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Title: *Halohasta litorea* gen. nov. sp. nov., and *Halohasta litchfieldiae* sp. nov., isolated from the Daliang aquaculture farm, China and from Deep Lake, Antarctica, respectively

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Halohasta litorea gen. nov. sp. nov., and *Halohasta litchfieldiae* sp. nov., isolated from the Daliang aquaculture farm, China and from Deep Lake, Antarctica, respectively

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Abstract

Two halophilic archaeal strains, R30^T and tADL^T, were isolated from an aquaculture farm in Dailing, China, and from Deep Lake, Antarctica, respectively. Both have rod-shaped cells that lyse in distilled water, stain Gram-negative and form red-pigmented colonies. They are neutrophilic, require > 2M NaCl and 0.5-0.7 M $MgCl_2$ for growth but differ in their temperature optima (30 °C, tADL^T *vs* 40 °C, R30^T). The major polar lipids were typical for members of the *Archaea* but also included a major glycolipid chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1). The 16S rRNA gene sequences of the two strains are 97.4 % identical, show most similarity to genes of the family *Halobacteriaceae*, and cluster together as a distinct clade in phylogenetic tree reconstructions. The *rpoB′* gene similarity between strains R30^T and tADL^T is 92.9 % and less to other halobacteria. Their DNA G+C contents are 62.4 - 62.9 mol% but DNA–DNA hybridization gives a relatedness of only 44 %. Based on phenotypic, chemotaxonomic and phylogenetic properties, we describe two new species of a novel genus, represented by strain R30^T (= CGMCC 1.10593^T = JCM 17270^T) and strain tADL^T (= JCM 15066^T = DSMZ 22187^T) for which we propose the names *Halohasta litorea* gen. nov., sp. nov. and *Halohasta litchfieldiae* sp. nov., respectively.

Keywords:

Halohasta litorea gen. nov., sp. nov. · *Halohasta litchfieldiae* sp. nov. · Halophilic archaea · Deep Lake · marine solar saltern

Introduction

Artificial and natural hypersaline environments, such as marine solar salterns and salt lakes with salt concentrations exceeding 150–200 g/l, are common habitats for halophilic archaea, members of the family *Halobacteriaceae* within the order *Halobacteriales* (Oren 2006). In the last three years, many new isolates representing novel taxa have been isolated and described from such habitats all over the world, such as *Halonotius pteroides* from an Australian solar saltern (Burns et al. 2010), *Halogranum rubrum*, *Halorussus rarus*, *Halolamina pelagica* and *Halorubellus salinus*, all five from Chinese solar salterns (Cui et al. 2010a, 2010b, 2011a, 2012), and *Haloarchaeobius iranensis* and *Halovenus aranensis* from an Iranian salt lake (Makhdoumi-Kakhki et al. 2012a, 2012b). This rapid expansion in new taxa indicates that the members of family *Halobacteriaceae* are more diverse than was previously recognized.

During our surveys on halophilic archaeal diversity of marine solar salterns of Eastern China and Deep Lake, Antarctica, two haloarchaeal strains, R30^T and tADL^T, were recovered. In this study, we characterize these two strains and propose they represent two novel species of a novel genus, *Halohasta*.

Materials and methods

Isolation and cultivation of halophilic archaeal strains

Strains R30^T was isolated from a brine sample taken from an aquaculture farm at Daliang, Liaoning Province, China (38°55'57" N, 121°12'25" E) and stored at 4°C during transport to the laboratory in 2009. The pH of the brine was 7.5, and the

specific gravity 1.21. Strain tADL^T was isolated from a surface water sample taken in late 2006 from Deep Lake, Antarctica (68° 33' 35.6" S, 78° 11' 44.9" E) and stored at 4°C during transport to the laboratory in 2007. The pH of the water was 7.1, and the specific gravity 1.17.

