Denitrificimonas halotolerans sp. nov., a novel species isolated from landfill leachate

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Abstract

A polyphasic taxonomic approach was employed to characterize the strain JX-1\(^T\) isolated from landfill leachate samples in Wuhan city, Hubei province. Compared to the five most closely related type species, strain JX-1\(^T\) and *Denitricimonas caeni* HY-14\(^T\) exhibited the highest sequence similarity (96.83\%) for 16S rRNA gene, nearly reaching the maximum value of 20.3\% for digital DNA-DNA hybridization (DDH), with the highest value of 76.06\% for average nucleotide identity (ANI) and the highest value of 78.89\% for average amino acid identity (AAI). The genome size of strain JX-1\(^T\) was 2,778,815 bp with a G + C content of 46.12\%. It had a total of 2059 clusters, 2710 proteins, and 498 singletons. The major fatty acids in strain JX-1\(^T\) were Summed Feature 8 (C18:1\(\omega_7c/C18:\omega_6c\))(18.28\%), iso-C15:0 (28.52\%), and anteiso-C15:0 (13.54\%). The respiratory quinones were predominantly Q9 (91.92\%) with a minor presence of Q8 (8.08\%). The major polar lipids included aminolipid, aminophospholipid, diphosphatidylglycerol, glycolipid, phosphatidylethanolamine, phosphatidylglycerol, and phospholipid. This bacterium showed resilience to elevated salinity and high levels of ammonia nitrogen in physiological experiments. These results of this polyphasic study enabled the genotypic and phenotypic differentiation of the strain analyzed from the closest related species, which confirmed that the strain represented a novel species within the genus *Denitricimonas*, for which the name *Denitricimonas halotolerans* sp. nov. was proposed with JX-1\(^T\) (MCCC 1K08958\(^T\) = CCTCC M2023896\(^T\)) as the type strain. The strain JX-1\(^T\), despite being classified as *Denitricimonas*, lacked denitrification-related genes and was solely capable of ammonia assimilation.

Introduction

The family *Pseudomonadaceae* was initially proposed by Winslow (Winslow et al., 1917). As of the time of writing, there were currently 29 genera within the family (https://lpsn.dsmz.de/family/Pseudomonadaceae) Notably, *Pseudomonas, Oblitimonas, Thiopseudomonas* and *Denitricimonas* were all taxonomically classified within this family. The initial classification of certain bacteria within the family *Pseudomonadaceae* was attributed to the genus *Pseudomonas*. However, advancements in analytical techniques and scientific progress had revealed significant differences in morphological characteristics, genome size, G + C proportion, and functional attributes among these bacteria compared to those of *Pseudomonas*. Consequently, they were reclassified as a distinct genus.

For instance, the strain HY-14\(^T\) was initially assigned to the genus *Pseudomonas* in 2009 (Xiao et al., 2009), but later reclassified as *Denitricimonas* in August 2021 (Saati-Santamaria et al., 2021). Concurrently in 2021, there was also a proposal suggesting the classification of *Pseudomonas caeni* under the genus *Thiopseudomonas* (Rudra and Gupta et al., 2021). The strain B4199\(^T\) was initially identified as belonging to a novel genus and species, for which the name “*Oblitimonas alkaliphila*” gen. nov. sp. nov. was proposed in 2015 (Lauer et al., 2015). Subsequently, it was officially classified as a novel species (*Oblitimonas alkaliphila*) of a new genus (*Oblitimonas*) within the family.
Pseudomonadaceae in 2016 (Drobish et al., 2016). *Thiopseudomonas denitrificans* was proposed to be a novel genus and species within the family *Pseudomonadaceae* by Tan in 2015 (Tan et al., 2015). However, in 2021, some researchers suggested the integrating of *Pseudomonas caeni*, *Oblitimonas alkaliphila* and *Thiopseudomonas denitrificans* into the emended genus *Thiopseudomonas*, which took priority over the taxon name *Oblitimonas* (Rudra and Gupta et al., 2021). Currently, the genus *Thiopseudomonas* comprises three species: *Thiopseudomonas alkaliphila*, *Thiopseudomonas caeni* and *Thiopseudomonas denitrificans* (https://lpsn.dsmz.de/search?word=Thiopseudomonas). Therefore, *Pseudomonas caeni* was associated with three synonymous taxonomic names, namely *Pseudomonas caeni*, *Thiopseudomonas caeni*, and *Denitrificimonas caeni* (https://lpsn.dsmz.de/species/Pseudomonas-caeni). Similarly, *Oblitimonas alkaliphila* was linked to two synonymous taxonomic names, *Oblitimonas alkaliphila* and *Thiopseudomonas alkaliphila* (https://lpsn.dsmz.de/species/oblitimonas-alkaliphila).

The alteration of microbial genus names may somewhat impede the classification of newly discovered species within specific genera. The genus name of the newly discovered species in this paper had undergone three revisions: *Pseudomonas*, *Thiopseudomonas* and *Denitrificimonas*. Therefore, it was crucial to be cautious when determining the appropriate genus for this species.

The objective of this study was to conduct a comprehensive analysis of the characteristics, including phenotypic traits, chemotaxonomic features and genomic properties, of a newly discovered species isolated from landfill leachate with remarkable salt tolerance. Furthermore, the taxonomic classification and genus investigation of this innovative species had also been addressed.

