



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

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Abstract A Gram-stain-negative, facultative anaerobe, rod-shaped strain JX-1^T was isolated from UASB sludge treating landfill leachate in Wuhan, China. The isolate is capable of growing under conditions of pH 6.0–11.0 (optimum, pH 7.0–8.0), temperature 4–42 °C (optimum, 20–30 °C), 0–8.0% (w/v) NaCl (optimum, 5.0%), and ammonia nitrogen concentration of 200–5000 mg/L (optimum, 500 mg/L) on LB plates. The microorganism can utilize malic acid, D-galactose,

L-rhamnose, inosine, and L-glutamic acid as carbon sources, but does not reduce nitrates and nitrites. The major fatty acids are C_{18:1ω7c}/C_{18:1ω6c}, iso-C_{15:0}, and anteiso-C_{15:0}. The respiratory quinones are Q9 (91.92%) and Q8 (8.08%). Polar lipids include aminolipid, aminophospholipid, diphosphatidylglycerol, glycolipid, phosphatidylethanolamine, phosphatidylglycerol, and phospholipid. Compared with other strains, strain JX-1^T and *Denitrificimonas caeni* HY-14^T have the highest values in terms of 16S rRNA gene sequence similarity (96.79%), average nucleotide identity (ANI; 76.06%), and average amino acid identity (AAI; 78.89%). Its digital DNA-DNA hybridization (dDDH) result is 20.3%. The genome of strain JX-1^T, with a size of 2.78 Mb and 46.12 mol% G + C content, lacks genes for denitrification and dissimilatory nitrate reduction to ammonium (DNRA), but contains genes for ectoine synthesis as a secondary metabolite. The results of this polyphasic study allow genotypic and phenotypic differentiation of the analysed strain from the closest related species and confirm that the strain represents a novel species within the genus *Denitrificimonas*, for which the name *Denitrificimonas halotolerans* sp. nov. is proposed with JX-1^T (=MCCC 1K08958^T=KCTC 8395^T) as the type strain.

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Introduction

The genus *Denitrificimonas* belongs to the family *Pseudomonadaceae* with the type species *Denitrificimonas caeni* (Xiao et al. 2009; Saati-Santamaría et al. 2021). Currently, the genus *Denitrificimonas* comprises only one species: *Denitrificimonas caeni* (<https://lpsn.dsmz.de/species/denitrificimonas-caeni>). The genus most closely related to *Denitrificimonas* is *Thiopseudomonas* (Tan et al. 2015; Rudra and Gupta 2021). The genus *Thiopseudomonas* consists of three species: *Thiopseudomonas denitrificans*, *Thiopseudomonas alkaliphila*, and “*Thiopseudomonas acetoxidans*” (<https://lpsn.dsmz.de/search?word=Thiopseudomonas>) (Tan et al. 2015; Rudra and Gupta 2021; An et al. 2024). *D. caeni* HY-14^T was isolated from the sludge of an anaerobic ammonium oxidizing-bioreactor (Xiao et al. 2009), while “*T. acetoxidans* CY1220”, *T. alkaliphila* B4199^T and *T. denitrificans* X2^T were isolated from the sludge of anaerobic fermentation liquid of food waste, human deep liver and an anaerobic, denitrifying, sulfide removal bioreactor, respectively (An et al. 2024; Drobish et al. 2016; Tan et al. 2015). *D. caeni* is a denitrifier that can reduce nitrite to nitrogen, which could play an important role in wastewater treatment and environmental remediation. *D. caeni* is also a bacterium that can grow in alkaline and saline environments and has the ability to degrade caprate and malate (Xiao et al. 2009). Therefore, studying the taxonomy and functional characteristics of *Denitrificimonas* can provide new ideas and methods for environmental pollution control. During an investigation of the microbial community structure in landfill leachate, strain JX-1^T was isolated from landfill leachate treated by UASB in Wuhan, China. It was identified as a potential novel species within the genus *Denitrificimonas* based on the 16S rRNA gene sequence. The culture experiment showed that strain JX-1^T had the ability to tolerate salt, alkaline conditions and high ammonia nitrogen concentration for growth. The objective of this study is 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. It is expected to offer new insights into the function, ecology and distribution of *Denitrificimonas* members.

