Halomonas salinarum sp. nov., a moderately halophilic bacterium isolated from saline soil in Yingkou, China

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Abstract
Strain G5-11T, a Gram-negative, moderately halotolerant, facultatively aerobic, motile bacterium was isolated from saline soil collected from Yingkou, Liaoning, China. The cells of strain G5-11T grew in the presence of 3–15% (w/v) NaCl (optimum 5%), at between 4 and 35 °C (optimum 30 °C), and at a pH of 6.0–9.0 (optimum 8.0). The major respiratory quinone was Q-9 and the dominant cellular fatty acids were summed feature 8 (C18:1ω7c/C18:1ω6c), C16:0, and summed feature 3 (C16:1ω7c/C16:1ω6c). The major components of the polar lipid profile were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and unidentified aminolipid. The G+C content of the strain G5-11T genome was 61.0 mol%. The isolated strain G5-11T showed the highest 16S rRNA gene similarity to Halomonas niordiana LMG 31227T and Halomonas taeanensis DSM 16463T, both reaching 98.3%, followed by Halomonas pacifica NBRC 102220T. The results from phenotypic, chemotaxonomic, and phylogenetic analyses showed that strain G5-11T represented a novel species of the genus Halomonas, for which the name Halomonas salinarum sp. nov. was proposed. The type strain of Halomonas salinarum is G5-11T (= CGMCC 1.12051T = LMG 31677T).

Keywords Halomonas salinarum sp. nov. · Marine bacteria · Moderately halotolerant · Alkaliphilic

Abbreviations
DPG Diphosphatidylglycerol
PE Phosphatidylethanolamine
PG Phosphatidylglycerol
PC Phosphatidylcholine
UAL Unidentified aminolipid

Introduction
The genus Halomonas, the species of which are known for their versatility, is in the family Halomonadaceae of the class Gammaproteobacteria, as proposed by Vreeland et al. (1980) and later amended by Dobson and Franzmann (1996). At the time of writing, the genus comprised 116 species with valid published names (http://www.bacterio.net/halomonas.html). The species of the genus Halomonas are Gram-negative, rod-shaped, aerobic, non-spore forming bacteria. Highly halotolerant, they can tolerate salinities of up to 20% (Kämpfer et al., 2018) and are mostly associated with saline environments.

The environment around the Yingkou Saltworks, Liaoning Province, Northeastern China, is rich in halophilic or halotolerant microbes. The functional cellular physiology, metabolic mechanisms, and community structure of these organisms are determined by environments with high salinity and high osmosis (Oren 2002, 2008). While investigating the microbial diversity of saline soil from the salt mine site, we discovered a moderately halotolerant strain designated G5-11T that could grow under a wide range of salt concentrations. Phenotypic, molecular, and chemotaxonomic

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The GenBank accession number for the 16S rRNA gene sequence of strain G5-11T is JQ010842. The GenBank accession number for the 23S rRNA gene sequence of strain G5-11T is MT901368. The whole genome of strain G5-11T has been deposited at DDBJ/ENA/GenBank under accession number WWNB00000000.

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evidence confirmed that the strain represented a novel species of the genus Halomonas.

Materials and methods

Isolation of the bacterial strain and the culture conditions

The strain was isolated from soil collected from a salt mine at Yingkou, Liaoning Province, China (40°28′N, 122°12′E), in July 2010. The soil had a pH of 8.0–8.5 and a salinity of 0.6–5.0%. The strain was isolated by suspending a sample of the soil in an aseptic saline solution [containing 5% (w/v) NaCl]; platting onto a basal Gibson medium (pH of 8.0) containing (L−1) 10 g yeast extract powder, 5.0 g casein, 5.0 g peptone, 3.0 g trisodium citrate, 20.0 g MgSO₄·7H₂O, 2.0 g KCl, 50 g NaCl, and 15 g agar, and incubating for 5 days at 30 °C. After incubation, several single colonies were transferred onto new Gibson agar plates and incubated again. This step was repeated several times until a single colony type was purified by subculturing. The pure culture was maintained at −80 °C in 30% glycerol. Unless otherwise indicated, this article describes the morphological, physiological, and biochemical characteristics of cells grown on Gibson medium supplied with 5% (w/v) NaCl at pH 8.0 and 30 °C. The type strain Halomonas taeanaensis DSM 16463ᵀ, obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) was used for comparative purposes. Unfortunately, we could not obtain the type strain of H. niordiana because of the coronavirus pandemic. As all the strains were cultured under the same conditions, we have quoted the experimental data instead.