For strain R30, the neutral oligotrophic haloarchaeal medium (NOM) was used for the isolation procedure, and contained the following ingredients (g/L): yeast extract (Oxoid), 0.05; fish peptone (Sinopharm Chemical Reagent Co., Ltd.), 0.25; sodium pyruvate, 1.0; KCl, 5.4; K_2HPO_4, 0.3; $CaCl_2$, 0.25; NH_4Cl, 0.25; $MgSO_4 \cdot 7H_2O$, 26.8; $MgCl_2 \cdot 6H_2O$, 23.0; NaCl, 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The medium was solidified with 2.0 % agar. The strain was routinely grown aerobically at 37 °C in NOM-3 medium (NOM series medium) with the following modifications (g/L): yeast extract, 1.0; fish peptone, 0.25; sodium formate, 0.25; sodium acetate, 0.25; sodium lactate, 0.25; sodium pyruvate, 0.25. For the isolation of tADL^T, the liquid end-point dilution method (as described by Burns et al. 2004) was used, with DBCM2 medium (Dyall-Smith 2009).

Phenotypic determination

Phenotypic tests were performed for all species in the same basic medium, NOM, according to the proposed minimal standards for description of new taxa in the order *Halobacteriales* (Oren et al. 1997). The type strains *Halonotius pteroides* 1.15.5^T and *Haloferax volcanii* CGMCC 1.2150^T were selected as reference strains, and used in all tests. The Gram stain was performed following the method outlined by Dussault (1955). Cell morphology and motility in exponentially growing liquid cultures was examined using a Nikon microscope equipped with phase-contrast optics (model: E400). The

minimum salt concentration preventing cell lysis was determined by suspending washed cells in serial sterile saline solutions containing NaCl ranging from 0 to 150 g/L and the stability of the cells was detected by light microscopic examination. Growth and gas formation with nitrate as an electron acceptor were tested in 9-ml stoppered tubes (with Durham tubes) completely filled with liquid NOM medium, and to which $NaNO_3$ (5 g/L) had been added. The formation of gas from nitrate was detected by the presence of gas bubbles in Durham tubes, and the formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of L-arginine or DMSO (5 g/L) was tested in completely filled 9-ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with 2 g/L soluble starch, and detected by flooding the plates with Lugol's iodine solution. Gelatin hydrolysis was performed by growing colonies on NOM agar plates amended with 5 g/L gelatin, and detected by flooding the plates with Frazier's reagent (McDade and Weaver 1959). Esterase activity was measured as outlined by Gutiérrez and González (1972). Tests for catalase and oxidase activities were performed as described by Gonzalez et al (1978). Production of H_2S was tested by growing the isolates and reference strains in a tube containing NOM liquid medium supplemented with 5 g/L sodium thiosulfate, and detected using a filter-paper strip impregnated with lead acetate (Cui et al. 2007). To test for growth on single carbon sources, fish peptone and sodium pyruvate were omitted from the NOM medium, and the compound to be tested was added at a concentration of 5 g/L. Antimicrobial susceptibilities were determined by the Gutiérrez et al. (2008) methods on NOM agar plates with antimicrobial compound discs.

Chemotaxonomic characterization

Polar lipids were extracted using a chloroform / methanol system and analysed using one- and two-dimensional TLC, as described previously (Cui et al. 2010b). Merck silica gel 60 F_{254} aluminium-backed thin-layer plates were used for TLC analyses. In two-dimensional TLC, the first solvent was chloroform–methanol–water (65:25:4, by vol.) and the second solvent was chloroform–methanol–acetic acid–water (80:12:15:4, by vol.). The latter solvent mixture was also used in one-dimensional TLC. Two specific detection spray reagents were used; phosphate stain reagent for phospholipids and α-naphthol strain for glycolipids. The general detection reagent, sulfuric acid–ethanol (1:2, by vol.), was also used to detect total polar lipids.

Phylogenetic analysis

Genomic DNA from halophilic archaeal strains was prepared as described previously (Cui et al. 2011b). The 16S rRNA genes were amplified, cloned and sequenced according to the protocol described previously (Cui et al. 2009). PCR-mediated amplification and sequencing of the *rpoB′* genes were carried out according to Minegishi et al. (2010). Multiple sequence alignments were performed using the ClustalW program integrated in the MEGA 5 software (http://www.megasoftware.net/). Phylogenetic trees were reconstructed using Neighbour-Joining, Maximum-Parsimony and Maximum-Likelihood algorithms in the MEGA 5 software (Tamura et al 2011). Gene sequence similarity among halophilic archaea was calculated using the Pairwise-Distance computing function of MEGA 5. The DNA G+C content was determined from the mid-point value (T_m) of the thermal denaturation method (Marmur and Doty 1962) at 260 nm with a Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high-performance temperature controller. The type strain *Halomicrobium mukohataei* JCM 9738^T was selected as reference strain for these

