Halorubrum Salipaludis Sp. Nov., Isolated From the Saline–Alkaline Soil

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Abstract

Strain WN019^T, an aerobic, motile, and pleomorphic rods bacterium, was isolated from the natural saline-alkali wetland soil of Binhai new district, Tianjin, China. Cells of strain WN019^T were 0.5-0.8 μm in width and 2.0-2.5 μm in length, and the growth occurred optimally at 33-37 °C, pH 7.5-8.0, and in the presence of 15.0-20.0 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that the isolate belonged to the genus *Halorubrum* and exhibited high sequence similarity of 97.8 % to *Halorubrum saccharovorum* JCM 8865^T. The major respiratory quinone of strain WN019^T were MK-8 and MK-8 (H_2), and the major polar lipids were Glycolipid (GL), Phospholipid (PL), Phosphatidylglycerol-Sulfate (PGS), Phosphatidylglycerol (PG) and Phosphatidylglycerol-Phosphate-Methyl Ester (Me-PGP). The DNA G+C content of the strain was 67.3 mol%. The average nucleotide identity (ANI) based on whole genome sequences of strain WN019^T and *Halorubrum saccharovorum* JCM 8865^T was 87.5 %, and the digital DNA-DNA hybridization (dDDH) value between them was determined to be 35.4 %. Phenotypic, chemotaxonomic, phylogenetic, and genomic analyses suggested that strain WN019^T represent a novel species of the genus *Halorubrum*, for which the name *Halorubrum salipaludis* sp. nov. is proposed. The type strain is WN019^T (= KCTC 4269^T = ACCC 19977^T).

Introduction

The genus *Halorubrum* was first proposed by McGenity and Grant belonging to the family Halorubraceae, and the type strain of *Halorubrum saccharovorum* was affiliated to strain JCM 8865^T (McGenity and Grant 1995). At the time of writing, the genus *Halorubrum*, which is the largest genus within the family Halorubraceae, consists of 37 validly published species that are phylogenetically and phenotypically heterogeneous listed on the LPSN (http://www.bacterio.net/). It has been reported that the genus *Halorubrum* are widely distributed in various hypersaline environments, such as salt lakes, hypersaline soda lakes, coastal sabkhas, salt mine, saline soil, salt-fermented seafood, and marine salterns (Chen et al. 2017a, b; de la Haba et al. 2018; Ochsenreiter et al. 2002). *Halorubrum* species, which grow fast and are active in the conversion of different kinds of substrates, may play a major role in carbon and nitrogen cycling of several hypersaline environments (Feng et al. 2004). Saline-alkali land is a special and major resource with high salinity and alkalinity. In this study, we reported the novel strain WN019^T of the genus *Halorubrum*, which can live in the saline-alkaline habitat, according to their phenotypic, biochemical, and genotypic characteristics.

Materials And Methods

Isolation and Culture Conditions

The marine sediment samples were collected from the natural saline-alkali wetland soil of Binhai new district, Tianjin, China (38°46'N, 117°13'E), and were transferred to the laboratory with ice. Then they were purified into a single colony on the modified R2A (MR2A) medium using the traditional dilution

inoculation method, for 1 liter medium containing the following ingredients: 0.5 g casamino acids (Difo), 0.5 g of yeast extract (Difo), 0.5 g of sodium pyruvate, 0.5 g of peptone, 0.5 g of glucose, 3.0 g of trisodium citrate, 2.0 g of KCl, 0.3 g of K_2HPO_4, 0.5 g of $CaCl_2$, 20.0 g of $MgSO_4 \bullet 7H_2O$, 180 g of NaCl, pH 7.0-7.5, agar 15.0 g, 121 ºC, autoclaving 20 min (Reasoner and Geldreich 1985). The purified strain was preserved in cryotubes at -80 ºC by adding 250 µL glycerol/SW 30 (80:20, v/v) to 750 µL fresh culture (OD_{600} 0.8-1.0) (Dyall-Smith, 2009) for further characterization. *Halorubrum saccharovorum* JCM 8865^T, *Halorubrum persicum* JCM 30541^T and *Halorubrum kocurii* JCM 14978^T were incubated on MR2A medium with 18.0 % (w/v) NaCl and used as the reference strains for all experiments.