Material and method

Source, isolation, cultivation and preservation

Strain JX-1^T was isolated from a sludge sample obtained from a UASB reactor used for landfill leachate treatment in Wuhan, China, after 1000-fold dilution followed by dilution plating technique on 2216E or LB (Difco) agar plates under aerobic conditions. During continuous operation of the UASB reactor, the influent COD (chemical oxygen demand) concentration ranged from 20,000 to 45,000 mg/L and the effluent concentration ranged from 6000 to 18,000 mg/L. The influent ammonia nitrogen concentration varied between 1200 and 1900 mg/L with an effluent concentration ranging from 1300 to 2200 mg/L. Salinity was maintained in the range of 0.7% to 1.2% (w/v) throughout the year. The inlet pH was measured to be 7.6 and the outlet pH was measured to be 8.4.

Strain JX-1^T was cultured in both liquid LB medium and solid agar at a temperature of 28 °C. The strain JX-1^T was then sent to the Third Institute of Oceanography, Ministry of Natural Resources for evaluation, which included the determination of respiratory quinones, fatty acid content, and polar lipid composition. In the meantime, the strain was sent to both the Marine Culture Collection of China (MCCC) and the Korean Collection for Type Cultures (KCTC) for long-term storage, while another aliquot of the strain was cryopreserved in a laboratory freezer at – 80 °C using a 40% glycerol solution as cryoprotectant. Lastly, the remainder of the JX-1^T stock was retained for use in numerous upcoming experiments.

Phenotypic, physiological, biochemical characteristics

The growth of strain JX-1^T was evaluated under varying NaCl concentrations of 0%, 1%, 2%, 5%, 7.5%, and 10% (w/v), ammonia nitrogen concentrations (200, 500, 1000, 2000, 5000, 10000 mg/L, expressed as N), pH (5, 6, 7, 8, 9, 10, and 11), and temperature (4 °C, 10 °C, 20 °C, 30 °C, 37 °C, and 42 °C) on LB medium for up to 1 week. The pH was adjusted with buffers containing HAc-NaAc (pH 5.0–6.0), PIPES (pH 6.5–7.0), HEPES (pH 7.5–8.0), and CAPSO (pH 9.0–11.0) at 28 °C. Transmission electron microscopy (TEM) (HT7700; Hitachi) was used to observe the

morphology and size of the strain, as well as the presence or absence of flagella or spores. To observe cells under TEM, cells were grown on liquid LB medium and suspended in saline. A small drop of the suspension was placed on a carbon-coated copper grid and the cells were negatively stained with 1% phosphotungstic acid for observation under TEM.

Motility was assessed using a semi-solid 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 10 days utilizing the anaerobic incubator system (Longyue Instrument Equipment Co., Ltd, Shanghai). Catalase activity was determined by observing bubble formation in a 3% (v/v) solution of H₂O₂, whereas cytochrome c oxidase activity was analyzed using N,N,N,N-tetramethyl-p-phenylenediamine. Hydrolysis assays were performed using modified LB medium except that the concentration of trypticase peptone (BD) was decreased to 1g/L and different substrates were supplemented, including casein, gelatin, starch (all at 5 g/L), and 0.3% (v/v) Tweens 20, 40, 60 and 80 (Zhang et al. 2015). 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.

Chemotaxonomic analysis

Samples for chemotaxonomic analysis of strain JX-1^T were obtained from cultures grown in LB medium at 28 °C for 48 h (during the logarithmic phase) and then immediately lyophilised.