analyses, and the formula G+C mol% $_{\text{unknown strain}}$ = G+C mol% $_{\text{reference strain}}$ + 2.08 × ($T_{\text{m unknown strain}} - T_{\text{m reference strain}}$) was used to calculate the G+C content from the known T_{m} value (Owen and Pitcher 1985). DNA–DNA hybridizations were performed in a Beckman-Coulter DU800^{TM} spectrophotometer equipped with a high performance temperature controller, and were carried out according to the thermal denaturation and renaturation method of De Ley et al. (1970), as modified by Huß et al. (1983). DNA–DNA hybridizations were carried out in 2 × SSC at 81 ºC and each determination was carried out in triplicate.

Results and discussion

Cells of strains R30^{T} and tADL^{T} were motile and rod-shaped when grown in NOM-3 liquid medium (Supplementary Fig. S1). They stained Gram-negative and their colonies were red-pigmented. Strain R30^{T} was able to grow at 25–50 ^{o}C (optimum 40 ^{o}C), in the presence of 2.1–4.8 M NaCl (optimum 2.6 M NaCl), with 0–1.0 M $MgCl_2$ (optimum 0.7 M $MgCl_2$) and at pH 5.5–9.0 (optimum pH 7.0), while strain tADL^{T} was able to grow at 25–45 ^{o}C (optimum 30 ^{o}C), in the presence of 2.6–4.3 M NaCl (optimum 3.1 M NaCl), with 0.1–1.0 M $MgCl_2$ (optimum 0.5 M $MgCl_2$) and at pH 6.0–8.0 (optimum pH 7.5). The cells of both isolates lyse in distilled water and the minimum NaCl concentrations that prevented cell lysis were 12 % (w/v) for strain R30^{T} and 15 % (w/v) for strain tADL^{T}. Both strains produced H_2S from sodium thiosulfate, did not hydrolyze Tween 80, gelatin and casein and did not produce indole from tryptophan. The main phenotypic characteristics differentiating strain R30^{T} and strain tADL^{T} are shown in Table 1. More detailed results of phenotypic tests and nutritional features of strain R30^{T} and strain tADL^{T} are given in the species descriptions.

The major polar lipids of the two strains are PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and one major glycolipid (GL) chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether). The 1D and 2D chromatograms of the lipids of the two strains were essentially identical (Supplementary Fig. S2), and similar to the polar lipid profiles of *Halonotius pteroides* 1.15.5^T (Supplementary Fig. S2).

Sixteen complete 16S rRNA gene sequences of strain R30^T and strain tADL^T were obtained (1474 and 1473 bp in length, respectively), and these indicated that each strain has one kind of 16S rRNA gene sequence. They share 97.4 % sequence identity and were most similar to the sequence of *Halonotius pteroides* 1.15.5^T (93.5 % and 92.4 %, respectively), with related genera showing lower values, e.g. *Halorubrum* (89%), *Halogranum* (89%) *and Haloferax* (89%). Phylogenetic tree reconstructions using the Neighbour-Joining (NJ) algorithm revealed that strain R30^T and strain tADL^T tightly clustered with each other forming a distinct clade, separate from other recognized genera of the *Halobacteriaceae*. This clade regularly branched near *Halonotius pteroides* 1.15.5^T (Fig. 1a). Their phylogenetic position was also confirmed in other trees generated using the Maximum-Parsimony (MP) and Maximum-Likelihood (ML) algorithms (data not shown).

The 16S rRNA genes of isolates R30^T and tADL^T, and *Hnt. pteroides,* were examined for genus-specific signature bases (Kamekura et al. 2004), and the results are given in Table 2. *Hnt. pteroides* possesses several signature bases not shared by strains R30^T and

tADL^T, while the latter strains share a signature base not found in *Hnt. pteroides*. These results are consistent with the two isolates being distinct from the genus *Halonotius*.

