Halomonas salipaludis sp. nov., isolated from a saline-alkali wetland soil

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Abstract
Strain WRN001T, a Gram-staining-negative, strictly aerobic, non-motile bacterium was isolated from the natural saline-alkali wetland soil of Binhai new district, Tianjin, China (38°46' N, 117°13' E). Cells of strain WRN001T were 0.3–0.5 µm in width and 1.5–2.5 µm in length, and the growth occurred optimally at 33–37 °C, pH 7.5–8.0, and in the presence of 8–10% (w/v) NaCl. Based on 16S rRNA gene sequence analysis, the isolate could be affiliated to the genus Halomonas, and the highest 16S rRNA gene sequence similarity of strain WRN001T to its closest relative Halomonas qiaohouensis DSM 26770T was 97.5%. The size of the genome as presented here was 5,475,884 bp with a G + C content of 63.8 mol %. The major respiratory quinone of strain WRN001T was Q-9, and the dominant fatty acids were summed feature 8, summed feature 3, C10:0, C12:0, C12:0 3-OH, C16:0, and C17:0 cyclo. The major polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), two phospholipids (PL), aminolipid (AL), and three unidentified lipids (L). These data combined with the low digital DDH values between strain WRN001T and the close relative, Halomonas alkalitolerans CGMCC 1.9129T (42.2%) and based on comparisons with currently available genomes, the highest average nucleotide identity (ANIm) value was 91.4% to Halomonas alkalitolerans CGMCC 1.9129T (GenBank accession No. GCA_001971685.1). Therefore, we propose a novel species in the genus Halomonas to accommodate this novel isolate: Halomonas salipaludis sp. nov. (type strain WRN001T = KCTC 52853T = ACCC 19974T).

Keywords Halomonas salipaludis · Gammaproteobacteria · Saline-alkali wetland soil

Introduction
Halomonas, as a large genus, was first described by Vreeland et al. (1980), with Halomonas elongata as the type species, and most of the species of the genus Halomonas were isolated from saline habitats, such as sea sediments, salt lakes, brines, salty foods, deep sea hydrothermal vent environments, as well as saline sand and soils (https://www.bacterio.net/). At the time of writing, the genus Halomonas includes 96 species with validly published names (http://www.bacterio.net/halomonas.html). In this paper, we described the isolation, identification, and physio-biochemical characteristics of novel strain WRN001T and proposed the name Halomonas salipaludis for this bacterium.

Materials and methods
Isolation and culture conditions
Strain WRN001T was isolated from the natural saline-alkali wetland soil of Binhai new district, Tianjin, China (38°46' N, 117°13' E) in June 2015. The in-situ temperature, salinity and pH of the samples were measured as 30 °C, 4.0–13.5% and 7.8–9.3, respectively. To isolate halophilic
heterotrophic microorganisms, 1.0 g of soil was placed in sterile 30 ml glass tube for enrichment using Difco™ marine 2216 amended with final concentration of 10% (w/v) NaCl and cultivated for 3 days and subsequently purified into single colonies.

### Morphological, physiological and biochemical characterization

Cell size, morphology and motility of strain WRN001^T were established 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% H₂O₂ solution onto bacterial colonies and observing bubble production. Reduction of nitrate and hydrolysis of starch, casein, gelatin, and Tween 80 were analyzed according to the methods of Smibert and Krieg (1994) and Dong and Cai (2001). The optimal growth temperature of strain WRN001^T was determined after incubation on Difco™ marine 2216 agar (8.0% NaCl, w/v) and shaking in Difco™ marine 2216 liquid medium (8.0% NaCl, w/v) at 4, 10, 15, 20, 25, 30, 33, 40, 45, and 50 °C (at pH 7.5). Bacterial growth was measured as increase in turbidity at 600 nm, and in LB liquid medium amended with 0.0–25.0% NaCl (w/v). Similarly, the pH range for growth was measured by adjusting the final pH to 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 (at 8.0% NaCl, w/v, 33 °C) with the appropriate buffers (Na₂HPO₄/NaH₂PO₄ for pH 5.0–7.0 and Na₂CO₃/NaHCO₃ for pH 8.0–12.0). Anaerobic growth was determined through measuring the OD₆₀₀ at 33 °C with 8.0% NaCl (w/v) in the tubes with the butyl rubber stopper and screw cap.