The fatty acids of cells cultured aerobically in modified marine agar (BD) containing CH₃COONa (1.0 g/L) and trisodium citrate (0.5 g/L) at pH 7.5 for 72 h at 28 °C 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 1990).

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 aluminium-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 molybdotophosphoric acid as a detection agent, while specific functional groups were identified through customized spray reagents targeting distinct functional groups (Kates 1986).

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

Gene extraction, sequencing, and assembly

Amplification of the 16S rRNA gene of strain JX-1^T using universal bacterial primers (27F and 1492R) was performed by polymerase chain reaction (PCR). Genomic DNA of strain JX-1^T was extracted using FastPure Bacteria DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd, Nanjing). After extraction, the bacterial genomic DNA was assessed for sample integrity and purity by agarose gel electrophoresis and NanoDrop™ 8000 spectrophotometer. The extracted DNA was then 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). After successful sequencing, the obtained 16S rRNA gene sequence and whole genome sequence of strain JX-1^T were submitted to the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov>). Following this submission, the draft 16S rRNA gene sequence of strain JX-1^T was deposited in NCBI GenBank (accession number: OR878460), and its whole genome sequence was assigned the accession number JAXIVU000000000 in DDBJ/EMBL/GenBank under BioProject PRJNA1048099.

Phylogenetic trees and genome relatedness analyses

Phylogenetic trees were constructed based on the 16S rRNA genes and a set of 20 validated bacterial core genes (VBCG), respectively, while a phylogenomic tree was constructed based on protein-coding genes. Phylogenetic analysis was performed utilizing MEGA version 11 software (Tamura et al. 2021) based on the nearly complete 16S rRNA gene sequences. 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. Software version 1.3 of the up-to-date 20 validated bacterial core genes (VBCG) was used for the genome-based phylogeny of strain JX-1^T and reference species (Tian and Imanian 2023). The Type Strain Genome Server (TYGS) utilized the Genome-BLAST Distance Phylogeny (GBDP) method to perform nucleotide-level comparisons of whole genome sequences, enabling the construction of a phylogenomic tree based on protein-coding genes (Meier-Kolthoff et al. 2013; Meier-Kolthoff and Göker 2019). The strain *Acinetobacter albensis* ANC 4874^T was used as an outgroup strain for the construction of two different phylogenetic trees and a phylogenomic tree.

For each of the reference strains mentioned, the corresponding 16S rRNA gene sequences, protein amino acid sequences, gene annotation information, and whole genome sequence, were retrieved individually by downloading from the NCBI website. These data were used for comparative analysis of G + C content (mol%), gene size, digital DNA-DNA hybridization (dDDH), average nucleotide identity (ANI), and average amino acid identity (AAI) values between strain JX-1^T and the above four strains. For digital DNA-DNA hybridization (dDDH), the genome-to-genome distance was calculated online (<https://ggdc.dsmz.de/>) (Meier-Kolthof et al. 2013). Orthologous average nucleotide identity (ANI) values and DNA G + C content (mol%) were determined using the EzBioCloud server (<https://www.ezbiocloud.net/>). Average amino acid identity (AAI) calculations were performed by computing the average similarity values for all pairwise homologous protein sequences within each genome pair (<http://enve-omics.ce.gatech.edu/aai/index>) (Rodriguez-R and Konstantinidis 2014).

Genome annotation and analysis

The whole gene sequences of strain JX-1T and its closely related strains were individually annotated using RAST 2.0 (Rapid Annotation using Subsystem Technology) (<https://rast.nmpdr.org/>) (Overbeek et al. 2014; Brettin et al. 2015; Aziz et al. 2008) 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). The GhostKOALA tool was used to annotate the genome based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (<https://www.kegg.jp/ghostkoala/>) (Kanehisa, et al. 2016).