**Material and method**

**1.1 Source, isolation, cultivation and preservation**

The strain JX-1^T^ was isolated from a sludge sample obtained from a UASB reactor used for landfill leachate treatment in Wuhan, China, using the dilution-plating technique on 2216E and LB (Difco) agar plates under aerobic conditions. During the continuous operation of the UASB reactor, the influent COD (chemical oxygen demand) concentration ranged from 20,000 to 45,000 mg/L with an effluent concentration ranging between 6,000 and 18,000 mg/L. The influent ammonia nitrogen concentration varied between 1200 and 1900 mg/L, while the effluent concentration ranged from 1300 to 2200 mg/L. The salinity level had been consistently maintained at a range of 0.7–1.2% (w/v) throughout the year.

The strain JX-1^T^ was extensively cultured in LB liquid and solid medium at 28°C. A portion of the strains had been preserved in a laboratory refrigerator at -80°C with 40% glycerol. Under LB liquid culture conditions, a subset of the bacterium had undergone expansion, freeze-drying, and subsequent shipment to the Third Institute of Oceanography for chemical composition determination. Some strains had been cultured on solid media and sent to both Marine Culture Collection of China (MCCC) and the China Center
for Type Culture Collection (CCTCC) for long-term storage. The remaining strains would be utilized for various additional experiments.

1.2 Phenotypic, physiological, biochemical characteristics

The growth of Strain JX-1\textsuperscript{T} was evaluated under varying NaCl concentrations (0%, 1%, 2%, 5%, 7.5% and 10% w/v), pH values (5, 6, 7, 8, 9 and 10) and temperatures (4°C, 10°C, 20°C, 30°C, 37°C and 42°C) on LB medium for up to one week. Transmission electron microscopy (HT7700; Hitachi) was used to observe the strain's morphology and size, as well as presence or absence of flagella or spores in a sample stained with ammonium molybdate at a concentration of 2%.

Motility was assessed using semisolid LB medium supplemented with 0.3% agar. Gram staining was performed following the standardized method (Shen et al., 2010). Anaerobic growth was examined on LB medium at 28°C for a duration of 10 days utilizing the Longyue Anaerobic Incubator system (Dahal et al., 2018). The activities of oxidase, catalase, amylase, protease, and lipase (Tween 20,40,60 and 80) were quantified using established protocols (Tindall et al., 2007). The following four commercial kits, namely API ZYM, API 20E, API 20NE (bioMérieux) and the GEN III MicroPlate (Biolog) with protocol A, were employed for subsequent phenotyping in accordance with the manufacturer's instructions.

1.3 Chemotaxonomic analysis

The samples for chemotaxonomic analysis of strain JX-1\textsuperscript{T} were obtained from cultures grown in LB medium at 28°C for 48 h (during the logarithmic phase) and subsequently subjected to immediate freeze-drying.

The fatty acids of cells cultured aerobically in modified Marine agar (BD) containing CH\textsubscript{3}COONa (1.0 g/L) and trisodium citrate (0.5 g/L), with a pH of 7.5 at 28°C for 72 hours, were extracted, saponified and esterified. Subsequently, the fatty acid methyl esters were analyzed by GC using the MIDI system instructions (Sherlock Version 6.0B; TSBA6 Method) (Sasser et al., 1990).

The polar lipids were extracted utilizing a chloroform/methanol system and subjected to one- and two-dimensional thin-layer chromatography (TLC) analysis according to the established protocol. For TLC analysis, Merck silica gel 60 F254 aluminum-backed plates were employed. The sample-dotted plate underwent two-dimensional development using an initial solvent mixture of chloroform-methanol-water (65:25:4, v/v), followed by a subsequent solvent composition of chloroform-methanol-acetic acid-water (85:12:15:4, v/v). Total lipid content was detected using molydbatophosphoric acid as a detection agent, while specific functional groups were identified through customized spray reagents targeting distinct functional groups (Kates et al., 1986).

Respiratory quinones were extracted from freeze-dried biomass using solid-phase extraction and subsequently analyzed via high-performance liquid chromatography (HPLC) following the method described by Vieira et al (Vieira et al., 2021). The analysis of respiratory quinones was performed using...
reversed-phase HPLC coupled with a Diode-Array Detection detector. Absorption spectra were recorded in the range of 220 to 400 nm, and ubiquinones were relatively quantified at 270 nm.

1.4 Phylogenetic Analysis based on 16S rRNA Gene and VBCG gene

The phylogenetic analysis was performed utilizing the MEGA version 11 software (Tamura et al., 2021), based on the nearly complete 16S rRNA gene sequences obtained from separate downloads on NCBI (https://www.ncbi.nlm.nih.gov/). The sequences were aligned using the ClustalW program, and the best evolutionary distance method was determined by identifying optimal DNA models as implemented in MEGA 11. The up-to-date 20 validated bacterial core genes (VBCG) version 1.3 software (Tian et al., 2023) was used for the genome-based phylogeny of the strain JX-1<sup>T</sup> and reference species. The strain Acinetobacter albensis ANC4874<sup>T</sup> was used to determine a root of the phylogenomic tree.

1.5 Genome sequencing, assembly, relatedness analyse

The bacterial cells were collected through centrifugation, followed by amplification of the 16S rRNA gene using universal primers (27F and 1492R, 27F: 5’-AGAGTTTGATCCTGGCTCAG-3’; 1492R: 5’-GTTACCTTGTTACGACTT-3’). The PCR methodology was employed to amplify the 16S rRNA gene sequence of strain JX-1<sup>T</sup>. Subsequently, the resulting amplification products were sent to Wuhan Genecreate Biological Engineering Co., Ltd for analysis of the 16S rRNA gene sequence. The complete genome of strain JX-1<sup>T</sup> was extracted using the bacterial genome extraction kit provided by Nanjing Vazyme Biotech Co., Ltd. After extraction, the bacterial genomic DNA was assessed for sample integrity and purity through agarose gel electrophoresis and NanoDrop™ 8000 spectrophotometer. Subsequently, the extracted DNA was sent to Novogene, Ltd. for whole genome sequencing. Finally, the sequencing results were processed and assembled using the SPAdes 3.15.5 genome assembler tool on a Linux system (Bankevich et al., 2012).