For all physiological experiments, Halomonas giaoohoensis DSM 26770^T and the closely related strain Halomonas pantelleriensis DSM 9661^T were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as the reference strains, and Halomonas socia CCTCC AB 2011033^T was obtained from China Center for Type Culture Collection (CCTCC). Unless otherwise stated, all the strains mentioned above were incubated at 30 °C in Difco™ marine 2216 medium amended with final concentration of 10.0% NaCl (w/v) for strain WRN001^T and final concentration of 10.0% NaCl (w/v) for reference organisms.

Carbon source utilization and enzyme activities were tested using the Biolog GEN III MicroPlates, API 20E, API 20NE and API ZYM (bioMérieux, Biolog Inc.) according to the manufacturer’s instructions, with the exception that salinity was adjusted to 8.0% (w/v) NaCl. Susceptibility to antibiotics was assessed on Difco™ marine 2216 agar (8.0% NaCl, w/v) using the disk-diffusion plate method (Fraser and Jorgensen 1997) with disks containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg), vancomycin (30 μg), gentamicin (10 μg), or polymyxin B (30 μg). Antibiotic-containing disks were placed on the Difco™ marine 2216 agar (8.0% NaCl, w/v) plate surfaces, and the bacterial cultures (200 μL) that were spread on the plate were checked for clearing zones after 3 day at 33 °C.

### Chemotaxonomic characterization

Cells of strain WRN001^T and the reference strains were harvested during the late exponential growth phase in Difco™ marine 2216 liquid medium (8% NaCl for strain WRN001^T and 10% NaCl for reference strains) at 33 °C for characterization of respiratory quinones, cellular fatty acids, and polar lipids. 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 et al. 1984). The fatty acids were identified and quantified by the Sherlock Microbial Identification System with standard MIS Library Generation Software (VERSION 6.0 and Date 4, Microbial ID Inc., Newark, DE, USA) and a 6890 N gas chromatograph (Agilent) according to the method of Sasser (1990). Polar lipids were extracted from 200 mg of freeze-dried cell material using a chloroform:methanol:aqueous NaCl mixture (0.3%, w/v) with the ratio of 1:2:0.8 (v/v), modified after Bligh and Dyer (Bligh and Dyer 1959), recovered into the chloroform phase by adjusting the mixture to a ratio of 1:1:0.9 (v/v), and separated by two-dimensional silica gel thin-layer chromatography. The first dimension was developed in a chloroform:methanol:water (65:25:4, v/v/v) mixture and the second was developed in a chloroform:acetate:acid:water (80:12:15:4, v/v/v/v) mixture. Total lipid material was detected using molybdovanadophosphoric acid and specific functional groups detected using spray reagents specific for defined functional groups (Tindall 2007).

### Molecular characterization

The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set 27F and 1492R (Lane
1991; Weisburg et al. 1991). The PCR product was purified using the PCR purification kits (MinElute PCR Purification Kit, QIAGEN) and sequenced by Sangon Biotech (Shanghai) Co., Ltd., China. The 16S rRNA gene sequence of strain WRN001\textsuperscript{T}, as determined in this study, was submitted to GenBank, and the 16S rRNA gene sequences of microorganisms’ related taxa were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed by maximum-likelihood method, and Neighbor-joining (NJ) and maximum parsimony (MP) phylogenetic trees were also constructed to corroborate the phylogenetic position of the strain WRN001\textsuperscript{T} in software MEGA 7.0 (Kumar et al. 2016).

Genomic DNA was extracted using a commercial kit (TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0) and subsequently sequenced on the Illumina HiSeq 2000 platform in 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 v1.05 (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 3.02) (Lowe and Eddy 1997).

