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Halomonas jincaotanensis sp. nov., a 1-naphthylamine degrading bacterium isolated from Pamir

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The GenBank/EMBL/DDBJ accession number for the genome and 16S rRNA gene sequence of strain TRM 85114^{T} are JAHCLU000000000 and MW584241, respectively.

Abstract

A Gram-stain-negative, aerobic, rod-shaped bacterium, designated strain TRM85114^T, was isolated from Jincaotan in Pamir, PR China. We found it has the ability to degrading 1-naphthylamine. Strain TRM85114^T grows at 4-40 °C (optimum, 30 °C), at pH 6.0-9.0 (optimum, pH 6.0-7.0) and with 3%-15% (w/v) NaCl (optimum, 3%-6%). Phylogenetic analysis of 16S rRNA gene sequences revealed that strain TRM85114^T is affiliated with the genus *Halomonas*, sharing high sequence similarity (97.3%) with the type strain of *Halomonas korlensis* CGMCC 1.6981^T. The major fatty acids of strain TRM85114^T are $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c, summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c) and summed feature 8 ($C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c). The predominant respiratory quinone is Q-7. The genomic DNA G + C content of strain TRM85114^T was determined to be 61.6 mol%. Calculating the average nucleotide identities and the digital DNA-DNA hybridization values between strain TRM85114^T and the related type *Halomonas* strains further revealed that TRM85114^T represented a novel species of the genus *Halomonas*. The name *Halomonas jincaotanensis* sp. nov. is proposed. The type strain is TRM85114^T (CCTCC AB 2021006^T =LMG 32311^T).

Keywords *Halomonas jincaotanensis* sp. nov.; Polyphasic taxonomy; 1-naphthylamine

Introduction

Halophilic microorganisms, especially *Halomonas* spp., which accumulate polyphosphate (Nguyen et al. 2012), produce biodegradable polyhydroxy-alkanoates (Tuma et al. 2020; Jiang et al. 2018) and various metabolic chemicals (Du et al. 2020; Jiang et al. 2021), have been the candidates for producing multiple products used in various industrial fields. Vreeland et al. (1980) initially proposed *Halomonas,* which belongs to the family Halomonadaceae of the phylum Proteobacteria. At present, the genus contains more than 140 validly named species. Members of the genus *Halomonas* have been isolated from diverse terrestrial and aquatic habitats, such as lake water (Kazemi et al. 2020), saline-alkali land (Dou et al. 2015), tidal flat (Koh et al. 2017), Arctic tundra soil (Zhou et al. 2019), deep-sea sediment (Xu. et al. 2013) and hypersaline wetland (Ramezani et al. 2020). A study to culture bacteria from plateau soil collected from the Pamir obtained a *Halomonas*-like strain, designated TRM85114^{T}. In this study, we explore the taxonomic characterization and the ability degrading 1 -naphthylamine of strain TRM85114^{T} and propose to classify it as the representative of a novel species in the genus *Halomonas*, named here as *H. jincaotanensis* sp. nov..

1-Naphthylamine, one of the top priority contaminants and carcinogens (Hu et al. 2011), is a derivative of polycyclic aromatic hydrocarbon used as an intermediate for synthesis industries (Yang et al. 2020). The degree of human activity in industrial applications determines the concentration of carbon and the

transport of these ubiquitous and persistent compounds. The genus of *Halomonas* has shown the degradation ability of polycyclic aromatic hydrocarbon (Govarthanan et al. 2019; Farraj et al. 2020), a tremendous potential capability in environmental remediation.

Materials and methods

Sample collection, isolation and preservation

We sterilely collected the Mud-water mixture from a wetland named Jincaotan (37°47′ N, 75°16′E) from Pamir, at 3100 m. Bacterial strains were isolated from the sample using the general dilution-plating method. In brief, approximately 2 g sediment sample was suspended into 20 mL sterile 12%MGM liquid medium (Smith MD, 2009) containing sterile glass drops and shaken at 15 °C for 2 h. The resulting suspension was serially diluted (ten-fold) with distilled water and 100 μL of the diluted suspension was spread on 12%MGM medium plates, which contained 5 g/L peptone (Oxoid), 1 g/L yeast extract (Oxoid), 400 mL/L salt water (30% stock), 567 mL/L pure water and 1.5% agar, plates to be incubated for 2 weeks at 15 °C. Colonies on the plates showing morphological difference were picked and purified by repeated streaking on fresh 12% MGM plates, among which strain TRM85114^{T} was routinely grown in 12%MGM agar at 15 °C and preserved in 12%MGM containing 50% (v/v) glycerol at - 80 °C.

The reference strain *H. korlensis* CGMCC 1.6981^{T} was obtained from China General Microbiological Culture Collection Center (CGMCC) and cultured under

64 the same conditions, as specified, for comparative purposes.