Morphological, Physiological and Biochemical Characterization

Cell size, morphology, and motility of strain WN019^T were established by using a Leica microscope equipped with phase–contrast optics (Leica DM 6000 B) during exponential growth phase. Cell morphology was also assessed by transmission electron microscopy (TEM), i.e., cells were harvested from exponentially growing culture, and the cells were negatively stained with 0.5 % uranyl acetate and the grids were examined at the microscope (Tecnai Spirit, FEI, Hillsboro, OR, USA). Gram staining was performed using BD Gram staining kits according to the manufacturer's instructions. Oxidase activity was tested using the oxidase reagent kit (bioMérieux) according to the manufacturer's instructions. Catalase activity was determined by pouring a 3.0 % H_2O_2 solution onto bacterial colonies and observing bubble production. The optimal growth temperature of strain WN019^T was determined after incubation on MR2A medium at 4, 10, 15, 20, 25, 30, 33, 37, 40, 45, and 50 °C (at pH 7.5). NaCl tolerance was tested in MR2A medium amended with 0.0-25.0 % NaCl (w/v) at intervals of 1.0 %. The pH range for growth was measured by adjusting the final pH value to 4.0-13.0 at intervals of 0.5 (at 18.0 % NaCl, w/v, 37 °C) with the appropriate buffers (Na_2HPO_4/NaH_2PO_4 for pH 5.0-7.0 and $Na_2CO_3/NaHCO_3$ for pH 8.0-12.0). Bacterial growth was measured as increase in turbidity at 600 nm, using a DU 800 spectrophotometer (Beckman Coulter). Anaerobic growth was determined through measuring the OD_{600} nm at 37 °C with 18.0 % NaCl (w/v) in the tubes with the butyl rubber stopper and screw cap.

For all physiological experiments, we selected *Halorubrum saccharovorum* JCM 8865^T, *Halorubrum persicum* JCM 30541^T, and the closely related strain *Halorubrum kocurii* JCM 14978^T as reference organisms. Unless otherwise stated, all the strains mentioned above were incubated at 37 °C in MR2A medium amended with final concentration of 18.0 % NaCl for strain WN019^T and final concentration of 18.0 % NaCl for reference organisms.

Biochemical activities and use of organic substrates as sole carbon and energy sources were evaluated using API 20E, API 20NE kits (bioMérieux) and Biolog GENIII MicroPlates (bioMérieux) according to the manufacturer's instructions, except for a salinity adjustment to 18.0 % (w/v) NaCl. This involved supplementing with (l-1) 2.0 g of KCl, 0.3 g of K_2HPO_4, 0.5 g of $CaCl_2$ and 20.0 g of $MgSO_4 \bullet 7H_2O$ (pH between 7.0-7.5). Susceptibility to antibiotics was assessed on MR2A (18.0 % NaCl) using the disc–diffusion plate method (Fraser and Jorgensen 1997) with discs containing ampicillin (10 µg),

chloramphenicol (30 µg), erythromycin (15 µg), penicillin G (10 µg), streptomycin (10 µg), vancomycin (30 µg), gentamicin (10 µg), polymyxin B (30 µg), neomycin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), aphidicolin (20 µg), norfloxacin (10 µg), nitrofurantoin (300 µg), trimethoprim (5 µg), mycostatin (100 µg), novobiocin (30 µg), and bacitracin (0.04 IU per disc). Antibiotics–containing discs were placed on MR2A (18.0% NaCl) plate surfaces, and the bacterial cultures (200 µL) that were spread on the plate were checked for clearing zones after 3 d at 37 °C.