The *rpoB′* genes of both strains were sequenced and found to be identical in length 1827 bp, and the nucleotide sequences were 92.9 % similar to each other. The most closely similar sequence was the corresponding gene of *Halonotius pteroides* 1.15.5^T, but the level of similarity to the two isolates was much lower (85.5 % and 84.5 %, respectively). In phylogenetic tree reconstructions using *rpoB′* gene sequences, strains R30^T and tADL^T clustered tightly with each other, forming a distinct clade, and like the 16S rRNA gene trees, this clade branched nearby *Halonotius pteroides* 1.15.5^T (Fig. 1b). The phylogenetic position was also confirmed in trees generated using the Maximum-Parsimony (MP) and Maximum-Likelihood (ML) algorithms (data not shown). The results of the *rpoB′* gene analyses supported the close similarity of strains R30^T and tADL^T. A recent taxonomic study of the *Halobacteriaceae* has proposed that a similarity value less than 86.2% between *rpoB* genes be used to distinguish genera (Minegishi et al. 2010), supporting the proposal that strains R30^T and tADL^T be placed outside the genus *Halonotius* .

The DNA G+C content of strains R30^T and tADL^T were 62.4 mol% and 62.9 mol%, respectively. These values are significantly higher than that of *Halonotius pteroides* 1.15.5^T (58.4 mol%) (Burns et al. 2011). The DNA–DNA hybridization value between strain R30^T and strain tADL^T was 44 %, much lower than the accepted threshold value (70 %) to separate two species (Stackebrandt and Goebel 1994). The two strains showed only weak signals with *Halonotius pteroides* 1.15.5^T.

Based on these phenotypic, chemotaxonomic and phylogenetic properties, we believe these two isolates should be accommodated in species within a novel genus. We propose the names *Halohasta litorea* gen. nov., sp. nov. and *Halohasta litchfieldiae* gen. nov., sp. nov. Characteristics that distinguish strains R30^T and tADL^T from each other are shown in Table 1. The significant differences between *Halohasta* and related genera of the *Halobacteriaceae* is provided in Table 2.

Description of *Halohasta* gen. nov.

Halohasta (Ha.lo.has'ta. Gr. n. hals halos, salt; L. fem. n. hasta, a rod; N.L. fem. n. *Halohasta*, rod-shaped cells living in saline conditions).

Cells are rods under optimal growth conditions and stain Gram-negative. Aerobic heterotrophs. Cells lyse in distilled water. Catalase test is positive, and the oxidase test may be positive or not. Extremely halophilic, with growth occurring in media containing 2.1–4.8 M NaCl; most strains grow best at 2.6–3.1 M NaCl. The optimum magnesium concentration is 0.5–0.9 M. Temperatures between 25 and 45 °C and pH between 6.0 and 8.5 may support growth. Sugars are metabolized, in some cases with the formation of acids. The polar lipids are PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and one major glycolipid chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether). The genomic DNA G+C content is between 62.4–62.9 mol%. Isolated from marine solar salterns. The type species is *Halohasta litorea*. Recommended three-letter abbreviation: *Hht*.

Description of *Halohasta litorea* sp. nov.

Halohasta litorea (li.to're.a. L. fem. adj. *litorea*, of or belonging to the sea-shore).

Cells are motile, rod-shaped (0.4–0.5×1–4 μm) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 2.6 M NaCl are red, elevated and round. Chemoorganotrophic and aerobic. Growth occurs at 25–50 ^{o}C (optimum 40 ^{o}C), at 2.1–4.8 M NaCl (optimum 2.6 M), at 0–1.0 M $MgCl_2$ (optimum 0.7 M) and at pH 5.5–9.0 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 12 % (w/v). Catalase- and oxidase-positive. Weakly grow under anaerobic conditions with arginine, but do not with nitrate or DMSO. Nitrate reduction to nitrite and formation of gas from nitrate are not observed. H_2S is produced from sodium thiosulfate. Indole formation is negative. Do not hydrolyze starch, gelatin, casein and Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, lactose and pyruvate, DL-lactate, succinate, L-malate and fumarate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-glutamate and L-arginine. No growth occurred on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, starch, acetate, glycerol, D-mannitol, D-sorbitol, citrate, glycine, L-alanine, L-aspartate, L-lysine and L-ornithine. Acid is produced from D-glucose, D-mannose and D-galactose. Sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): bacitracin (0.04 IU per disc), rifampin (5) and nitrofurantoin (300). Resistant to the following antimicrobial compounds: novobiocin (30), trimethoprim (5), mycostatin (100), erythromycin (15), ampicillin (10), penicillin G (10 IU per disc), chloramphenicol (30), neomycin (30), ciprofloxacin (5), streptomycin (10), kanamycin (30), vancomycin (30), norfloxacin (10), tetracycline (30), gentamicin (10) and nalidixic acid (30). The polar lipids are

PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and one major glycolipid chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether). The DNA G+C content of R30^T is 62.4 mol% (T_m).