65 **Phenotypic and physiological identification**

66 Cellular morphology was examined by light microscopy (Leica DM1000) and
67 scanning electron microscope (SEM) (Quanta; FEI) when cells were growing on
68 12%MGM agar for 4 days. Motility was observed by stab inoculation in the
69 12%MGM medium with 5 g/L agar. Growth at different temperatures (4, 8, 12, 16,
70 20, 25, 28, 32, 37, 40, 45, 50 and 55 °C) was also assessed in 12%MGM liquid
71 medium. Growth in different NaCl concentrations (0, 3, 6, 9,12, 15, 20, and 25
72 w/v%) and pH range (4-11 at 1.0 pH unit intervals) was examined in 12%MGM
73 liquid medium at 30°C. The catalase, oxidase, urease, and phenylalanine
74 deaminase activity, hydrolysis of starch, Gelatin, aesculin, and Tween (20, 40, 60,
75 and 80), H_2S production, nitrate reduction, and the use of sole carbon source and
76 nitrogen source (0.5%, w/v) were used the technique described by Gerhardt (1994)
77 and Ederer (1971). Acid production was tested by using the method of Zhang
78 (2015). After 4 days of culture, the optical density was measured at 600 nm using
79 a Microplate Reader (BIO-RAD) to determine whether the inoculation vial had
80 grown compared to the control group. Antibiotic susceptibility tested on
81 12%MGM agar using filter paper discs containing the following compounds
82 (μg/disc): ampicillin (10), vancomycin (30), carbenicillin (100), lincomycin (2),
83 amikacin (30), and nalidixic acid (30).

84 **Chemotaxonomic characterization**

85 Biomass used for studies was obtained by culture in liquid 12%MGM medium for

4 days in shake flasks at 30 °C. The cells collected by centrifugation were washed with distilled water and then freeze-dried. Menaquinones were extracted from freeze-dried biomass according to the method proposed by Collins et al. (1977), and analyzed by high-performance liquid chromatography (HPLC) (Collins 1985). Cellular fatty acids were extracted from the fresh cells according to the method proposed by Sasser (1990), and analyzed by gas chromatography using the Microbial Identification System (Sherlock version 6.1; MIDI database: RTSBA6). Polar lipids were detected by two-dimensional thin-layer chromatography and identified by the method proposed by Minnikin et al. (1984).

Sequence similarity and phylogenetic analysis

Genomic DNA of strain TRM85114^T was extracted using a TIANGEN (Beijing, China) bacterial DNA extraction kit. The 16S rRNA gene of strain TRM85114^T was PCR-amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). The PCR product was purified, ligated to the vector by using a commercial vector kit (Tsingke Biology and Technology Company), and transferred into *Escherichia coli* DH5α competent cells. The plasmid DNA was extracted, and pair-end sequenced using primers M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGC-TATGACC-3′) (Tsingke Biology and Technology). The sequence was then compared with the available sequences in the EzBioCloud database (https://www.ezbiocloud.net/identify) using Identify Analysis (Yoon et al. 2017) and the NCBI database by using BLAST (Altschul et al. 1990). Phylogenetic trees

of the 16S rRNA gene were performed using MEGA version 7.0 (Kumar et al. 2016) with the neighbor-joining (Saitou et al. 1987), maximum-likelihood (Felsenstein 1981), and maximum-parsimony (Fitch 1971) methods. Bootstrap analysis was performed for 1000 replicates to estimate the confidence of branches in the phylogenetic trees generated (Felsenstein 1985). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura 1980).

Genome sequencing and analysis

Genomic DNA sequence was sequenced by the Illumina Hiseq platform and assembled by ABySS (version 2.0) assembler (Jackman et al. 2017). We used the software CheckM to evaluate the completeness and contamination of the genome (Parks et al. 2015), online prodigal to predict assembled genome (Hyatt et al. 2010), software tRNAscan-SE to predict the tRNA in the genome (Lowe et al. 1997), and the software Infernal 1.1 (Nawrocki et al. 2013) to predict the rRNA in the genome based on the Rfam (Nawrocki et al. 2015) database. Genome annotation obtained by Personal Biotechnology Co., Ltd (Shanghai, China). The DNA G + C content was calculated based on the whole genome sequence. Calculations of the digital DNA-DNA hybridization (dDDH) and the average nucleotide identity (ANI) values was performed by using the Genome-to-Genome Distance Calculator (GGDC, version 2.1; http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff et al. 2013) and the ChunLab's online ANI Calculator (www.ezbiocloud.net/tools/ ani), respectively.

Detection of 1-Naphthalamide degradation capacity

Take 0.02 g 1-nathalamine in 100 mL measuring flask and add methanol to scale. Then diluted with methanol to make 1-naphamide final content of 120 mg/L, 100 mg/L, 80 mg/L, 60 mg/L, 40 mg/L and 20 mg/L. Using 10% to 100% methanol gradient elution 0–40 min by using HPLC, the detection wavelength of 222 nm to detect the peak area of 1-naphthalamide, the 1-naphthalamine standard curve was drawn with the 1-naphthalamide absorb peak area as the abscissa and the content as the longitudinal coordinate.

