-likelihood phylogenetic reconstruction based on 16S rRNA gene sequences showing the relationships between strain S1CR25-10^T, members of the genus *Natrinema*, and other related species within the family *Natrialbaceae*. The species *Halorubrum saccharovorum* JCM 8865^T was used as an outgroup. Sequence accession numbers are shown in parentheses. Bootstrap values (%) are based on 1000 pseudoreplicates and only those ≥70 % are shown at branch points. Black circles indicate branches that were recovered in the maximum-likelihood, neighbor-joining, and maximum-parsimony phylogenetic trees. Bar represents expected substitutions per nucleotide position.

Figure 2. Neighbor-joining phylogenetic reconstruction based on *rpoB'* gene sequences of strain S1CR25-10^T and related species of the family *Natrialbaceae*. The species *Halorubrum saccharovorum* JCM 8865^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 neighbor-joining and maximum-likelihood algorithms. Bar, 0.01 expected substitutions per nucleotide position.

Figure 3. Approximately maximum-likelihood phylogenomic tree based on the comparison of 1524 core-orthologous proteins showing the relationships between strain S1CR25-10^T, members of the genus *Natrinema*, and other related species within the family *Natrialbaceae*. 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 nucleotide position.

Figure 4. Heatmap displaying Orthologous Average Nucleotide Identity (OrthoANI) and digital DNA-DNA hybridization (dDDH) percentages among strain S1CR25-10^T, members of the genus *Natrinema*, and other related species of the family *Natrialbaceae*.

Figure 5. Heatmap showing Average Amino acid Identity (AAI) percentages among *Natrinema* species, including strain S1CR25-10^T, and other related species of the family *Natrialbaceae*.