The circular genome map of strain JX-1T and the schematic map of the Basic Local Alignment Search Tool (BLAST) comparison between strain JX-1T and the genomes of closely related strains were generated using the CGView server (<http://cgview.ca/>) (Grant and Stothard 2008; Stothard et al. 2019). The orthology analysis Venn diagram and pairwise orthology analysis heatmap among the five genomes were generated 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 performed by analyzing the presence of biosynthetic gene clusters (BGCs) in the genome using the antiSMASH tool, version 7.1.0 (<https://antismash.secondarymetabolites.org/#/!start>) (Blin et al. 2023).

Results and discussion

Phenotypic characteristics

After 2 to 3 days of growth on solid LB medium in an aerobic environment, strain JX-1^T formed pale yellow, round colonies approximately 1.5 mm in diameter (Fig. S1). The strain was observed under the microscope (Fig. 1) and the bacteria were 2–3.3 µm long and 0.9–1.0 µm wide, with single, paired, or stacked rod cells containing polar flagella. The isolate was able to grow at pH 6.0–11.0 (optimum, pH 7.0–8.0), 4–42 °C (optimum, 20–30 °C), and in the presence of 0–8.0% (w/v) NaCl (optimum, 5.0% (w/v)) (Fig. S2) and 200–5000 mg/L ammonia nitrogen concentration

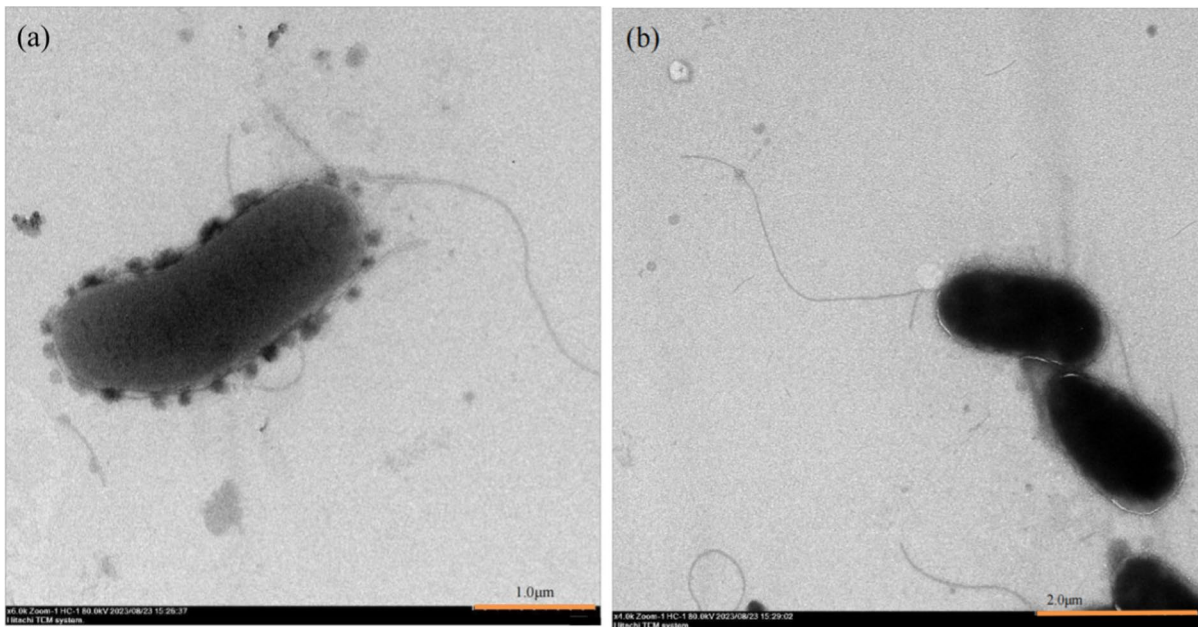


Fig. 1 Transmission electron micrographs of strain JX-1^T grown on LB for 48 h. Bars, 1.0 μm (a), 2.0 μm (b)

(optimum, 500 mg/L) (Fig. S3). After 2 weeks of culture in the anaerobic incubator system, strain JX-1^T was found to grow slowly on the plate, indicating its facultative growth. The differential enzyme activities, carbon source utilization, and other physiological and biochemical characteristics of strain JX-1^T and other members of the genera *Denitrificimonas* and *Thiopseudomonas* are provided in Table 1. The test results for strain JX-1^T are presented in Tables S1, S2, S3, and S4, respectively, corresponding to API 20NE, API 20E, ZYM, and GEN III MicroPlate (Biolog).