Prepare a 5 mg/mL 1-naphthaline ethanol solution and filter with a 0.45 μm microporous filter. TRM 85114^T single colony was selected in a shake flask containing sterilized 100 mL 12%MGM liquid medium, adding 5 mg/mL 1-naphthaline 100 μL to three parallel, and blank medium containing 1-naphamide without TRM 85114^T colony, the shaking temperature was set at 30 ℃. Sampling 1 mL 2 days apart for 14 days, detecting content changes of 1-naphthaline at different sampling times using HPLC.

Results and discussion

Phenotypic, physiological characterization

Strains of TRM85114^T were aerobic, Gram-stain-negative, short-rod shape, non-motile. The cell size of 0.9–1.0 × 0.4–0.5 μm (Fig. 1). After 4 days of incubation at 30 °C, cream-white colonies grew on 12% MGM agar plates. The strain can tolerate 3% to 15% (w/v) NaCl (optimum, 3–6%), grow at 4–40 °C (optimum, 30 °C), and pH of 6.0–9.0 (optimum, 6.0–7.0). Strain has the ability to

making catalase, oxidase, and phenylalanine deaminase, hydrolyzed gelatin and Tween 20, reduced nitrate, but negative for urease activity, hydrolyzed starch, aesculin, Tweens 40, 60 and 80, and H_2S production. Strains can produce acid from monosaccharides, especially D-glucose, not D-fructose. D-glucose, D-fructose, D-galactose, D-mannose, lactate, maltose, formate, gluconate, malonate, ethanol, and *myo*-inositol can be used as the sole carbon source for the growth of the strain. The strain can use L-alanine, L-isoleucine, and L-valine as a sole nitrogen source. The cells were susceptible to vancomycin not for ampicillin, carbenicillin, lincomycin, amikacin, and nalidixic acid.

Characteristics allowing differentiation of strain TRM85114^T from several similar species in the genus *Halomonas*, including colony color, the temperature ranges for growth, the abilities to hydrolyze Tween and to assimilate various substrates, several enzyme activities, and the antibiotic sensitivity, are shown in Table 1.

Chemotaxonomic characteristics

The predominant respiratory quinone of strain TRM85114^T is Q-7, not consistent with the ubiquinone systems of members of the genus *Halomonas.* As shown in Table 2, strain TRM85114^T differed from related bacteria in the composition of cellular fatty acids. The primary cellular fatty acids of strain TRM85114^T are $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c, summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c) and summed feature 8 ($C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c). The polar lipids of strain TRM85114^T were diphosphatidyl glycerol, phosphatidylglycerol, phosphatidyl-

ethanolamine, and phosphatidyl methyl ethanolamine, hydroxy phosphatidyl ethanolamine, phosphotidyl inositol, lyo-phosphatidylglycerol, phosphatidylcholine, phospholipids of the unknown structure containing glucosamine, phosphotidylinositol mannosidase, phosphotidylinositol dimannoside, and three unidentified lipids (Supplementary Fig. S1).

Phylogenetic analysis of the 16S rRNA gene sequences

Based on the EzBioCloud analysis, the 16S rRNA gene sequence of strain TRM85114^T (GenBank accession no. MW584241) had high similarity with members of the genus *Halomonas*, and the closest phylogenetic neighbor was *H. korlensis* CGMCC 1.6981^T (97.3%). TRM85114^T shares high 16S rRNA gene sequence similarity with the type strain of *H. korlensis* CGMCC 1.6981^T (Li et al. 2008) (97.2%), followed by three other *Halomonas* species, including *H. tibetensis* CGMCC 1.15949^T (Lu et al. 2018) (96.4%). Sequence similarities of strain TRM85114^T with other recognized *Halomonas* species and known species in another genus in the family Halomonadaceae were less than 96.0%. In the neighbor-joining, maximum-likelihood, and maximum-parsimony phylogenetic trees (Fig. 2 and Supplementary Fig. S2, S3), strain TRM85114^T clustered tightly with *H. korlensis* CGMCC 1.6981^T, forming an independent sub-cluster supported by very high bootstrap values (99%) within the genus *Halomonas* cluster.

Genomic features and analysis

The obtained draft genome of TRM85114^T (accession no. JAHCLU000000000) is

4,126,476 bp in length with a high sequence, which comprise of 60 contigs (N50 = 190,960 bp) and 52 scaffolds (N50 = 208,915 bp). The content of DNA G + C is 61.6 mol%. The annotated genome encodes a total of 3902 genes, including 1666 protein-coding genes. A total of 3 rRNAs and 57 tRNAs were identified in the genome. Sequences of the two complete 16S rRNA genes found in the genome were identical to those obtained through the Sanger sequencing.