The 16S rRNA gene sequences, whole gene sequences, protein sequences, and gene annotation information of the six model strains (Denitrificimonas caeni HY-14<sup>T</sup>, Oblitimonas alkaliphila B4199<sup>T</sup>, Thiopseudomonas denitrificans X2<sup>T</sup>, Pseudomonas alcaliphila AL15-21<sup>T</sup>, Pseudomonas toyotomiensis HT-3<sup>T</sup> and Pseudomonas chengduensis MBR<sup>T</sup>) most closely related to strain JX-1<sup>T</sup> were obtained from NCBI (National Center for Biotechnology Information).

These data were utilized for comparative analysis of G + C content, gene size, digital DNA-DNA hybridization (DDH), average nucleotide identity (ANI), and average amino acid identity (AAI) values between strain JX-1<sup>T</sup> and the aforementioned six strains. The Type Strain Genome Server (TYGS) utilizes the Genome-BLAST Distance Phylogeny method (GBDP) to perform nucleotide-level comparisons of whole genome sequences, enabling the calculation of dDDH values (Meier-Kolthoff et al., 2013; Meier-Kolthoff et al., 2019). Orthologous average nucleotide identity (ANI) values and the DNA G + C content (%) was determined using the EzBioCloud server (https://www.ezbiocloud.net/) The average amino acid identity (AAI) calculations were performed by computing the mean similarity values for all pairwise
homologous protein sequences within each pair of genomes (Rodriguez-R et al., 2014).

1.6 Genome annotation and Analysis

The complete gene sequences of strain JX-1T and its six closest model strains were individually annotated using RAST 2.0 (Rapid Annotation using Subsystem Technology) (Aziz, et al., 2008; Overbeek, et al., 2014; Brettin, et al., 2015) and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/)(Tatusova, et al., 2016; Haft, et al., 2018). KofamKOALA tool was used to annotate the genome based on the KEGG (Kyotto Encyclopedia of Genes and Genomes) database (https://www.genome.jp/tools/kofamkoala/)(Aramaki, et al., 2019).

The draft genome sequence of the strain JX-1T was deposited in DDBJ/EMBL/GenBank under the BioProject PRJNA1048099 (accession number JAXIVU000000000).

The circular genome map of the strain JX-1T and the schematic map of BLAST (Basic Local Alignment Search Tool) comparison among closest type strain genomes was generated using the CGView server (http://cgview.ca/)(Grant, et al., 2008; Stothard, et al., 2019). Comparative genome-wide analysis of orthologous clusters and gene ontology analysis among all predicted protein-coding genes was performed using Ortho Venn3(https://orthovenn3.bioinfotoolkits.net/home)(Sun, et al., 2023).

Carbohydrate-active enzyme analysis was performed using the dbCAN3 meta server https://bcb.unl.edu/dbCAN2/(Zheng, et al. 2023). Secondary metabolite production potential was carried out analyzing the presence in the genome of biosynthetic gene clusters (BGCs) within the tool antiSMASH version 7.1.0 (https://antismash.secondarymetabolites.org/#!/start) (Blin, et al., 2023).

Results and discussion

2.1 Cultivation and preservation

A portion of the strains were cultured on solid media and subsequently submitted to the Marine Culture Collection of China (MCCC) under deposit number MCCC 1K08958T, as well as to the China Center for Type Culture Collection (CCTCC) under deposit number CCTCC M2023896T for preservation. The remaining strains were used for the other various experiments.

2.2 Phenotypic, physiological, biochemical characteristics

After 2 to 3 days of growth and reproduction on LB solid medium in an aerobic environment, strain JX-1T formed pale yellow round colonies with a diameter of about 1.5mm (Figure S1). The strain was observed under the microscope (Figure S1), and the bacteria were 2-3.3 µm long and 0.9-1.0µm wide, with single, paired or stacked rod cells containing polar flagella showing negative Gram staining results. In anaerobic experiments, the strains grew slowly.
The bacteria manifest optimal growth at pH 6 and 7, while their growth was significantly reduced under other conditions within the pH range of 5.0–10.0. Moreover, temperature plays a crucial role in bacterial growth, with the highest rates observed at 30°C among the tested temperatures of 4, 10, 20, 30, 37, and 42°C. The physiological and biochemical characterization, based on the Biolog Gen microwell identification plate (Table S4), revealed that the strain JX-1T can grow at sodium chloride concentrations of 1%, 4%, and 8%. Particularly, the strain displayed optimal growth at a concentration of 1%, while no significant disparity was observed between concentrations of 4% and 8%. The growth curves of JX-1T at various salt concentrations were depicted in Figure S2. Strain JX-1T had a salinity tolerance range of 0-7.5%. Under 0% salinity, the strains displayed limited growth during the early stage and sluggish growth during the late stage. At 10% salinity, no growth was observed for the strain. The highest growth rate was achieved at a salinity level of 5%. This bacterium demonstrated significant potential for application in high-salt wastewater treatment.

In this bacterial ammonia nitrogen resistance concentration test experiment, it was observed that bacterial growth was absent at an ammonia nitrogen concentration of 10000g/ml. However, at other concentrations, the bacteria grew normally with minimal variation. Noticeably, the growth rate at a concentration of 500mg/ml surpassed that of other concentrations slightly (Figure S3).