Chemotaxonomic analysis

Respiratory quinones were dominated by Q9 in both strain JX-1^T and *D. caeni* HY-14^T. Specifically, in strain JX-1^T, Q9 accounted for 91.92% of the total, with a small percentage of 8.08% for Q8 (Table S5).

The predominant fatty acids in strain JX-1^T were C16:0 (6.71%), summed feature 3 (C_{16:1}ω7c / C_{16:1}ω6c) (7.64%), summed feature 8 (C_{18:1}ω7c / C_{18:1}ω6c) (18.3%), iso-C_{15:0} (28.5%), and anteiso-C_{15:0} (13.5%) (Table S5).

The polar lipids of strain JX-1^T included aminolipid (AL), aminophospholipid (APL), diphosphatidylglycerol

(DPG), glycolipid (GL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phospholipid (PL) and an unknown polar lipid (L), as shown in Figure S4. Both strain JX-1^T and *D. caeni* HY-14^T had the same polar lipids, including aminolipid (AL), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phospholipid (PL) (Table S5).

Phylogenetic relationship and genomic relatedness analyses

The 16S rRNA gene sequence of strain JX-1^T was 1540 bp length and was deposited in the GenBank database (accession number: OR878460). Comparison of 16S rRNA gene sequences using EzBioCloud showed that strain JX-1^T was phylogenetically related to members of *Denitrificimonas* and *Thiopseudomonas*, and was most closely related to the strain *D. caeni* Y-14^T (96.79%) (Xiao et al. 2009; Saati-Santamaría et al. 2021), followed by *T. alkalicola* B4199^T (95.63%) (Drobish et al. 2016). None of the reference taxa displayed sequence identity to the new isolate above the recommended threshold of 98.65% for bacterial species delimitation (Kim et al. 2014). The phylogenetic trees based on 16S rRNA

Table 1 Comparison of characteristics between strain JX-1^T and its closely related species.

Characteristic	1	2	3	4	5
Motility	+	+	-	+	+
Oxidase	+	+	+	+	-
Oxygen tolerance	Facultative anaerobe	Aerobic	Aerobic	Facultative anaerobe	Facultative anaerobe
Temperature range for growth (°C) (optimum)	4–42 (20–30)	4–42 (30)	10–42 (20–35)	10–42 (30)	20–40 (30)
NaCl tolerance (%) (optimum)	0–8 (5)	0–3 (0.5–1)	0–8 (4–6)	0–3 (0–1)	0–5 (2)
pH range for growth (optimum)	6.0–11.0 (7.0–8.0)	6.0–11.0 (7.0–8.0)	6.0–11.0 (7.5)	6.0–10.0 (8.0)	6.0–11.0 (8.0)
Utilization of:					
Malate	-	+	-	+	-
Acetate	-	-	-	-	+
Sodium butyrate	-	-	-	-	W
D-mannose	W	-	+	-	-
α-D-glucose	-	-	+	-	-
API 20NE:					
Denitrification	-	+	-	+	-
D-glucose fermentation	-	-	-	-	-
Gelatin hydrolysis	-	-	-	+	-
Aesculin hydrolysis	+	-	-	-	+
Enzyme activity					
Esterase lipase (C8)	+	+	+	W	+
Lipase (C14)	+	-	-	-	+
Valine arylamidase	+	-	-	-	+
Cystine arylamidase	W	-	-	-	W
Acid phosphatase	W	-	-	-	W
Naphtol-AS-BI-phosphohydrolase	W	-	-	+	+
DNA G+C content (mol%)	46.1%	48.3%	47.4%	59.0%	48.6%