The type strain is R30^T (=CGMCC 1.10593^T = JCM 17270^T), and was isolated from a marine solar saltern at Daliang, Liaoning Province, China.

Description of *Halohasta litchfieldiae* sp. nov.

Halohasta litchfieldiae (litch.fi.el.di'a.e. N.L. fem. n. *litchfieldiae*, of Litchfield, named after Carol D. Litchfield, a prominent microbial ecologist).

Cells are motile, rod-shaped (0.5–0.6×1–6 μm) under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 3.1 M NaCl are red, elevated and round. Chemoorganotrophic and aerobic. Growth occurs at 25–45 ^oC (optimum 30 ^oC), at 2.6–4.3 M NaCl (optimum 3.1 M), at 0.1–1.0 M $MgCl_2$ (optimum 0.5 M) and at pH 6.0–8.5 (optimum pH 7.5). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 15 % (w/v). Catalase-positive and oxidase-negative. Do not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite and formation of gas from nitrate are not observed. H_2S is produced from sodium thiosulfate. Indole formation is negative. Do not hydrolyze starch, gelatin, casein and Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, maltose, sucrose, pyruvate, DL-lactate, L-malate, fumarate and citrate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-alanine and L-aspartate. No

growth occurred on D-fructose, L-sorbose, D-ribose, D-xylose, lactose D-mannose, D-galactose, starch, acetate, glycerol, D-mannitol, D-sorbitol, succinate, glycine, L-lysine, L-glutamate, L-arginine and L-ornithine. Acid is produced from D-glucose. Sensitive to the following antimicrobial compounds (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), rifampin (5) and nitrofurantoin (300). Resistant to the following antimicrobial compounds: trimethoprim (5), mycostatin (100), erythromycin (15), ampicillin (10), penicillin G (10 IU per disc), chloramphenicol (30), neomycin (30), ciprofloxacin (5), streptomycin (10), kanamycin (30), vancomycin (30), norfloxacin (10), tetracycline (30), gentamicin (10) and nalidixic acid (30). The polar lipids are PA (phosphatidic acid), PG (phosphatidylglycerol), PGP-Me (phosphatidylglycerol phosphate methyl ester), PGS (phosphatidylglycerol sulfate) and one major glycolipid chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether). The DNA G+C content of tADL^T is 62.9 mol% (T_m).

The type strain is tADL^T (= JCM 15066^T = DSMZ 22187^T), and was isolated from Deep Lake, Antarctica.

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Fig. 1. Neighbour-Joining phylogenetic tree reconstructions based on 16S rRNA gene (a) and *rpoB'* gene (b) sequences, showing the relationships between strain R30^T, strain tADL^T and related members within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 50 % bootstrap support. Bar represents expected changes per site.