The test results for strain JX-1T were presented in Table S1, Table S2, Table S3, and Table S4 respectively, corresponding to the API20NE, API20E, ZYM, and GEN III MicroPlate (Biolog).
### Table 1
Protologue description of *Denitrificimonas halotolerans* sp. nov.

<table>
<thead>
<tr>
<th>Genus name</th>
<th><em>Denitrificimonas</em></th>
</tr>
</thead>
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<tr>
<td>Species name</td>
<td><em>Denitrificimonas halotolerans</em></td>
</tr>
<tr>
<td>Specific epithet</td>
<td>halotolerans</td>
</tr>
<tr>
<td>Species status</td>
<td>sp. nov.</td>
</tr>
<tr>
<td>Species etymology</td>
<td><em>Denitrificimonas halotolerans</em> sp. nov. (ha.lo.to'le.rans. Gr. masc. n. hals, salt; L. pres. part. tolerans, tolerating; N.L. part. adj. halotolerans, referring to the organism's ability to tolerate high salt concentrations)</td>
</tr>
</tbody>
</table>

**Description of the new taxon and diagnostic traits**

Cells were Gram-stain-negative, strictly aerobic, motile, slim, slightly pleomorphic rods (2–3.3 µm long and 0.9–1.0 µm wide) occurring mainly in groups or singly. One or more polar flagella can be observed. Colonies were lightly yellow, circular with regular margins, smooth, shiny, convex, reaching about 1.5 mm in diameter after cultivation on LB at 30°C for 24 h. The cultures grow well on LB and 2216E agar. The bacteria grow well in temperature range from 5 to 30°C after 24–48 h. They grow weakly also at 1°C after 10 days of cultivation, but not at −2°C after 14 days. The bacteria grow well in a presence of 0–8% of NaCl and at a pH ranging from 5–8. Positive reactions were observed for Tweens 20, 60, 80, catalase, cytochrome oxidase. Nitrate and nitrite reduction were negative.

API ZYM kit revealed positive reactions for alkaline phosphatase, esterase (C4), esteraselipase (C8), lipase (C14), Leucine arylamidase, Valine arylamidase, while cystine arylamidase, acid phosphatase and naphthol-AS-Bl-phosphatase gave weak reactions. Negative tests were obtained for trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

API 20NE kit gave positive reactions for utilization of β-glucosidase, malic acid and weak reaction for D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, Potassium gluconate, Adipic acid, Phenylacetic acid. Negative reactions were observed for Reduction of nitrate to nitrite, Denitrification, Indole production, D-glucose fermentation, Arginine dihydrolase, UREase, Gelatinase, β-galactosidase, Capric acid, Trisodium citrate.

The API20E test results showed weak reactions for mannitol, rhamnose, amygdalin, and negative reactions for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease tryptophane deaminase, indole production, acetoin production (Voges Proskauer), gelatinase, glucose, inositol, sorbitol, saccharose, arabinose.
Denitrificimonas

Carbon source utilization ability via respiration, as determined in the Biolog GEN III MicroPlate test panel, was positive for 1% NaCl, 1% Sodium Lactate, Rifamycin SV, Guanidine HCl, L-Malic acid, Lithium Chloride. It showed weak utilization for pH 6, N-acetyl-D-galactosamine, 1% NaCl, 4% NaCl, 8% NaCl, D-galactose, L-rhamnose, Inosine, 1% Sodium lactate, D-serine, D-glucose-6-PO$_4$, Rifamycin SV, L-glutamic acid, Lincomycin, Guanidine HCl, Niaproof 4, D-galacturonic acid, L-galactonic acid Lactone, D-Gluconic acid, D-glucuronic acid, Glucuronamide, Quinic acid, Vancomycin, Tetrazolium violet, Tetrazolium blue, Methyl pyruvate, D-Lactic acid methyl ester, L-lactic acid, D-malic acid, Acetoacetic acid, Propionic acid, Acetic acid; and negative for Dextrin, D-maltose, D-trehalose, Cellulose, Gentiobiose, Sucrose, D-turanose, Stachyose, pH 5, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-Acetyl-β-D-Mannosamine, N-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, 3-methyl glucose, D-fucose, L-fucose, Fusidic acid, D-sorbitol, D-mannitol, D-arabitol, Myo-inositol, Glycerol, D-fructose-6-PO$_4$, D-aspartic acid, D-serine, Troleandomycin, Minocycline, Gelatin, Glycyl-L-prolin, L-alanine, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid, Serine, Pectin, Mucic acid, D-saccharic acid, α-hydroxy-phenylacetic acid, Citric acid, α-Keto-glutaric acid, Bromo-succinic acid, Nalidixic acid, Potassium Tellurite, Tween40, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-Butyric acid, α-Keto-Butyric acid, Formic acid, Aztreonam, Sodium butyrate, Sodium bromate.

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**16S rRNA gene accession.** OR878460

**Genome accession numbers** JAXIVU000000000

**Genome status** Draft

**Genome size** 2,778,815 bp
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<td>Strain Collection Numbers</td>
<td>MCCC 1K08958&lt;sup&gt;T&lt;/sup&gt; = CCTCC M2023896&lt;sup&gt;T&lt;/sup&gt;</td>
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</table>

**Table 2. The Chemotaxonomical Characteristics of strain JX-1<sup>T</sup> and the reference strains.**

1=strain JX-1<sup>T</sup>; 2=Denitrificimonas *caeni* HY-14<sup>T</sup>; 3=*Oblitimonas alkaliphila* B4199<sup>T</sup>; 4=*Thiopseudomonas denitrificans* X2<sup>T</sup>; 5=*Pseudomonas alcaliphila* AL15-21<sup>T</sup>; 6=*Pseudomonas toyotomiensis* HT-3<sup>T</sup>; 7=*Pseudomonas chengduensis* MBR<sup>T</sup>. The following data taken from: (Xiao et al., 2009), (Drobish et al., 2016), (Tan et al., 2015), (Yumoto et al., 2001), (Hirota et al., 2011), (Tao et al., 2014).