Phylogenetic and genotypic analysis

The 16S rRNA gene was amplified using the universal primer set 27F/1492R (5′-AGA GTT TGA TCC TGG CTC AG-3′/5′-TACGGYTACCTTGTTACGACTT-3′) and cloned into the pMD-18T vector (TaKaRa) for sequencing (Cui et al. 2017). The sequence of strain G5-11 T was aligned using sequences in EzBioCloud server databases (Yoon et al. 2018). Evolutionary distance matrices of the phylogenetic trees were calculated with Kimura’s two-parameter model (Kimura 1980). The topology of each tree was evaluated by bootstrap analysis with 1000 replications (Felsenstein 1985).

The genome of strain G5-11ᵀ was extracted with a bacterial genomic DNA Rapid Extraction Kit (Beijing Huitian Oriental Technology Co. Ltd.), following the manufacturer’s instructions. The whole genome of strain G5-11ᵀ was sequenced using a sequencing platform (Illumina NovaSeq PE150) at the Beijing Novogene Bioinformatics Technology Co. Ltd. The genome was assembled and the gaps were filled with SOAPdenovo (version 2.04) and Gap Closer (version 1.12), respectively (Li et al. 2010). Gene prediction was performed using GeneMarkS 4.17 (Besemer et al. 2001). The functional genes were analyzed with the GO, KEGG, NR, Pfam, and Swiss-Prot general functional databases. tRNAscan-SE (version 1.3.1) (Lowe and Eddy 1997) and Rfam (version 1.2) (Lagesen et al. 2007) were used to identify tRNAs and rRNAs. To confirm the phylogenetic status of G5-11ᵀ, phylogenetic relationships of the genomes were explored with UBCG (Na et al. 2018) using the default settings. The whole genome sequences of the reference strains in the phylogenomic tree were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome/). The average nucleotide identity (ANI) values were calculated with the EzBioCloud online tool (https://www.ezbiocloud.net/tools/ani) (Lee et al. 2016) and the digital DNA–DNA hybridization (dDDH) values were calculated with the Genome-to-Genome Distance Calculator from https://ggdc.dsmz.de/ (Meier-Kolthoff et al. 2013). The dDDH values were calculated with Formula 2.

Because of strain G5-11ᵀ was slightly halophilic and alkaliphilic in nature, the gene/gene clusters were analyzed further. The amino acid sequence of the target species was compared with the TCDB (Saier et al. 2016), NR (Non-Redundant Protein Database), Swiss-Prot, and KEGG databases with Diamond software (Buchfink et al. 2015), and the annotation result was obtained by combining the gene strain with the corresponding functional annotation information.

Phenotypic and biochemical characterization

Various phenotypic and biochemical characteristics of strain G5-11ᵀ were examined. The cell morphology of strain G5-11ᵀ after 2 days of growth was examined using transmission electron microscopy (JEM01230, JEOL), after preparation as described by Ming et al. (2012). Gram-staining was performed as described by Smibert and Krieg (1994). The motility of the cells was examined on semisolid agar. The facultative anaerobic activity was determined by incubating in Gibson liquid medium for 7 days and observing the distribution of the strain G5-11ᵀ. A range of phenotypic characteristics of strain G5-11ᵀ, strain H. taeanaensis DSM 16463ᵀ, and strain H. niordiana