Based on the KEGG orthology-based annotation, a total of 3544 genes (90.94%) were annotated and assigned to putative functions, of which 1389 genes were annotated into metabolism-associated pathways, and 249 genes were annotated into environmental information processing pathways. There are 44 ORFs of enzymes involved in aromatic hydrocarbon degradation ability (Table S1), three (*frmA*, *ADH5*, *adhC*) of them with naphthalene degradation function. The ANI value between strain TRM85114^{T} and *H. korlensis* CGMCC 1.6981^{T} is 87.9%, the dDDH value between them is 34.6%. These data are all significantly lower than the threshold values for ANI (95–96%) and DDH (70%) used to discriminate bacterial species, respectively.

1-Naphthalamide degradation rate

The results showed that the retention time of 1-naphthylamine was 26 min, and the absorption peak area of 1-naphthylamine decreased significantly with the increase of days (Fig. 3). According to the standard curve of 1-naphthylamine drawn (Fig. 4), the content of 1-naphthylamine in the fermentation broth of TRM85114^{T} in different culture days was determined. Test results show that the

degradation of 1-naphthylamine could reach up to 21.6 mg/L on the 4th day and 32.0 mg/L on the 14th day (Table 3). These results showed that TRM85114^T had a solid ability to degrade 1-naphthylamine. It is a strain with environmental remediation ability, treating wastewater containing 1-naphthylamine in a wide temperature range.

Description of *Halomonas jincaotanensis* sp. nov.

Halomonas jincaotanensis (jin.caotan.en´ sis. NL fem. adj. jincaotanensis were pertaining to Jincaotan, Pamir, China, from where the type strain was isolated).

Cells are Gram-stain-negative, aerobic, short-rod shape, non-motile. The cell size of 0.9–1.0 × 0.4–0.5 μm. Colonies on 12%MGM agar incubated at 30 °C for 4 days are circular, cream-white, and convex with smooth surfaces. Grows at 4–40 °C (optimum, 30 °C), at pH 6.0–9.0 (optimum, pH 6.0–7.0) and with 3-15% (w/v) NaCl (optimum, 3–6%). Strains were catalase, oxidase, and phenylalanine deaminase positive. The cells can hydrolyse gelatin and Tween 20, reduced nitrate, produce acid from D-glucose. D-glucose, D-fructose, D-galactose, D-mannose, lactate, maltose, formate, gluconate, malonate, ethanol, and *myo*-inositol can be utilized as sole carbon sources, L-alanine, L-isoleucine, and L-valine for sole nitrogen source for growth. But the cells cannot produce urase, H_2S, hydrolyze starch, aesculin, Tweens 40, 60, 80, and produce acid from D-fructose. Be sensitive to vancomycin not for ampicillin, carbenicillin, lincomycin, amikacin, and nalidixic acid. The predominant respiratory quinone is Q-7. The major fatty acids of the cells are $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c, summed

239 feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c) and summed feature 8 ($C_{18:1}$ ω6c and/or
240 $C_{18:1}$ ω7c). The major polar lipids are diphosphatidyl glycerol,
241 phosphatidylglycerol, phosphatidylethanolamine, and phosphatidyl methyl
242 ethanolamine, hydroxy phosphatidyl ethanolamine, phosphotidyl inositol,
243 lyso-phosphatidyl glycerol, phosphatidylcholine, phospho- lipids of the unknown
244 structure containing glucosamine, phosphotidylinositol mannosidase,
245 phosphotidylinositol dimanno- side, and three unidentified lipids. The DNA G +
246 C content of the strain is 61.6 mol %. The degradation of 1-naphthylamine could
247 reach up to 32.0 mg/L in 14 days.

248 The type strain, TRM85114^T (= CCTCC AB 2021006^T =LMG 32311^T), was
249 isolated from the wetland soil of Jincaotan in Pamir. The GenBank accession
250 numbers for the 16S rRNA gene and the draft genome sequence of TRM85114^T
251 are MW584241 and JAHCLU000000000, respectively.

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258 **Author contributions**

259 XB performed the experiment, analyzed the data, and drafted the manuscript. ZL
260 contributed to guiding the degradation test of 1-naphthylamine. ZX, CW， MR

261 critically revised the manuscript. LZ contributed to the creation of the project. All
262 authors read and approved the manuscript.

263 **Disclosure statement**

264 **Conflict of interest**

265 All authors declare no conflict of interest.

266 **Ethical approval**

267 No specific ethical or institutional permission is required for sampling, and
268 experimental studies do not involve endangered or protected species.

269

270 **The table title and Figure caption**

271 **Table 1** Differential characteristics of strain TRM85114^T and the type strains of
272 closely related species.

273 **Table 2** Fatty acid compositions of strain TRM85114^T and closely related type strains of
274 the genus *Halomonas*.