Chemotaxonomic Characterization

To characterize respiratory quinones and polar lipids of strain WN019^T and the reference strains, cells were grown in medium mentioned above (37 °C) and harvested during late exponential growth phase. Respiratory quinones were extracted with chloroform/methanol (2:1) (v/v) from lyophilized cells (300 mg) and purified using high performance liquid chromatography (HPLC) (Minnikin DE 1984). Analysis of respiratory quinones were carried out by the Identification Service, DSMZ, Braunschweig. Germany. Polar lipids were extracted from 200 mg of freeze-dried cell material using a chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture with the ratio of 1:2:0.8 (v/v/v), modified after Bligh and Dyer (1959), recovered into the chloroform phase by adjusting the mixture to a ratio of 1:1:0.9 (v/v/v), and separated by two-dimensional silica gel thin-layer chromatography (Qingdao. Haiyang Chemical Co.; silica gel GF254, 0.25-mm thick, China). The first dimension was developed in a chloroform/methanol/water (65:25:4, v/v/v) mixture and the second was in a chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v) mixture. Total lipid materials were detected using molybdatophosphoric acid and specific functional groups were detected using spray reagents specific for defined functional groups. Polar lipid analysis was performed by the Identification Service, DSMZ, Braunschweig, Germany.

Molecular Characterization

Genomic DNA was extracted using a commercial kit (TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0) based on the manufacture's protocol. The amplification of the 16S rRNA fragment was done by PCR with 27F and 1492R as primers and genomic DNA as template. The genome was sequenced on the Illumina HiSeq2000 platform at Shanghai Personal Biotechnology Co., Ltd. China. Filtering and trimming of the genomic raw data were done with PRINSEQ v0.20.4 (Schmieder and Edwards 2011), and the trimmed reads were assembled using SOAPdenovo v.2.3 (Li et al. 2008, 2010) with default parameters. The genome completeness (100 %) was assessed using CheckM (version 1.03) (Parks et al. 2015). Protein-coding open reading frames were predicted by Glimmer (version 3.02) (Delcher et al. 2007). For RNA prediction, rRNAs were predicted by RNAmmer (version 1.2) (Lagesen et al. 2007), and tRNAs were predicted by tRNAscan-SE (version 1.21) (Lowe and Eddy 1997). The 16S rRNA gene sequence and the genome of strain WN019^T was submitted to GenBank (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignments of strain WN019^T and the most closely related taxa were carried out using CLUSTALXv1.81 (Thompson et al. 1997). Phylogenetic trees were constructed with the maximum-likelihood (ML) method using MEGA v7.0 (Kumar et al. 2016), and neighbour-joining (NJ) and maximum parsimony (MP) phylogenetic trees were also constructed to

confirm the phylogenetic position of the strain WN019^T. The resultant tree topologies were evaluated by bootstrap analyses (1000 replications). Average nucleotide identity (ANI) values of the total genomic sequences shared between the genomic sequences of strain WN019^T and closely related genomic sequences from GenBank were performed using the ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms in JSpeciesWS (https ://jspecies.ribohost.com/jspeciesws/) (Richter et al. 2016). As a proposed complement to ANI values, digital DNA–DNA hybridization (dDDH) values were calculated using Genome-to-Genome Distance Calculator (GGDC2.1) (Meier-Kolthoff et al. 2013) using the BLAST+ method. Results were recommended based on the recommended formula 2 (identities/ HSP length), which was useful when dealing with incomplete draft genomes.

Results And Discussion

Morphological, Physiological and Biochemical Characteristics

The cells of strain WN019^T were aerobic, motile, pleomorphic rods, and 0.5-0.8 μm in width and 2.0-2.5 μm in length (Supplementary Fig. S1). Gram-staining-variable: in young cultures, most cells are Gram-staining-negative, while a few cells are observed as Gram-staining-positive. Cells of strain WN019^T were resistant to ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), penicillin G (10 μg), streptomycin (10 μg), vancomycin (30 μg), gentamicin (10 μg), polymyxin B (30 μg), neomycin (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), aphidicolin (20 μg), norfloxacin (10 μg), nitrofurantoin (300 μg), trimethoprim (5 μg) and mycostatin (100 μg), but sensitive to novobiocin (30 μg), bacitracin (0.04 IU per disc). Some other physiological and biochemical characteristics of strain WN019^T and comparison with the close relatives were presented in the species description and Table 1.

Chemotaxonomic Characteristics

MK-8 and MK-8 (H_2) were identified as the major respiratory quinone of strain WN019^T, which is the same as *Halorubrum saccharovorum* JCM 8865^T, *Halorubrum persicum* JCM 30541^T and *Halorubrum kocurii* JCM 14978^T. The only difference between them lied in the percentage of each respiratory quinone. More specifically, WN019^{T}contains 83 % of MK-8 and 17 % of MK-8 (H_2), *Halorubrum saccharovorum* JCM 8865^T contains 95 % of MK-8 and 5 % of MK-8 (H_2), *Halorubrum persicum* JCM 30541^T contains 96 % of MK-8 and 4 % of MK-8 (H_2), while 96 % and 4 % for *Halorubrum kocurii* JCM 14978^T.