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LMG 31227T were compared. The growth of strain G5-11T was investigated at various temperatures (0, 4, 10, 15, 20, 30, 35, 37 and 45 °C) over 7 days and at different pH values, from pH 5.0–11.0 (at 1.0 pH unit intervals), using the buffers described by Xu et al. (2005). The growth was also tested for its NaCl tolerance at various concentrations of NaCl (1%, 3%, 5%, 7%, 10%, 15%, and 25%, w/v). The catalase activity and oxidase activity were tested by assessing the formation of bubbles when 3% (v/v) H₂O₂ was added and the oxidation of tetramethyl-p-phenylenediamine, respectively (Kovacs 1956). The antibiotic susceptibility was tested after incubating on MA medium at 30 °C and a 5% salt concentration for 2 days, as described by Chen et al. (2018a, b). The physiological and biochemical characteristics were examined using GEN III MicroPlates (Biolog), and API 50 CHB, API ZYM, and API 20NE strips (bioMérieux), following the manufacturers’ instructions. All suspension media were supplemented with 5% (w/v) NaCl and incubated at 30 °C. The experiments were carried out in triplicate.

Chemotaxonomic characterization

Respiratory quinones were extracted from freeze-dried cells (Collins et al. 1977) and analyzed by HPLC (Tamaoka 1986). Polar lipid profiles of strain G5-11T were extracted, separated, and analyzed by two-dimensional TLC, as described by Minnikin et al. (1984). Cellular fatty acids were methylated, separated, and identified with the Sherlock Microbial Identification System (MIDI, Sherlock version 6.0B).

Results and discussion

Phenotypic characteristics

Cells strain G5-11T were Gram-stain-negative, facultatively aerobic, motile by peritrichous flagella, and rod-shaped (Fig. 1). The colonies on the surface of Gibson medium with 5% NaCl were creamy, smooth, and slightly irregular after incubating for 2 days at 30 °C. They were

![Fig. 1 Genome-based phylogenetic tree of G5-11T reconstructed using a set of 92 UBCGs. NCBI genome accession numbers are given in parentheses. Bar, 0.050 substitutions per position](image-url)
positive for d-maltose, d-trehalose, sucrose, d-turanose, α-d-glucose, d-fructose, d-galactose (weak), d-sorbitol, d-arabitol, myo-inositol (weak), glycerol L-alanine, L-arginine (weak), L-glutamic acid, L-pyroglutamic acid (weak), L-serine, pectin, d-gluconic acid, methyl pyruvate, l-lactic acid, d/l-malic acid, bromo-succinic acid, propionic acid, acetic acid, γ-hydroxy-butyric acid, β-hydroxy-d,l-butyric acid, and formic acid (weak) in the Biolog GEN III MicroPlate system. The antibiotic sensitivity of strain G5-11T and its reference strain are shown in Table S1. Other detailed biochemical and physiological characteristics of the strain are given in Table 1. All negative traits from commercial kits are shown in Table S2.

Phylogenetic and genotypic characteristics

Comparison with the results from the EzBioCloud server showed that members of family Halomonadaceae were the closest relatives of the strain G5-11T, and H. niordiana LMG 31227T (SDSD01000014) and H. taeanensis DSM 16463T (AY671975) were the most closely related species, with 16S rRNA gene sequence similarities of 98.3%. Phylogenetically, the strain G5-11T was moderately related to H. niordiana LMG 31227T and H. taeanensis DSM 16463T (Fig. 1, S2–S3) which suggests that strain G5-11T ought to be recognized as a novel species within the genus Halomonas.