**Abbreviations:** APL, aminophospholipid; AL, aminolipid; DPG, diphosphatidylglycerol; GL, glycolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; PI phosphatidylinositol. L, unknown polar lipid.
<table>
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<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>DPG, PE, PG, L</td>
<td>DPG, PE, PI, L</td>
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<td>-</td>
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<tr>
<td>respiratory quinone</td>
<td>Q9(91.92%)</td>
<td>Q9</td>
<td>Q9</td>
<td>Q8(90.38%)</td>
<td>Q9</td>
<td>Q9</td>
<td>Q9</td>
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<tr>
<td>Q8(8.08%)</td>
<td></td>
<td></td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>4.3</td>
<td>-</td>
<td>1.24</td>
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<td>iso-C_{18:0}</td>
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<td>C_{17:1 w8c}</td>
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<td>34.48</td>
<td>27.38</td>
<td>33.45</td>
<td>33.41</td>
<td>36.18</td>
</tr>
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</table>
Feature 8

Summed feature 3: (C16:1ω7c/C16:1ω6c). Summed Feature 8(18:1 w6c/18:1 w7c).

Table 3
Comparison of the genome of strain JX-1\textsuperscript{T} with its close model strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S</th>
<th>Size(bp)</th>
<th>G + C</th>
<th>DDH</th>
<th>ANI</th>
<th>AAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain JX-1\textsuperscript{T}</td>
<td>100.00%</td>
<td>2,778,815</td>
<td>46.12%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Denitrificimonas caeni HY-14\textsuperscript{T}</td>
<td>96.83%</td>
<td>3,022,325</td>
<td>48.26%</td>
<td>20.30%</td>
<td>76.06%</td>
<td>78.89%</td>
</tr>
<tr>
<td>Oblitimonas alkaliphila B4199\textsuperscript{T}</td>
<td>95.63%</td>
<td>2,494,031</td>
<td>47.36%</td>
<td>21.90%</td>
<td>71.20%</td>
<td>67.59%</td>
</tr>
<tr>
<td>Thiopseudomonas denitrificans X2\textsuperscript{T}</td>
<td>93.36%</td>
<td>2,841,088</td>
<td>58.98%</td>
<td>19.40%</td>
<td>69.03%</td>
<td>66.77%</td>
</tr>
<tr>
<td>Pseudomonas alcaliphila AL15-21\textsuperscript{T}</td>
<td>95.34%</td>
<td>5,327,273</td>
<td>62.89%</td>
<td>18.50%</td>
<td>68.37%</td>
<td>64.00%</td>
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<tr>
<td>Pseudomonas toyotomiensis HT3\textsuperscript{T}</td>
<td>95.13%</td>
<td>5,474,114</td>
<td>62.61%</td>
<td>18.30%</td>
<td>68.19%</td>
<td>64.00%</td>
</tr>
<tr>
<td>Pseudomonas chengduensis MBR\textsuperscript{T}</td>
<td>95.13%</td>
<td>5,407,645</td>
<td>62.32%</td>
<td>18.90%</td>
<td>68.40%</td>
<td>63.27%</td>
</tr>
</tbody>
</table>

2.3 Chemotaxonomic analysis

The respiratory quinones of strain JX-1\textsuperscript{T} were predominantly Q9 (91.92%) with a minor presence of Q8 (8.08%). In contrast, Thiopseudomonas denitrificans X2\textsuperscript{T} had a reversed distribution, with Q8 accounting for 90.38% and Q9 for 9.62% (Table 2). The main respiratory quinone in other strains was identified as Q9. Thiopseudomonas denitrificans X2\textsuperscript{T} displayed significant variations in its respiratory quinones compared to the other strains due to its unique ability to utilize sulfur compounds as electron donors for respiration under anaerobic conditions. Consequently, the respiratory quinones of this bacterium differ from those observed in other strains that typically employ common electron acceptors such as oxygen or nitrate (Tan et al., 2015).

The predominant fatty acids in strain JX-1\textsuperscript{T} were C16:0 (6.71%), Summed Feature 3(C16:1ω7c/C16:1ω6c) (7.64%), Summed Feature 8 C18:1ω7c/C18:1ω6c (18.28%), iso-C15:0 (28.52%), and anteiso-C15:0 (13.54%).
The polar lipids of strain JX-1\textsuperscript{T} comprise aminolipid (AL), aminophospholipid (APL), diphosphatidylglycerol (DPG), glycolipid (GL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phospholipid (PL) and an unknown polar lipid (L), as shown in Table 2 and Figure S4. Both strain JX-1\textsuperscript{T} and *Denitrificimonas caeni* HY-14\textsuperscript{T} had the same polar lipids, including aminolipid (AL), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phospholipid (PL).

### 2.4 Phylogenetic analysis and genome relatedness analyses

The phylogenetic evolutionary tree constructed using 16S rRNA genes and the VBCG genes both revealed that strain JX-1\textsuperscript{T} clusters together with the model strains *Denitrificimonas caeni* HY-14\textsuperscript{T} within the same clade, indicating their close genetic relationship (Figure 1 and Figure 2). *Oblitimonas alkaliphila* B4199\textsuperscript{T} and *Thiopseudomonas denitrificans* X2\textsuperscript{T}, despite both being grouped in the same clade, exhibit only 81\% support based on VBGG genes (Figure 1 and Figure 2).