Strain WN019^T contained the following major polar lipids: glycolipid (GL), phospholipid (PL), phosphatidylglycerol-sulfate (PGS), phosphatidylglycerol (PG) and phosphatidylglycerol-phosphate-methylester (Me-PGP). The polar lipid of Strain WN019^T is parallel to its close relative *Halorubrum persicum* JCM 30541^T, but different from *Halorubrum saccharovorum* JCM 8865^T and *Halorubrum kocurii* JCM 14978^T at PL (Supplementary Fig. S2).

Molecular Characteristics

The 16S rRNA gene sequence of strain WNO19^T is 1,465 bp long and has been deposited to NCBI under the accession number MF782426. The genome assembly of strain WN019^T has been deposited at DDBJ/EMBL/GenBank under the accession NSKC00000000 as presented here is 3,506,649 bp in size, and the G+C content is 67.3 mol%. We predicted a total of 3,217 proteins, 48 tRNAs, 5 rRNAs, 2 ncRNAs for strain WN019^T, and it has only one copy of the 16S partial rRNA gene. Based on the phylogenetic analysis of the nearly complete 16S rRNA retrieved from genome, strain WN019^T was clearly affiliated with the *Halorubrum* clade in the family Halorubraceae (Fig. 1, Supplementary Fig. S3 and S4). The similarity of 16S rRNA between strain WN019^T and the following species: *Halorubrum saccharovorum* JCM 8865^T, *Halorubrum persicum* JCM 30541^T, *Halorubrum kocurii* JCM 14978^T, *Halorubrum halophilum* JCM 18963^T, *Halorubrum lipolyticum* JCM 13559^T, *Halorubrum lacusprofundi* ATCC 49239^T was 97.8 %, 97.7 %, 97.0 %, 96.9 %, 96.8 % and 96.7 %, respectively. The levels of 16S rRNA gene sequence similarity between strain WN019^T and other type strains of the genus *Halorubrum* were all below 98.7 % (Chun et al. 2018), which suggesting strain WN019^T maybe represents a novel species distinct from all other members of the genus *Halorubrum*.

Halorubrum saccharovorum JCM 8865^T shows maximum similarity with strain WN019^T in terms of 16S rRNA, and its dDDH value with strain WN019^T is 35.4 %, and the dDDH values between strain WN019^T and another two closest related species *Halorubrum persicum* JCM 30541^T, *Halorubrum kocurii* JCM 14978^T was 34.2 % and 33.8 %, respectively. The ANI values between strain WN019^T and its close relatives, *Halorubrum saccharovorum* JCM 8865^T, *Halorubrum persicum* JCM 30541^T, *Halorubrum kocurii* JCM 14978^T, were 87.5 %, 87.0 %, and 86.8 %, respectively, which suggesting that strain WN019^T represents a novel species belonging to the genus *Halorubrum* based on the recommended minimum DNA-DNA relatedness value of 70 % (Wayne et al. 1987; Graham et al. 1991) and ANI value is 95 % (Richter and Rosselló-Móra 2009) for strains of the same species.

Taxonomic Conclusion

In this study, we isolated and described the novel strain WN019^T from the natural saline-alkali wetland soil. Based on phenotypic, phylogenetic, and chemotaxonomic analyses, strain WN019^T appears to represent a novel species of the genus *Halorubrum*, for which the name *Halorubrum salipaludis* sp. nov. is proposed.

Description of Halorubrum salipaludis **sp. nov.**

Halorubrum salipaludis (sa.li.pa.lu'dis. L. masc. n. *sal*, *salis* salt; L. fem. n. *palus*, *paludism* swamp, marsh; N.L. gen. n. *salipaludis* of a salt marsh).