Chemotaxonomic characteristics

Q-9 was identified as the major respiratory quinone of strain WRN001\textsuperscript{T}, which is consistent with the main ubiquinone reported for most other members of the genus Halomonas. Strain WRN001\textsuperscript{T} had the following cellular fatty acids: summed feature 8 (C\textsubscript{18:1}\textomega6c and/or C\textsubscript{18:1}\textomega7c.) (55.68%), summed feature 3 (C\textsubscript{16:1}\textomega6c and/or C\textsubscript{16:1}\textomega7c) (9.62%), C\textsubscript{10:0} (2.95%), C\textsubscript{12:0} (3.64%), C\textsubscript{12:0} 3-OH (6.02%), C\textsubscript{16:0} (16.36%), C\textsubscript{17:0} cyclo (1.60%) and C\textsubscript{19:0} cyclo\textomega8c (2.43%), C\textsubscript{18:1}(54.2%), C\textsubscript{18:0} (11.2%), C\textsubscript{16:0} (8.6%), 11-methyl C\textsubscript{18:1}\textomega7c (7.7%), C\textsubscript{19:0} cyclo\textomega8c (3.3%), and C\textsubscript{12:1} 3-OH (3.5%), which is consistent with those of the selected reference species: Halomonas pantelleriensis DSM 9661\textsuperscript{T}, Halomonas qiaohouensis DSM 26770\textsuperscript{T} and Halomonas sobria CCTCC AB 2011033\textsuperscript{T} (Table 2).

Molecular characteristics

Strain WRN001\textsuperscript{T} contained the following major polar lipids: diphasatidylglycerol (DGP), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), amino lipid (AL), phospholipid (PL), and an unidentified lipid (L). The polar lipids of the strain WRN001\textsuperscript{T} and its close relatives Halomonas qiaohouensis DSM 26770\textsuperscript{T} and Halomonas sobria CCTCC AB 2011033\textsuperscript{T} are very similar, however, their differences are mainly at aminolipid (AL), aminoglycolipid (GNL) and lipid (L) (Fig. S2).

Results and discussion

Morphological, physiological and biochemical characteristics

Cells of Strain WRN001\textsuperscript{T} are aerobic, Gram-staining-negative, size with 0.3–0.5 × 1.5–2.5 μm (Fig. S1), and was sensitive to chloramphenicol, erythromycin, streptomycin, vancomycin and polymyxin B, but resistant to ampicillin, kanamycin, penicillin G, tetracycline, and gentamicin. Strain WRN001\textsuperscript{T} and Halomonas sobria CCTCC AB 2011033\textsuperscript{T} were negative for nitrate reduction, which distinguished them from Halomonas pantelleriensis DSM 9661\textsuperscript{T} and Halomonas qiaohouensis DSM 26770\textsuperscript{T}. Moreover, compared with the three related type strains, strain WRN001\textsuperscript{T} can hydrolyse starch, Tween 80, and pectin. The physiological and biological characteristics of Strain WRN001\textsuperscript{T} are summarized in the species description and Table S1. And further selective and comparative characteristics of strain WRN001\textsuperscript{T} and the related type strains are shown in Table 1.
5,475,884 bp in size. It has a G+C content of 63.8% and consists of 33 contigs with a 200-fold coverage. We predicted a total of 4940 ORFs, 65 tRNAs, and 10 rRNAs. Digital DDH values between strain WRN001^T and its close relatives, *Halomonas alkalitolerans* CGMCC 1.9129^T, *Halomonas pantelleriensis* DSM 9661^T, and *Halomonas shengliensis* CGMCC 1.6444^T, were 42.2%, 35.1%, and 23.3%, respectively. And ANI values between strain WRN001^T and its close relatives, *Halomonas alkalitolerans* CGMCC 1.9129^T, *Halomonas pantelleriensis* DSM 9661^T, and *Halomonas shengliensis* CGMCC 1.6444^T, were 88.7%, 87.0%, and 77.7%, respectively (Table S2). These add evidence that strain WRN001^T represents a novel species of the genus *Halomonas*, based on the recommended minimum relatedness DDH value is 70% and ANI value is 95% for strains of the same species (Graham 1991; Wayne et al. 1987; Richter and Rosselló-Móra 2009).