Comparative analysis of 16S rRNA gene sequences in relation to other strains (Table 2 and Table S5), strain JX-1\textsuperscript{T} had the highest degree of homologous similarity (96.83\%) to the reference strain *Denitrificimonas caeni* HY-14\textsuperscript{T}, while less than 96\% sequence similarity to other reference strains, including *Oblitimonas alkaliphila* B4199\textsuperscript{T} (95.63\%), *Thiopseudomonas denitrificans* X2\textsuperscript{T} (93.36\%), *Pseudomonas alcaliphila* AL15-21\textsuperscript{T} (95.34\%), *Pseudomonas toyotomiensis* HT-3\textsuperscript{T} (95.13\%), and *Pseudomonas chengduensis* MBR\textsuperscript{T} (95.13\%).

Generally, when the sequence similarity of 16S rRNA genes between tested and known strains was below 95\%, a novel genus can be identified, while a similarity below 97\% suggests a new species rather than a new genus. As the 16S rRNA gene of this strain exhibited less than 97\% homology to known model strains in the database but higher than 95\%, it was indicated that it might represent a novel bacterial species.

The gene sizes of the strains JX-1\textsuperscript{T}, *Denitrificimonas caeni* HY-14\textsuperscript{T}, *Oblitimonas alkaliphila* B4199\textsuperscript{T}, *Thiopseudomonas denitrificans* X2\textsuperscript{T}, *Pseudomonas alcaliphila* AL15-21\textsuperscript{T}, *Pseudomonas toyotomiensis* HT-3\textsuperscript{T} and *Pseudomonas chengduensis* MBR\textsuperscript{T} were 2,778,815 bp, 3,022,325 bp, 2,494,031 bp, 2,841,088 bp, 5,327,273 bp, 5,474,114 bp, and 5,407,645 bp, respectively. The corresponding proportion of G+C content were 46.12\%, 48.26\%, 47.36\%, 58.98\%, 62.89\%, 62.61\% and 62.32\% (Table 2). Based on the gene size, strains JX-1\textsuperscript{T}, *Denitrificimonas caeni* HY-14\textsuperscript{T}, *Oblitimonas alkaliphila* B4199\textsuperscript{T} and *Thiopseudomonas denitrificans* X2\textsuperscript{T} exhibited closer phylogenetic relationships, while *Pseudomonas alcaliphila* AL15-21\textsuperscript{T}, *Pseudomonas toyotomiensis* HT-3\textsuperscript{T} and *Pseudomonas chengduensis* MBR\textsuperscript{T} showed a distinct separation from the former group. Based on G+C ratio difference (Table S6), the small differences with strain JX-1\textsuperscript{T} were *Denitrificimonas caeni* HY-
14T (2.14%) and Oblitimonas alkaliphila B4199T (1.24%), respectively, the difference of other bacteria was greater than 10%.

Compared to the other five model strains (Table 3, Table S7-S9), strain JX-1T and Denitrificimonas caeni HY-14T had almost the highest values of 20.3% (21.9%, 19.40%, 18.50%, 18.30%, 18.90%) for DDH, with the highest ANI values of 76.06% (71.20%, 69.03%, 68.37%, 68.19%, 68.40%), and the highest AAI values of 78.89% (67.59%, 66.77%, 64.00%, 64.00%, 63.27%). The three values of 20.3% (20% <DDH<70%), 76.06% (70% ANI 95%), and 78.89% (60% AAI 90%) were all below the recommended threshold for the species and above the recommended threshold for the genus (Richter and Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013; Rodriguez-R et al., 2014).

Based on the aforementioned analysis, strain JX-1T was not a new genus, but a new species and had a recent phylogenetic relationship with Denitrificimonas caeni HY-14T, distinguishing it from other strains. Thus strain JX-1T was classified as a novel species within the same genus as Denitrificimonas caeni HY-14T. However, according to the current classification in LPSN (List of Prokaryotic names with Standing in Nomenclature)(https://lpsn.dsmz.de/species/ Pseudomonas-caeni), Pseudomonas caeni, Thiopseudomonas caeni, and Denitrificimonas caeni were considered synonymous taxa. Therefore, further discussion was required to determine the appropriate genus name for strain JX-1T, whether it should be classified as Thiopseudomonas or Denitrificimonas.

In accordance with the analysis results of Rudra and Gupta (Rudra and Gupta 2021), Pseudomonas caeni HY-14T, Oblitimonas alkaliphila B4199T, and Thiopseudomonas denitrificans X2T were classified as members of the Thiopseudomonas genus. Therefore, it was reasonable to classify strain JX-1T as a member of the Thiopseudomonas genus. However, when comparing the 16S rRNA gene sequence similarities between strain JX-1T or Pseudomonas caeni HY-14T and Thiopseudomonas denitrificans X2T, they were found to be 93.36% and 93.45%, respectively (below the threshold of 95%). These results indicated that both strain JX-1T and Pseudomonas caeni HY-14T did not belong to the same genus as Thiopseudomonas denitrificans X2T. Hence, it was currently inappropriate to assign Pseudomonas caeni HY-14T to the genus Thiopseudomonas.