1 = Strain JX-1^T; 2 = *D. caeni* HY-14^T; 3 = *T. alkaliiphila* B4199^T; 4 = *T. denitrificans* X2^T; 5 = "*T. acetoxidans* CY1220". + positive; -, negative; W, weak positive

and VBCG gene sequences both revealed that strain JX-1^T was positioned within the genus *Denitrificimonas*, forming a separate and stable clade with *D. caeni* Y-14^T (Figs. 2, S5). The phylogenomic tree constructed using protein-coding genes via the Type Strain Genome Server (TYGS) and analyzed by the Genome-BLAST Distance Phylogeny (GBDP) method further supported the above conclusion (Fig. S6). These results strongly suggested that strain JX-1^T represented a distinct *Denitrificimonas* species.

When performing pairwise comparisons of genome sequences among the five closely related strains, as listed in Table 2 and detailed in Tables S6–S9, it was observed that strain JX-1^T and *D. caeni* HY-14^T exhibited the highest level of 16S

rRNA gene sequence similarity at 96.79%, which was higher than the respective values of 95.63%, 93.36%, and 95.18% for other strain comparisons. At the same time, they displayed the maximum ANI value of 76.06%, in contrastion with the lower values of 71.20%, 69.03%, and 71.56% found between different pairs. They also shared the maximum AAI value of 78.89%, exceeding the AAI values of 67.59%, 66.77%, and 68.72% between strain JX-1^T and other relevant strains. The estimated dDDH value for the comparison between strain JX-1^T and *D. caeni* HY-14^T was calculated to be 20.3 mol %. The four values of 96.79% (95% < 16S rRNA < 98.65%), 20.3% (20 mol% < dDDH < 70 mol%), 76.06% (70% < ANI < 95%), and 78.89%