275 **Table 3** The content of 1-naphthylamine in TRM85114^T fermentation broth
276 during 14 days.

277 **Fig. 1** Scanning electron microscope image of TRM85114^T cells grown on 12%
278 MGM at 30 ℃ for 3 days. Scale bar, 2 μm.

279 **Fig. 2** Neighbour-joining tree based on 16S rRNA gene sequences showing the
280 phylogenetic relationships between strain TRM85114^T and related taxa. Bootstrap
281 values over 50 % are shown on the nodes as percentages of 1,000 replicates.
282 *Pseudomonas frederiksbergensis* DSM 13022^T (NR028906) was used as an

outgroup. Bars indicate 0.01 changes per nucleotide position.

Fig. 3 HPLC analysis of TRM85114^T fermentation liquor with 1-naphthalamide under absorption wavelength of 222 nm. The absorb peak of 1-naphthalamide was at 26 min. TRM85114^T cultured with 1-naphthylamine at a, 0 day; b, at 2nd day; c, at 4th day; d, 6th day; e, 8th day; f, 10th day; g, 12th day; h, 14th day.

Fig. 4 Standard curve of 1-naphthylamine. 1-Naphthalamide absorb peak area as the abscissa and the 1-naphthalamide content as the longitudinal coordinate. Data obtained by HPLC under absorption wavelength of 222 nm.

Table S1 KEGG annotation analysis of enzymes involved in aromatic hydrocarbon degradation ability based on TRM85114^T genome.

Fig. S1 Two-dimensional thin-layer chromatograms showing the polar lipids of strain TRM85114^T with a different chromogenic agent. a, 10% ethanolic molybdophosphoric acid reagent; b, ninhydrin reagent; c, molybdenum blue reagent; d, anisaldehyde reagent. DPG, diphosphatidyl glycerol; PG, phosphatidyl glycerol; PME, phosphatidyl methyl ethanolamine; PE, phosphatidyl ethanolamine; OH-PE, hydroxy phosphatidyl ethanolamine; PI, phosphotidyl inositol; LPG, lyso-phospatidyl glycerol; PC, phosphatidyl choline; NPG, phospholipids of unknown structure containing glucosarmine; PIM, phosphotidylinositol mannosides; PIDM, phosphotidylinositol dimannoside; PL, unidentified lipids.

Fig. S2 Maximum-parsimony tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain TRM85114^T and related taxa.

Bootstrap values over 50% are shown on the nodes as percentages of 1,000 replicates. *Pseudomonas frederiksbergensis* DSM 13022^T (NR028906) was used as an outgroup.

Fig. S3 Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain TRM85114^T and related taxa. Bootstrap values over 50% are shown on the nodes as percentages of 1,000 replicates. *Pseudomonas frederiksbergensis* DSM 13022^T (NR028906) was used as an outgroup. Bars indicate 0.01 changes per nucleotide position.