According to the findings of the Saati-Santamari study (Saati-Santamaria et al. 2021), Pseudomonas caeni HY-14T was reclassified as Denitrificimonas, while Oblitimonas alkaliphila B4199T and Thiopseudomonas denitrificans X2T were not reclassified. These two articles were published almost simultaneously. However, they did not reference each other. Based on our comprehensive analyses, we provided support for the taxonomic classification of Pseudomonas caeni HY-14T as the type species of the novel genus Denitrificimonas. As a result, strain JX-1T was considered to represent a new species within the genus Denitrificimonas of the family Pseudomonadaceae. In addition, the bacterium had a relatively strong salt tolerance. We proposed that it could be named Denitrificimonas halotolerans JX-1T sp. nov.
2.5 Genome annotation and analysis

The draft genome of strain JX-1\textsuperscript{T} comprised 62 contigs (N50 = 132,445 bp; L50 = 7), encompassing a total genome length of 2,778,815 base bp, with a G+C content of 46.12%. The genome contained a total of 2761 genes and 2710 coding sequences, along with 44 tRNAs, 3 rRNAs, 4 ncRNAs, as well as one each of the 5S, 16S, and 23S (Table S10, Figure S5). The genomic comparison among the genomes of the strain JX-1\textsuperscript{T}, Denitrificimonas caeni HY-14\textsuperscript{T}, Oblitimonas alkaliphila B4199\textsuperscript{T} and Thiopseudomonas denitrificans X2\textsuperscript{T} was depicted in Figure S6, showing that certain distinctions were observed in the genome of strain JX-1\textsuperscript{T} compared to other bacterial strains.

The results of the comparative pan-genomic analysis using OrthoVenn3 were conducted within strain JX-1\textsuperscript{T} and its closely related three species. The strain JX-1\textsuperscript{T} comprised a total of 2059 clusters, 2710 proteins and 498 singletons, while Denitrificimonas caeni HY-14\textsuperscript{T} demonstrates 2227 clusters, 2750 proteins and 473 singletons (figure S7). Compared to the other two strains, the strain JX-1\textsuperscript{T} and Denitrificimonas caeni HY-14\textsuperscript{T} had a closer phylogenetic relationship. By conducting pairwise comparisons among the four bacteria using Venn diagrams, it was observed that the strain JX-1\textsuperscript{T} and Denitrificimonas caeni HY-14\textsuperscript{T} had the highest degree of cluster similarity, with a total of 1895 shared clusters (Figure S8). The analysis of four bacteria shows a “core” genome composed by 1439 clusters of orthologous (Figure S8), most of them annotated as clusters of proteins with functions associated to cellular metabolic processes, motility, colonization and membrane exchange specialized systems. We found 42 protein clusters, which had been identified only in the strain JX-1\textsuperscript{T}, whereas only 22 and 6 clusters were found, respectively, in its closest relative type strains of Denitrificimonas caeni HY-14\textsuperscript{T} and Oblitimonas alkaliphila B4199\textsuperscript{T} (Figure S8). The phylogenetic analysis using the Maximum Likelihood method and JTT+CAT (Jones Taylor Thornton+Classification and Adaptive) Evolution Model also revealed that strain JX-1\textsuperscript{T} and Denitrificimonas caeni HY-14\textsuperscript{T} were clustered together in the same clade, indicative of a high degree of similarity (Figure S9).

The gene sequences of the seven strains were annotated for analysis using the RAST pipeline, and the results were presented in Table S11. The strain JX-1\textsuperscript{T} had a total of 1171 Subsystem Features. The first four bacteria had approximately 1100 Subsystem Feature, while the last three bacteria in Pseudomonas had over 2000 Subsystem Features. This observation was directly proportional to the size of their respective genes. The comparative analysis will primarily focus on nitrogen metabolism, sulfur metabolism, and phosphorus metabolism within the Subsystem Features.

By employing annotated functional gene analysis of nitrogen metabolism (figure S10-S16), we made an intriguing discovery that the nitrogen metabolism had dissimilarities between strain JX-1\textsuperscript{T} and its closest bacterial relative. The strain JX-1\textsuperscript{T} and Oblitimonas alkaliphila B4199\textsuperscript{T} both lack genes associated with denitrification and dissimilatory nitrate reduction to ammonium, but only possess the gene for ammonia assimilation. Conversely, Denitrificimonas caeni HY-14\textsuperscript{T} and Thiopseudomonas denitrificans X2\textsuperscript{T} possess a plethora of denitrogenation-related genes, with Thiopseudomonas denitrificans X2\textsuperscript{T} having the highest
abundance. The three bacteria in *Pseudomonas* had no denitrification-related genes, however they possess the ability to dissimilatory nitrate reduction to ammonium.

The analysis of sulfur metabolism (Table S11) revealed a limited number of genes related to sulfur metabolism in *Denitrificimonas caeni* HY-14\(^T\) and *Oblitimonas alkaliphila* B4199\(^T\), while *Thiopeudomonas denitrificans* X2\(^T\) and strain JX-1\(^T\) had a higher abundance. Three strains among the *Pseudomonas* bacteria possessed the highest number of sulfur metabolism-related genes.

Through the analysis of phosphorus metabolism, it was found that the seven strains had little difference in phosphorus metabolism and shared 17 genes related to phosphorus metabolism (Table S12). Strain JX-1\(^T\) had only one gene, 2-aminoethylphosphonate ABC transporter ATP-binding protein (TC3.A.1.9.1), which was absent in other strains (Table S12).