Strain G5-11T possessed a genome of 3,395,587 bp that comprised 3084 predicted genes. The draft genome of strain G5-11T consisted of 50 contigs, with an N50 value

Table 1 Differences in the phenotypic characteristics of strain G5-11T and some related type strains from the genus Halomonas

| Characteristic                     | 1          | 2          | 3a         | 4b         |
|------------------------------------|------------|------------|------------|------------|
| Colonial morphology and pigmentation | Creamy and smooth | Creamy and smooth | Cream and round colonies | White, opaque colonies |
| Growth pH                           | 6.0–9.0 (8.0) | 6.0–9.0 (8.0) | 4.0–10.0 (8.0) | 5.0–9.0 (8.0) |
| Growth temperature (°C)             | 4–35 (30)   | 4–37 (30)  | 4–37 (30)  | 4–45 (30/37) |
| Growth NaCl (%, w/v)                | 3.0–15.0 (5.0) | 2.0–25.0 (5.0) | 3.0–25.0 (12.0–15.0) | 3.5–20.0 (3.5–8.0) |
| DNA G+ C content (mol%)             | 61.0       | 65.0       | 60.8       | 60.5± 0.5   |
| Facultatively anaerobic growth      | +          | –          | +          | +          |
| Nitrate reduction                   | +          | W          | +          | +          |
| indole production                   | –          | –          | –          | +          |
| Activities of                       | –          | –          | +          | –          |
| Urease                              | +          | +          | –          | –          |
| Trypsin                            | –          | –          | +          | –          |
| Hydrolysis of                       | –          | –          | –          | +          |
| Casein                              | +          | +          | +          | –          |
| Gelatin                             | –          | –          | –          | +          |
| Aesculin                            | –          | –          | –          | +          |
| Utilization of                      | –          | +          | –          | +          |
| Lactose                             | –          | +          | –          | +          |
| d-Mannitol                          | +          | +          | –          | +          |
| d-Mannose                           | –          | W          | –          | +          |
| d-Cellobiose                        | –          | W          | +          | +          |
| Biolog GENIII                       | –          | +          | NT         | NT         |
| Acetoacetic Acid                    | –          | +          | NT         | NT         |
| Tween 40                            | –          | +          | NT         | NT         |
| Inosine                             | W          | –          | NT         | NT         |
| D-Lactic acid methyl ester          | –          | +          | NT         | NT         |

Strains: 1, G5-11T; 2, H. taeanensis DSM 16463T; 3, H. niordiana LMG 31227T; 4, H. elongata ATCC33173T

All the data were from this study unless otherwise indicated

All four strains are Gram-stain-negative, motile (by means of several flagella), halophilic, and catalase-positive, and have the ability to reduce nitrate and ferment glucose. All four strains can utilize glycerol, d-glucose, and sucrose, and none can hydrolyze starch, tween 80 and d-salicin. All the strains were negative for β-galactosidase

+ positive; –, negative; W weakly positive, NT not tested, ND not determined or data not available in relevant literature

aData fromDieguez et al. (2020)

bData fromVreeland et al. (1980)
of 139,373 bp and an 909 contig length of 54,722 bp. The complete genome of strain G5-11T had 100-fold depth of sequencing coverage. The percentages for the nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) with respect to the related species of Halomonas are shown in Table 2. The average ANI values and dDDH between the G5-11T and the reference strains were all below the cut-off level for species delineation (95–96% and 70%, respectively).

When confronted with the problem of H+/Na+ passive permeation through the cytoplasmic membrane in extreme conditions, e.g., organic solvent or high salt (Adamiak et al. 2016), the Na+/H+ antiporters found in halophilic bacteria play an important role in intracellular Na+ excretion (Mesbah et al. 2009). The majority of prokaryotes cope with increasing osmolarity by taking up or synthesizing compatible solutes, which are important for salt stress resistance (Averhoff and Müller 2010). The NR (Non-Redundant Protein Database) annotation result suggests that the Na+/H+ antiporter NhaD and Multicomponent Na+/H+ antiporter NhaA-G found in G5-11T shared the highest similarity with those of H. elongata (76.4% and 93.8%, respectively); these may allow strain G5-11T to grow over a range of extracellular pH and Na+ concentrations. Moreover, Swissprot annotation analysis highlighted the presence of several putative genes for biosynthesizing the compatible solute ectoine (e.g., ectA, ectB, ectC) (Table S3), which shared the highest similarity with H. taeanensis, H. niordiana, and H. elongata (Fig. S4). The combination of these genes may provide strain G5-11T with a special mechanism for adapting to hypersaline habitats.