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Figures

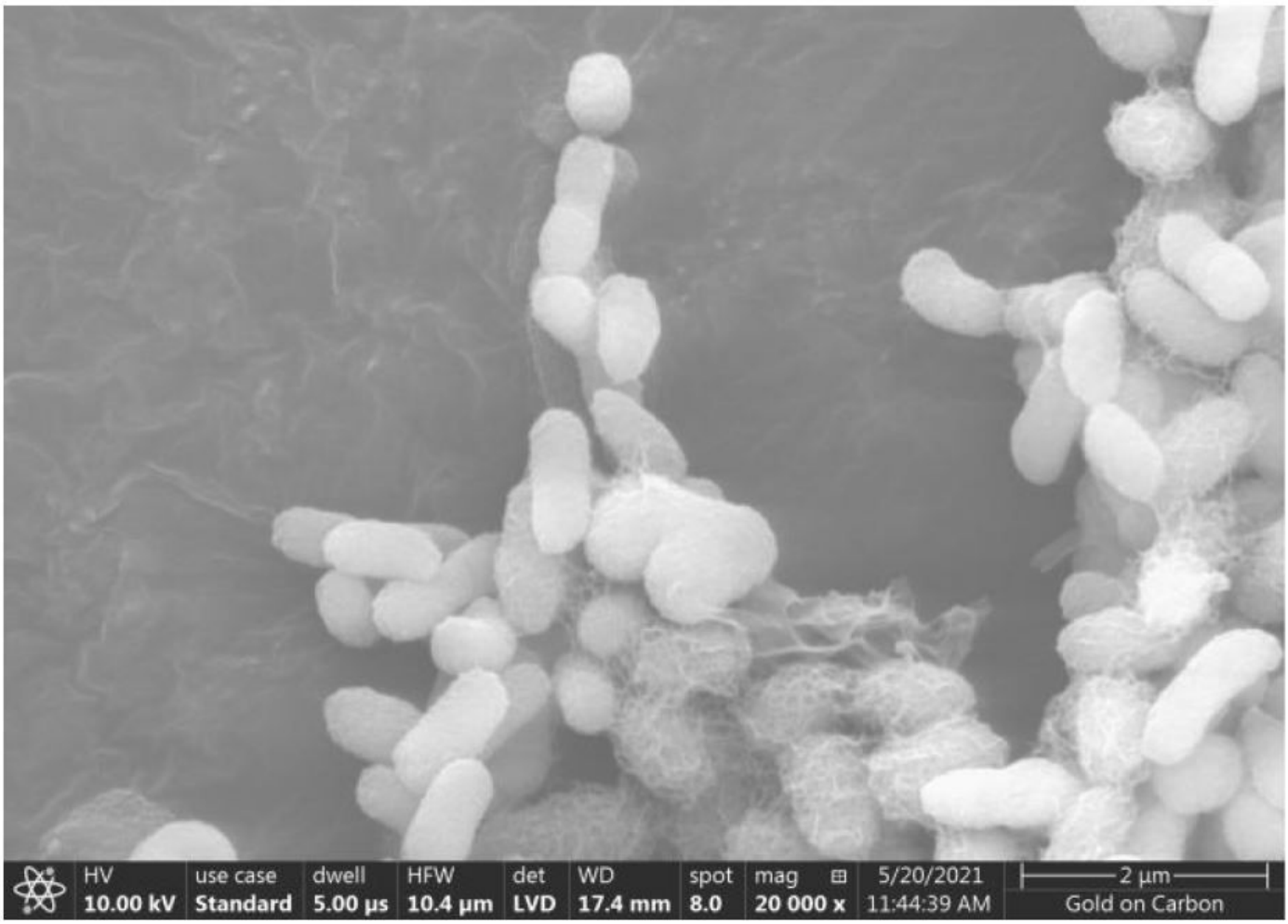

Figure 1

Scanning electron microscope image of Halomonas jincaotanensis cells grown on 12%MGM at 30 □ for 3 days. Scale bar, 2 µm.