### Table 1

| Characteristics | 1 | 2 | 3 | 4 |
|-----------------|---|---|---|---|
| Source          | Salt mine soil | Hard sand^a | Salt mine soil^b | Saline soil^c |
| Colony color    | Cream to yellow | Cream to pink^a | Cream-colored^b | Cream-colored^c |
| NaCl range (optimum) | 0.5–20 (8–10) | 1.25–15 (10)^a | 0.5–20 (2–6)^b | 0.5–26 (8)^c |
| Nitrate reduction | – | + | + | – |
| Oxidase         | + | + | + | – |
| Hydrolysis of   | – | – | – | – |
| Starch          | + | – | – | – |
| Tween 80        | + | – | – | – |
| Urea            | – | + | – | – |
| Pectin          | + | – | – | – |
| Utilization of  | – | – | – | – |
| l-araBinosine   | + | – | + | – |
| d-Galactose     | + | – | – | w |
| d-Mannose       | – | + | + | – |
| D-Salicin       | + | + | – | – |
| d-Fructose      | + | – | – | + |
| d-Sorbitol      | + | – | + | w |
| d-Mannitol      | + | – | – | + |
| l-Alanine       | + | – | – | – |
| l-Aspartic Acid | + | – | – | – |
| l-Pyroglutamic acid | + | – | – | – |
| Glycerol        | + | – | – | – |
| Acetate         | + | + | – | – |
| DNA G+C content (mol%) | 63.8 | 65.0^a | 64.6^b | 62.7^c |

Taxa: 1, WRN001^T; 2, *Halomonas pantelleriensis* DSM 9661^T; 3, *Halomonas qiaohouensis* DSM 26770^T; 4, *Halomonas socia* CCTCC AB 2011033^T.

^a + indicates positive, w indicates weakly positive, – indicates negative

^b Data from (Romano et al. 1996)

^c Data from (Wang et al. 2014)

^d Data from (Cao et al. 2013), and all other data were obtained from this study

### Table 2

| Fatty acid          | 1    | 2    | 3    | 4    |
|---------------------|------|------|------|------|
| C_{16:0}            | 16.36| 15.83| 13.61| 12.03|
| C_{12:0} 3-OH       | 6.02 | 6.13 | 7.05 | 6.60 |
| C_{12:0}            | 3.64 | 3.64 | 4.26 | 3.53 |
| C_{10:0}            | 2.95 | 2.75 | 3.03 | 2.88 |
| C_{19:0} cyclo \omega8c | 2.43 | 10.86 | 15.16 | 1.26 |
| Summed feature 3^a  | 9.62 | 4.61 | 3.46 | 8.10 |
| Summed feature 8^a  | 55.68| 49.45| 47.10| 63.17|

Taxa: 1, WRN001^T; 2, *Halomonas pantelleriensis* DSM 9661^T; 3, *Halomonas qiaohouensis* DSM 26770^T; 4, *Halomonas socia* CCTCC AB 2011033^T. The compositions of the fatty acids that less than 1.0% in all strains were not listed in the Table 2. All the data were taken from this study.

^a Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system, summed feature 3 contains C_{16:1}ω6c and/or C_{16:1}ω7c; summed feature 8 comprised C_{18:1}ω6c and/or C_{18:1}ω7c

5,475,884 bp in size. It has a G+C content of 63.8% and consists of 33 contigs with a 200-fold coverage. We predicted a total of 4940 ORFs, 65 tRNAs, and 10 rRNAs. Digital DDH values between strain WRN001^T and its close relatives, *Halomonas alkalitolerans* CGMCC 1.9129^T, *Halomonas pantelleriensis* DSM 9661^T, and *Halomonas shengliensis* CGMCC 1.6444^T, were 42.2%, 35.1%, and 23.3%, respectively. And ANI values between strain WRN001^T and its close relatives, *Halomonas alkalitolerans* CGMCC 1.9129^T, *Halomonas pantelleriensis* DSM 9661^T, and *Halomonas shengliensis* CGMCC 1.6444^T, were 88.7%, 87.0%, and 77.7%, respectively (Table S2). These add evidence that strain WRN001^T represents a novel species of the genus *Halomonas*, based on the recommended minimum relatedness DDH value is 70% and ANI value is 95% for strains of the same species (Graham 1991; Wayne et al. 1987; Richter and Rosselló-Móra 2009).
Taxonomic conclusion

In this study, we isolated and described the novel strain WRN001T from the natural saline-alkali wetland soil. Phylogenetic, phenotypic, and genetic analyses indicate that the strain WRN001T represents a novel species of the genus Halomonas, for which we propose the name Halomonas salipaludis sp. nov.