Cells are aerobic, motile, pleomorphic rods, and 0.5–0.8 μm in width and 2.0-2.5 μm in length. The colonies are circular, wet, smooth, and red on MR2A. Gram-staining-variable: in young cultures, most cells are Gram-staining-negative, while a few cells are observed as Gram-staining-positive. Growth occurs at

15–50 ℃ (optimum at 33–37 ℃), at pH 6.5–12.0 (optimum pH 7.5-8.0), and with 10.0–25.0 % (w/v) NaCl (optimum at 15.0–20.0 %, w/v). Oxidase and catalase activities are positive. The reaction of nitrate to nitrite is positive. Gelatin and Tween 80 are hydrolyzed, but casein and starch not. For the type strain, the following substrates are utilized for growth as sole source of carbon and energy: D-maltose, D-trehalose, sucrose, α-D-glucose, D-mannose, D-fructose, D-mannitol, glycerol, L-alanine, L-arginine and methyl pyruvate. The following compounds are not utilized: dextrin, D-cellobiose, gentiobiose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-arabitol, myo-inositol and pectin. The major respiratory quinones of strain are MK-8 and MK-8 (H_2). And the major polar lipids are glycolipid (GL), phospholipid (PL), phosphatidylglycerol-sulfate (PGS), phosphatidylglycerol (PG) and phosphatidylglycerol-phosphate-methyl ester (Me-PGP).

The type strain WN019^T (= KCTC 4269^T = ACCC 19977^T) was isolated from the natural saline-alkali wetland soil of Binhai new district, Tianjin China (38°46'N, 117°13'E). The GenBank accession numbers for the 16S rRNA genes and the genome sequence are MF782426 and NSKC00000000, respectively. The genome of the type strain is characterized by a size of 3,506,649 bp and a G + C content of 67.3 mol%.

Declarations

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Tables

Table 1. Differential phenotypic characteristics that distinguish strain WN019^T from other related species of the genus *Halorubrum*. Taxa: 1, WN019^T; 2, *Halorubrum saccharovorum* JCM 8865^T; 3, *Halorubrum persicum* JCM 30541^T; 4, *Halorubrum kocurii* JCM 14978^T; 5, *Halorubrum halophilum* JCM 18963^T. +, positive; -, negative.

Characteristics	1	2	3	4	5
NaCl range for growth (%, w/v)	10-25	20-25	15-30	15-30	15-30
Optimum NaCl (%)	15-20	25	20	20	25
Temperature range for growth (℃)	15-50	30-56	20-45	25-55	20-45
Optimum temperature (℃)	33-37	50	37	37	35-40
pH range for growth	6.5-12.0	7.0-9.0	7.0-8.0	6.0-9.0	7.0-9.0
Optimum pH	7.5-8.0	8.0	7.0-7.2	7.5	8.0
Mg^{2+} requirement	+	+	-	-	+
Nitrate reduction	+	-	+	+	+
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Hydrolysis of					
Gelatin	+	-	-	-	-
Tween 80	+	-	-	-	+
Utilization of					
D-Maltose	+	-	+	-	+
D-Trehalose	+	+	+	-	-
Sucrose	+	-	+	+	-
D-Turanose	-	+	-	-	-
D-Raffinose	-	-	+	-	-
α-D-Lactose	-	-	-	-	+
α-D-Glucose	+	+	+	-	+
D-Mannose	+	+	+	-	+
D-Fructose	+	-	-	-	+
D-Galactose	-	+	-	-	-
D-Sorbitol	-	-	+	-	+
D-Mannitol	+	-	+	-	+
D-Arabitol	-	+	-	-	-
Glycerol	+	-	+	-	+
D-Fructose-6-PO4	+	-	-	-	+
D-Aspartic Acid	+	-	-	-	-
L-Alanine	+	-	-	-	+
L-Arginine	+	+	-	-	-
L-Serine	+	-	+	-	-
Pectin	-	+	+	+	-
Glucuronamide	-	+	-	-	+
DNA G+C content (mol%)	67.3	71.2[a]	64.2[b]	69.4[c]	64.6 [d]

[a] Data from (McGenity and Grant 1995), [b] Data from (Corral et al. 2015), [c] Data from (Gutiérrez et al. 2008), [d] Data from (Yim et al. 2014) and all other data was obtained from this study.