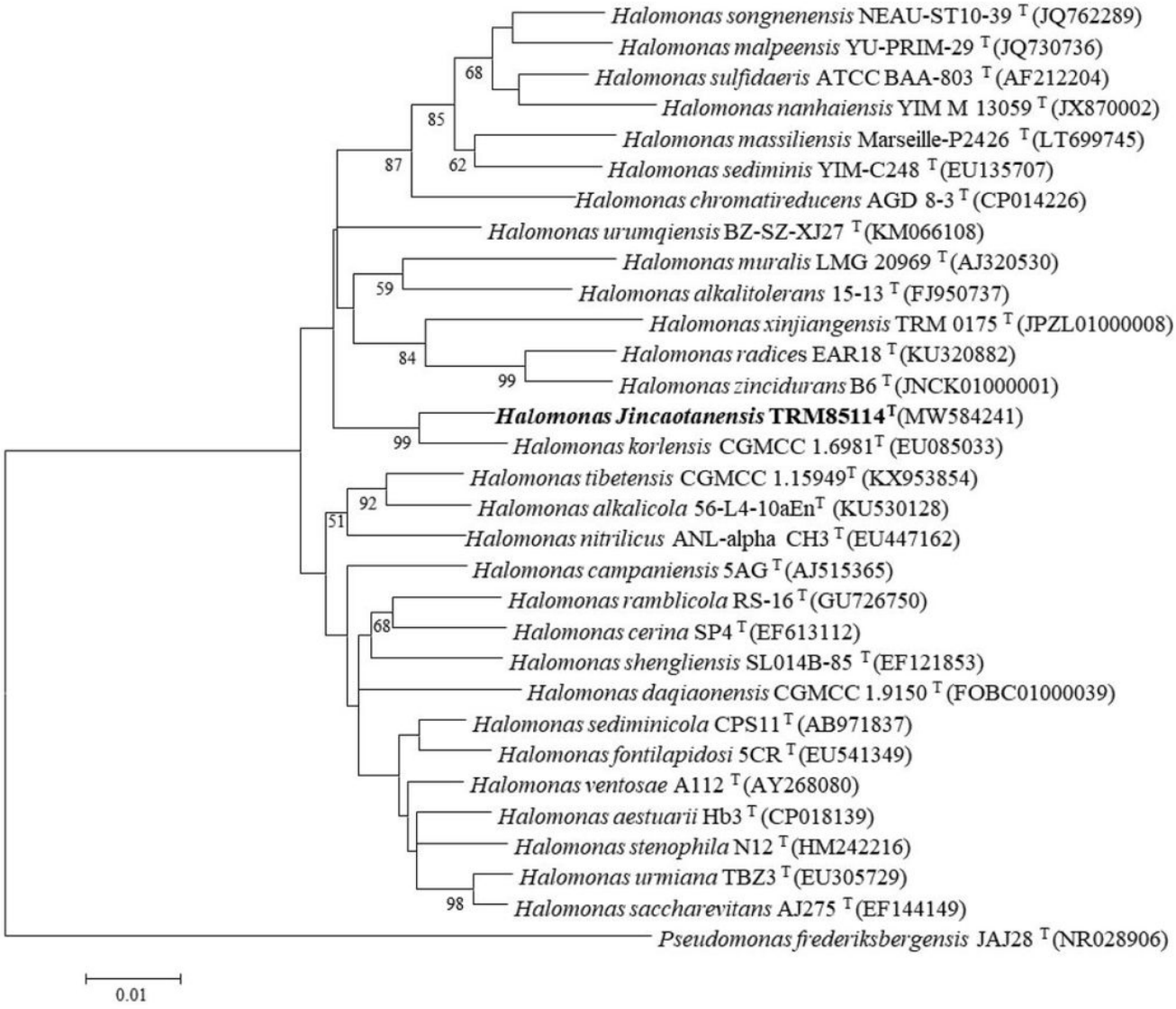

Figure 2

Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain TRM85114T and related taxa. Bootstrap values over 50 % are shown on the nodes as percentages of 1,000 replicates. Pseudomonas frederiksbergensis DSM 13022T (NR028906) was used as an outgroup. Bars indicate 0.01 changes per nucleotide position.

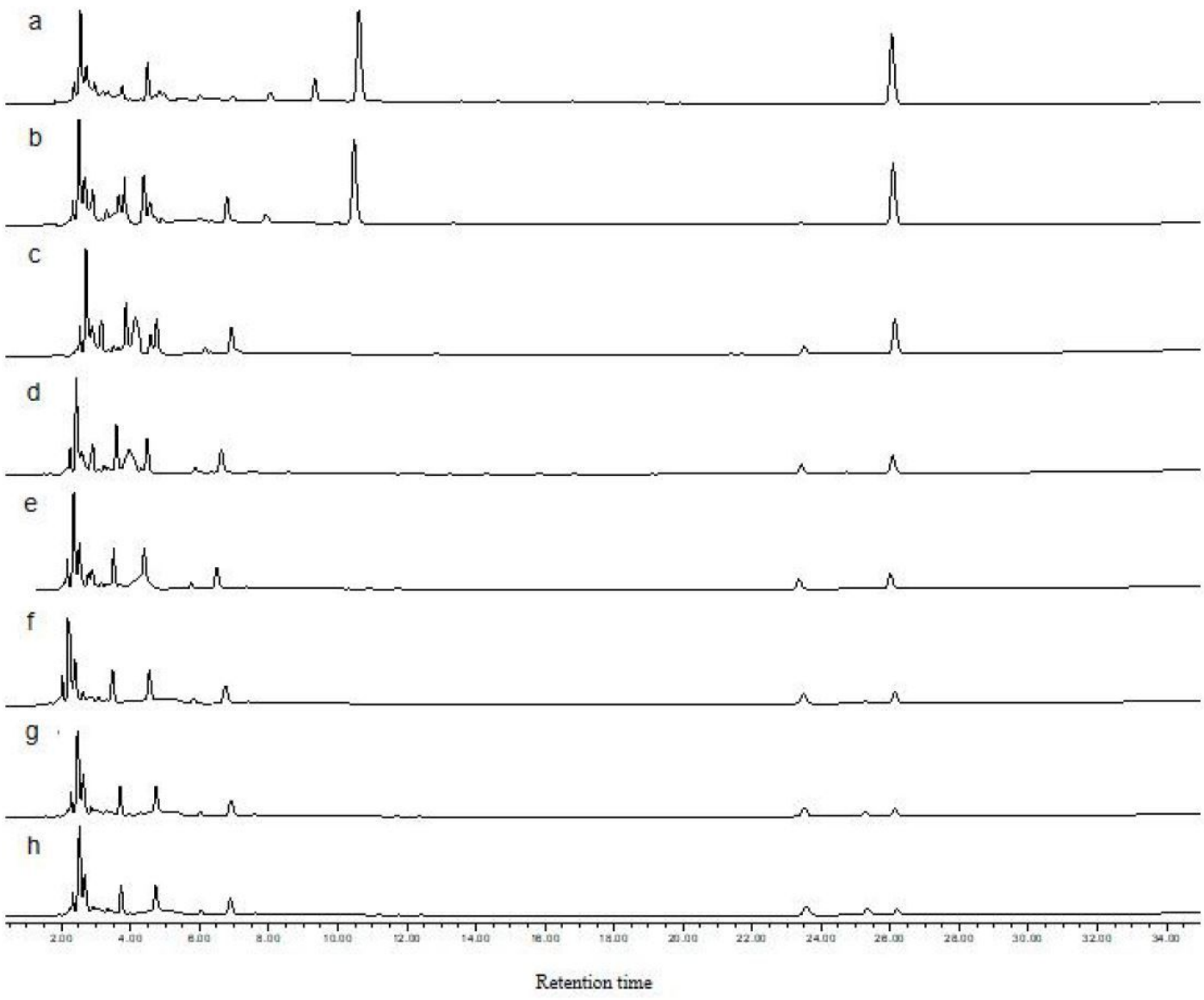

Figure 3

HPLC analysis of TRM85114T fermentation broth with 1-naphthalamide under absorption wavelength of 222 nm. The absorb peak of 1-naphthalamide was at 26 min. TRM85114T cultured with 1-naphthylamine at a, 0 day; b, at 2nd day; c, at 4th day; d, 6th day; e, 8th day; f, 10th day; g, 12th day; h, 14th day.