**Chemotaxonomic characteristics**

The isoprenoid quinone of strain G5-11T was Q-9, and was the same as for the H. niordiana DSM 31227T and H. taeanensis 16463T strains, which were closely related phylogenetically (Lee et al. 2005). The major fatty acids in strain G5-11T were summed feature 8 (C18:1ω7c/C18:1ω6c, 32.4%), C16:0 (24.1%), followed by summed feature 3 (C16:1ω7c/C16:1ω6c, 23.9%), which was consistent with the other members of genus Halomonas (Table S4). The fatty acid contents in strain G5-11T and H. taeanensis 16463T were comparable, while H. niordiana DSM 31227T had a larger amount of summed feature 8. The major components of the polar lipid profile were PC, PG, DPG, PE, and UAL (Fig. S5).

**Taxonomic conclusions**

The phylogenetic analysis and chemotaxonomic characteristics, including the isoprenoid quinone, major cellular fatty acid, and DNA G+C content, unequivocally support the placement of strain G5-11T within the genus Halomonas. Using a polyphasic taxonomic approach, we generated evidence that the strain represents a novel Halomonas species, proposed as Halomonas salinarum sp. nov.

**Description of Halomonas salinarum sp. nov.**

*Halomonas salinarum* (salina’rum. L. gen. pl. n. salinarum, of salt works)

Cells are Gram-stain-negative, facultatively aerobic, moderately halophilic, rod-shaped, and motile, and are 1.2–2.1 μm long and 0.7–1.1 μm wide. The colonies are creamy white to pale yellow. Growth occurs between 4 and 35 °C (optimum 30 °C), at pH 6.0–9.0 (optimum 8.0), and in 3–15% NaCl (optimum 5%). Growth occurs on Gibson, MA, LB, and R2A media at 5% NaCl. Acid is produced from D-ribose (weak), D-tagatose (weak), and potassium 5-ketogluconate (API 50CHB). Positive for catalase and oxidase. Positive for alkaline phosphatase, esterase lipase (C8) (weak), leucine aramidase, valine aramidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase (API ZYM). In API 20NE tests, the reductions of nitrate, urease, and arginine dihydrolase (weak), and the assimilation of D-glucose, L-arabinose, D-mannitol, D-maltose, and phenylacetic acid are positive. The isoprenoid quinone is Q-9, and the most abundant fatty acid is summed feature 8 (C18:1ω7c/C18:1ω6c) followed by C16:0 and summed feature 3 (C16:1ω7c/C16:1ω6c, 23.9%), which was consistent with the other members of genus Halomonas.

| Bacterial species | OrthoANIu value (%) | dDDH value (%) |
|-------------------|---------------------|----------------|
| H. salinarum G5-11T | 82.91               | 26.70          |
|                   | 93.05               | 50.70          |

Strains: 1. H. taeanensis DSM 16463T; 2. H. niordiana DSM 31227T

All the data were obtained in this study.

The type strain that was isolated from saline soil collected from Yingkou, Liaoning Province, China, was G5-11T (= CGMCC 1.12051T = DSM 31677T).

The GenBank accession number for the 16S rRNA gene sequence of strain G5-11T is JQ010842. The GenBank accession number for the 23S rRNA gene sequence of strain G5-11T is MT901368. The whole genome of strain G5-11T has been deposited at DDBJ/ENA/GenBank under accession number WWN00000000.
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Author contributions  Y-LY designed the research and the project outline. Y-LY and F-LL performed the isolation, and completed the deposition and polyphasic taxonomy. Y-LY performed the genome analysis. Y-LY and F-LL drafted the manuscript. LW revised the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest  All authors declare that there is no conflict of interest in this article.

Ethical statement  No experiments with humans or animals were carried out.

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