Figures

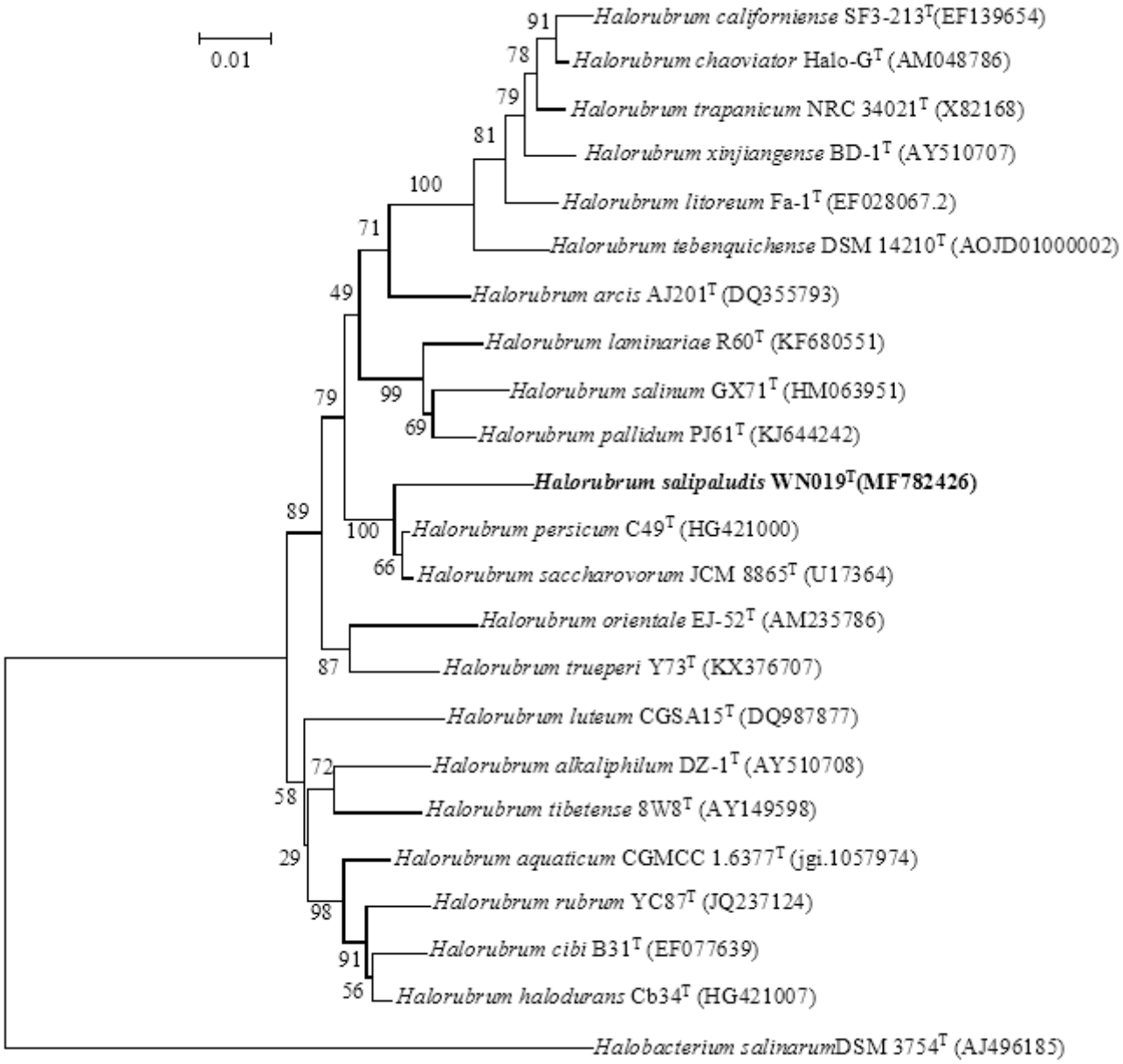

Figure 1

Neighbour-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain WN019T and related taxa. Bootstrap values > 50 % (1000 replicates) are shown. Scale bar indicates 0.01 substitutions per Halobacterium salinarum DSM 3754T (AJ496185) was used as an outgroup. GenBank accession numbers are indicated for each strain in parentheses.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.docx