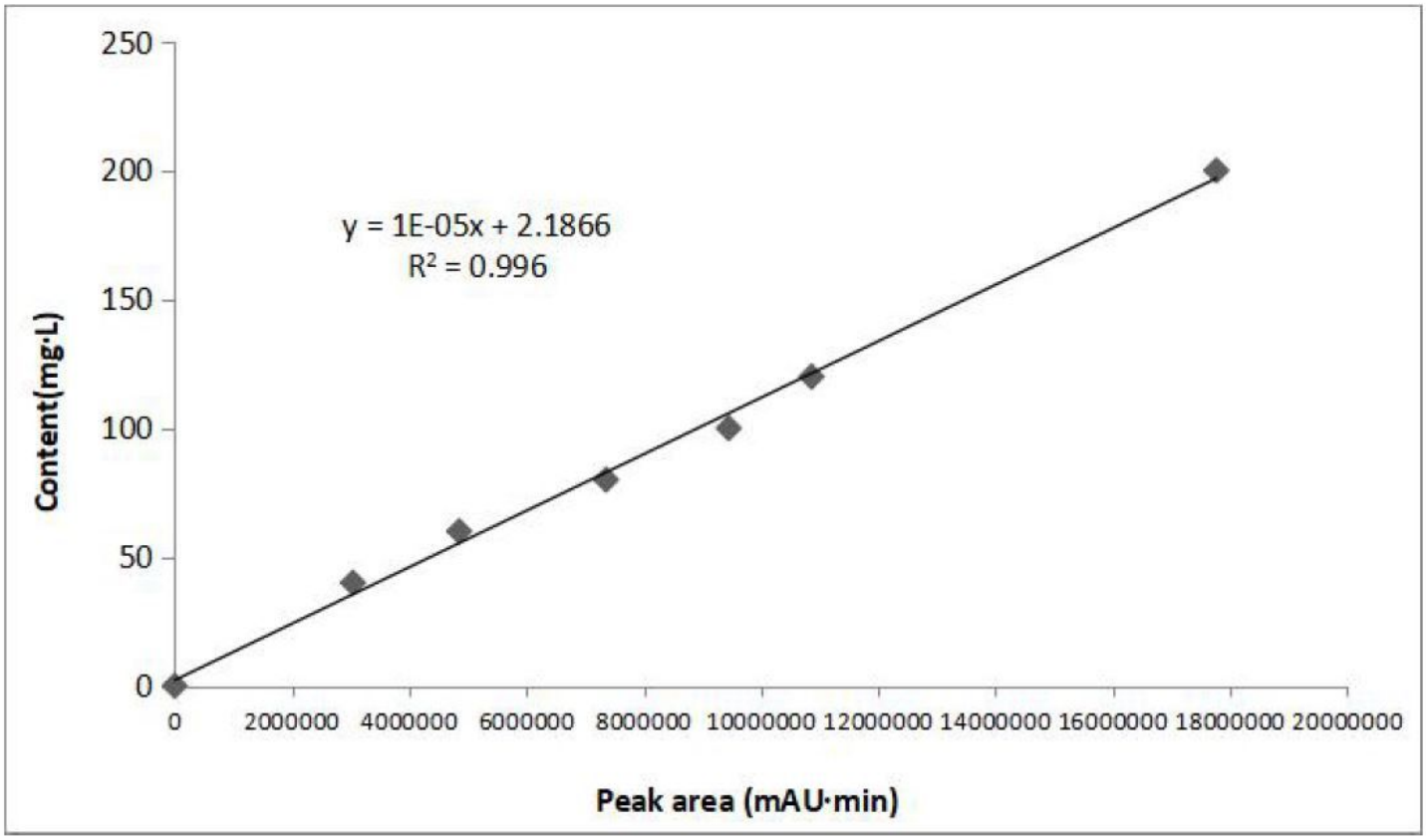

Figure 4

Standard curve of 1-naphthylamine. 1-Naphthalamide absorb peak area as the abscissa and the 1-naphthalamide content as the longitudinal coordinate. Data obtained by HPLC under absorption wavelength of 222 nm.

Supplementary Files

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

- Supplementary.pdf