We also identified salt-tolerant genes in the gene annotation results of strain JX-1\(^T\), including including Na\(^+\)/H\(^+\) antiporter NhaD and NhaB, as well as a Na\(^+\)-driven multidrug efflux pump. Additionally, we observed high ammonia tolerance-related genes such as Ammonium transporter, NAD-specific glutamate dehydrogenase (EC 1.4.1.2 / EC 1.4.1.4), Carbamoyl-phosphate synthase large/small chain (EC 6.3.5.5), Branched-chain amino acid ABC transporter ATP-binding protein LivF/LivG/LivM/LivH/LivJ (TC3.A.1.4.), Na\(^+\)(-)dependent branched-chain amino acid transporter, Signal transduction histidine kinase CheA, Sensor histidine kinase PrrB (RegB) (EC 2.7.3-), and Sensor histidine kinase Reut_B5169. The findings also align with previous physiological experiments on the bacterium's salt and ammonia tolerance.

Analysis of the genome sequence of strain JX-1\(^T\) with the dbCAN3 META server revealed a total of 93 genes encoding different CAZymes involved in degradation, modification, or synthesis of glycosidic bonds. These included 6 genes for Auxiliary Activities (AA), 3 genes for Carbohydrate Esterase (CE), 33 genes for glycosyl hydrolases (GH), 46 genes for glycosyl transferases (GT), and 5 genes for Carbohydrate-binding Module (CBM) (Table S13). Additionally, the dbCAN3 META server annotation also revealed the existence of 11 identified signal peptides.

The potential production of secondary metabolites of the strain JX-1\(^T\) was scanned with antiSMASH, and three possible gene clusters, involved in the biosynthesis of ectoine, RiPP-like and redox-cofactor, respectively, were found. In the comparative secondary metabolic analysis of strain JX-1\(^T\) and other six strains, strain JX-1\(^T\), *Denitrificimonas caeni* HY-14\(^T\), *Thiopeudomonas denitrificans* X2\(^T\) all encoded the ectoine gene cluster. On the other hand, *Oblitimonas alkaliphila* B4199\(^T\), *Pseudomonas alcaliphila* AL15-21\(^T\), *Pseudomonas toyotomiensis* HT3\(^T\) and *Pseudomonas chengduensis* MBR\(^T\) did not have the ectoine synthesis gene cluster. All the other six strains except for *Thiopeudomonas denitrificans* X2\(^T\) harbored the RiPP-like gene cluster. All seven strains encoded the redox-cofactor gene cluster.

**Conclusion**
The obtained results, including phylogenetic analysis using 16S rRNA gene and VBCG gene, whole-genome sequence relatedness and annotated analysis, respiratory quinones and fatty acid methyl ester analysis, and comprehensive phenotyping, demonstrated that the strain analyzed in this study represented a novel species within the genus *Denitrificimonas*. In addition, the bacterium displayed a substantial level of salt tolerance. Therefore, we proposed the name *Denitrificimonas halotolerans* sp. nov. After conducting an annotated analysis of the genome, we had identified a significant number of genes in the bacterium that exhibit salt and ammonia tolerance, which was consistent with its observed physiological characteristics. Compared to the closest related strain—*Denitrificimonas caeni* HY-14<sup>T</sup>, the strain JX-1<sup>T</sup> lacked the genes associated with denitrification and dissimilatory nitrate reduction to ammonium (DNRA), but only possessed the gene for ammonia assimilation and more sulfur metabolism genes. The strain JX-1<sup>T</sup> harbored a total of 2,059 clusters, 2,710 proteins and 498 singletons. It possessed 33 genes encoding glycosyl hydrolases (GH) and 46 genes encoding glycosyl transferases (GT), along with the identification of 11 signal peptides. Moreover, it demonstrated potential of producing secondary metabolites such as ectoine, RiPP-like compounds, and redox-cofactors. Despite the comprehensive genome analysis, the underlying mechanisms behind the adaptation of the investigated strains to the hostile conditions characterized by high levels of salt and ammonia nitrogen remained elusive. Compared to the nearest relative, *Denitrificimonas caeni*, the question why strain JX-1<sup>T</sup> lacked denitrification ability remained to addressed. The timing and reasons for the divergence in nitrogen-removing functions between these two strains remained unclear.

Type strain was JX-1<sup>T</sup> (MCCC 1K08958<sup>T</sup>=CCTCC M2023896<sup>T</sup>). The DNA content G + C was 46.12 mol%. A detailed description of the studied strain was presented in the Protologue table (Table 1).

**Declarations**

**Accession numbers**

The 16S rRNA gene sequences obtained in this study had been assigned GenBank accession numbers, including OR878460 for strain JX-1<sup>T</sup>. the whole-genome shotguns projects were deposited as JAXIVU000000000 for the strain JX-1<sup>T</sup>.

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**Data availability**

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://XXXXX

References


Figures
Figure 1

Maximum-likelihood tree based on 16S rRNA gene sequence comparison showing phylogenetic position of strains JX$^T$ and reference strains. Bootstrap probability values (percentages of 1000 tree replications) were shown at the branch points. Bar, 0.02 substitutions per nucleotide position. Filled circles indicate that the corresponding nodes were also obtained in the Neighbourjoining tree. *Acinetobacter albensis* ANC4874$^T$ (KR611798) was used as an outgroup. The GenBank accession numbers of the 16S rRNA gene sequences were shown in parentheses.
Figure 2

Maximum-likelihood tree based on VBCG gene sequence comparison showing phylogenetic position of strains JX\textsuperscript{T} and reference strains. The phylogenetic tree was constructed using the up-to-date bacterial core gene set (VBCG1.3; concatenated alignment of 20 core genes). \textit{Acinetobacter albensis} ANC4874\textsuperscript{T} was used out group. Assembly accession numbers were given in parentheses. Bar, 0.1 substitution per position.

Supplementary Files

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

- SupplementarydataofDenitrificimonashalotolerans.docx