Fig. 1a

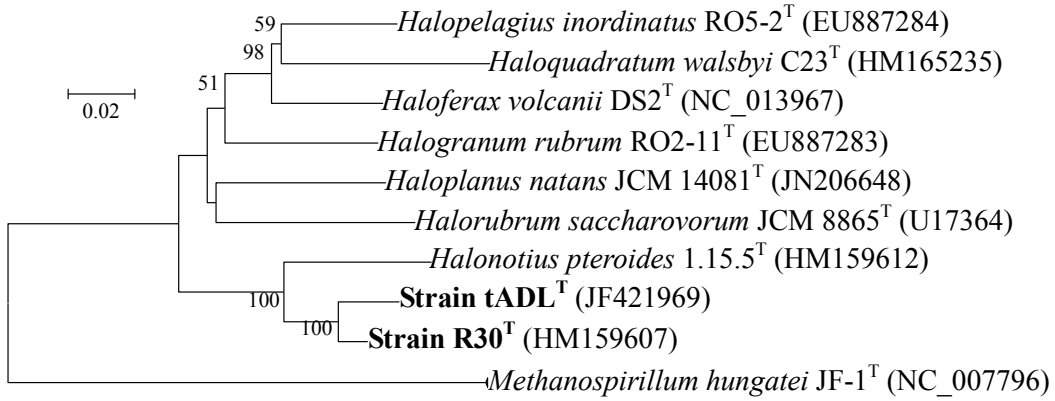

Fig. 1b

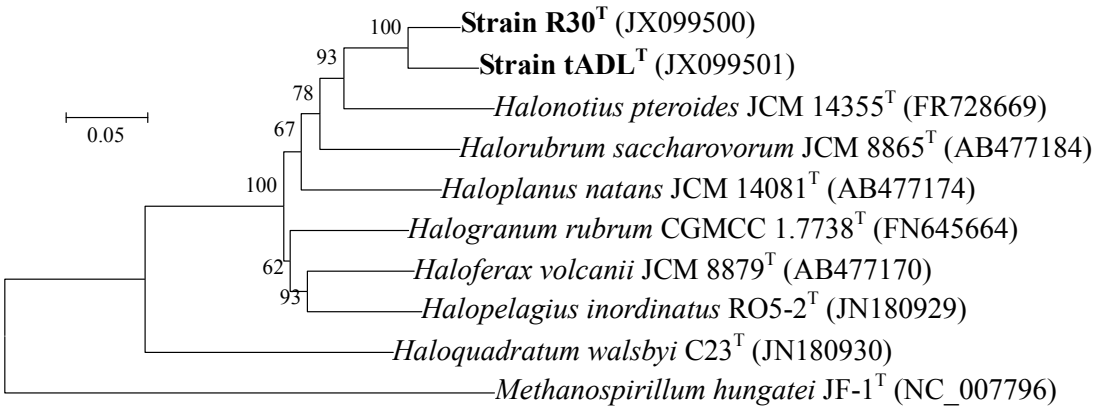

Table 1. Phenotypic and other characteristics that distinguish *Halohasta* from closely related genera within the order *Halobacteriales*.

Symbols: +, positive; -, negative; +/-, positive or negative; UG, unidentified glycolipid.

Table 1

Characteristic	***Halohasta***	***Halonotius***	***Halorubrum***	***Halobaculum***	***Haloferax***
Morphology	rod	pleomorphic	pleomorphic	rod	Pleomorphic
Cell size	0.4–0.6 × 1–6	0.7–1.5 × 2–6	0.12 – 1 × 0.5 – 7	0.5–1 × 5–10	0.4–3 × 0.5–3
Motility	+	+	+/–	+/–	+/–
NaCl optimum (M)	2.6 – 3.1	3.4 – 4.1	1.7 – 4.5	1.5 – 2.5	1.7 – 5.2
NaCl range (M)	2.1 – 4.8	2.7 – 6	0.5 – 4.5	1 – 2.5	1 – 5.2
pH optimum	7.0 – 7.5	neutral	neutral – 9.5	6 – 7	5.5 – 10
Temperature optimum (°C)	30 – 40	45	31 – 50	40	32 – 53
Nitrite from nitrate	–	–	+	+	+
Acid from carbohydrate	+	–	+/–	+	+/–
Anaerobic growth on nitrate	–	–	–	–	+/–
Hydrolysis of starch	–	–	+/–	+	+/–
Indole production	–	–	+/–	–	+/–
Major glycolipids	S-DGD-1	S-DGD-1	S-DGD-3	S-DGD-1	S-DGD-1
G+C content (mol%)	62.4 – 62.9	58	62.7 – 72.1	70	60 - 64

Table 2. Signature bases of 16S rRNA gene sequences

Species/Isolates	*E.coli* postions (nt)	Surrounding sequence
Hnt. pteroides	65	UAGUCG **A** ACGAGU
	143	CUUCAG **U** CGGCAA
	557	GAUGUC **C** CAUAUU
	673	UCAGAG **A** GUACGU
	749	AGACGG **C** UUCGAC
	780	GGUCUC **A** AACCGG
	1270	CUGAGA **A** GCGACG
t-ADL^{T} and R30^{T}	284	AAUAAU **U** GGUACG