Description of Halomonas salipaludis sp. nov.

Halomonas salipaludis (sa.li.pa.lu’dis. L. masc. n. salis salt; L. fem. n. palus, paludis swamp, marsh; N.L. gen. n. salipaludis of a salt marsh).

Cells are aerobic, Gram-stain-negative, non-motile, and oxidase-positive and catalase-negative with 0.3–0.5 × 1.5–2.5 μm. Colonies are circular, wet, smooth, and light yellow. Growth occurs at 0.5–20.0% NaCl (optimum growth at 8.0–10.0% NaCl, w/v), at 10–45 °C (optimum 33–37 °C), and pH 5.5–11.0 (optimum pH 7.5–8.0). Does not produce indole. Starch, casein, and Tween 80 can be hydrolyzed, but not gelatin. Nitrate was not reduced to nitrite. Positive for the following enzymatic activities: α-chymotrypsin, esterase (C4), esterase lipase (C8), lipase (C14), valine aryl amidase, β-glucosidase, α-mannosidase, α-glucosidase and N-acetyl-glucosaminidase, and negative for leucine arylamidase, α-galactosidase, β-fucosidase, cystinearyl amidase, β-galactosidase, alkaline phosphatase, β-glucuronidase, naphthol-AS-BI-phosphohydrolase and acid phosphatase. The following compounds are utilized as sole carbon and energy sources: D-glucose, esculin ferric citrate, L-arabinose, D-mannitol, N-acetyl-glucosamine.

Fig. 1 Neighbor-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain WRN001T and related taxa. Bootstrap values > 50% (1000 replicates) are shown. Scale bar, 0.01 substitutions per nucleotide position. Zymobacter palmae T109T (D14555) was used as an outgroup. GenBank accession numbers are indicated for each strain.
n-maltose, adipic acid, malic acid, trisodium citrate, starch, esculin, and Tween 80, however, l-arginine, urea, gelatin, and d-mannose are not. The major respiratory quinone of strain is Q-9, and the dominant fatty acids are summed feature 8, summed feature 3, C10:0, C12:0, C12:0 3-OH, C16:0, C17:0 cyclo, C19:0 cycloω8c. The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phophatidylcholine (PC), two phospholipids (PL), aminolipid (AL), and three unidentified lipids (L). The genomic DNA is a single circular chromosome (5,475,884 bp) with a G+C content of 63.8%.

The type strain WRN001T (= KCTC 52853T = ACCC19974T) was isolated from the natural saline-alkali wetland soil of Binhai new district, Tianjin, China (38°46′N, 117°13′E).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02560-8.

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Author contributions JX contributed to performing the experiments and writing the initial draft. QG provided samples of experiment and participated in the isolation and cultivation of strains. GZ and JZ contributed to the guidance of experimental operations. LT and JL contributed in the isolation and cultivation of strains. GZ and JZ contributed to reagents, instrumentation, and the financial support for this work.

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Data availability The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene of Halomonas salipaludis sp. nov. strain WRN001T is MF782428. The whole genome was deposited at GenBank/EMBL/DDBJ under the accession number NSKB00000000 for strain WRN001T. Transmission electron micrographs (TEM) of cells of strain WRN001T, thin-layer chromatograms of the polar lipids extracted from strain WRN001T and closely related species, additional phylogenetic trees, and the table containing the average nucleotide identity (ANI), and digital DDH values to closely related genomes are available as Supplementary Materials.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate All authors approved the manuscript.

Consent for publication Written informed consent for publication was obtained from all participants.

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