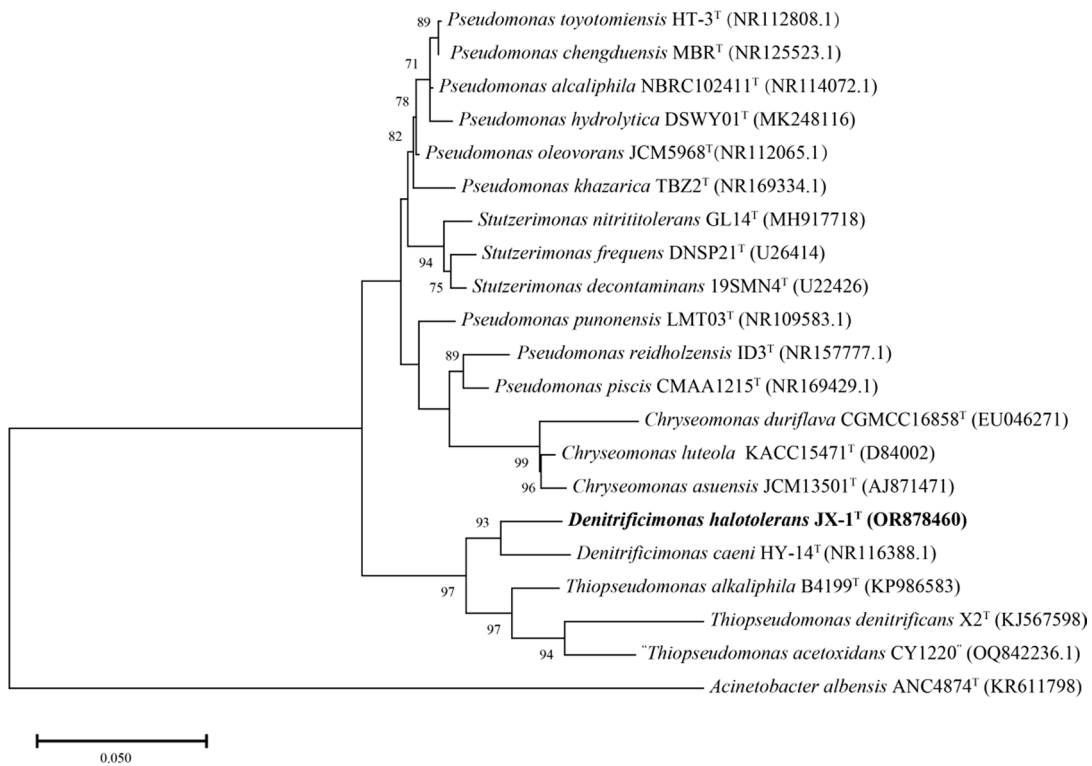


Fig. 2 Phylogenetic tree reconstructed using the maximum likelihood method based on the 16S rRNA gene sequences of strain JX-1^T and reference strains. The strain *Acinetobacter albensis* ANC 4874^T (KR611798) was used as an outgroup. Numbers at branch nodes represent confidence levels (val-

ues ≥ 70% are shown) based on 1000 replicate bootstrap samplings. GenBank accession numbers are given in parentheses. Bar, 0.05, represents number of substitutions per nucleotide site

Table 2 Comparison of the genome of strain JX-1^T with those of its closely related species

Species	16S rRNA gene	Size(bp)	G + C	dDDH	ANI	AAI
Strain JX-1 ^T	100%	2,778,815	46.12 mol%	100%	100%	100%
<i>D. caeni</i> HY-14 ^T	96.79%	3,022,325	48.26 mol%	20.30%	76.06%	78.89%
<i>alkaliphila</i> B4199 ^T	95.63%	2,494,031	47.36 mol%	21.90%	71.20%	67.59%
<i>denitrificans</i> X2 ^T	93.36%	2,841,088	58.98 mol%	19.40%	69.03%	66.77%
" <i>T. acetoxidans</i> CY1220"	95.18%	2,536,766	48.60 mol%	22.40%	71.56%	68.72%

(60% < AAI < 90%) were all below the recommended threshold for the species and above the recommended threshold for the genus (Kim et al. 2014; Richter and Rosselló-Móra et al. 2009; Meier-Kolthoff et al. 2013; Rodriguez-R and Konstantinidis 2014).

Based on the above comprehensive analysis, strain JX-1^T did not belong to a new genus, but rather represented a novel species and had the closest genetic relationship to *D. caeni* HY-14^T. Thus, strain JX-1^T

represented a novel species within the genus *Denitrificimonas* of the family *Pseudomonadaceae*. We proposed that it should be named *Denitrificimonas halotolerans* JX-1^T sp. nov.

Genome annotation and analysis

The draft genome of strain JX-1^T comprised 62 contigs (N50 = 132,445 bp; L50 = 7), encompassing a total genome length of 2,778,815 bp and a sequencing

depth of 100x, with a G+C content of 46.12 mol%. The genome contained a total of 2761 genes and 2710 coding sequences, as well as 44 tRNAs, 3 rRNAs, 4 ncRNAs, and one each of the 5S, 16S, and 23S (Table S10, Fig. S7). The genome was estimated at 87.06% completeness and 1.09% contamination. The 16S rRNA gene sequence (OR878460) obtained from the genomic data showed 100% identity with the sequence obtained through PCR amplification, confirming the authenticity of the final genome assembly (Chun et al. 2018). The genomic comparison between the genomes of strain JX-1^T and other strains is depicted in Fig. S8, showing that certain distinctions were observed in the genome of strain JX-1^T compared to other bacterial strains. This result also suggests that strain JX-1 may be a potential new species.

The results of the comparative pan-genomic analysis between strain JX-1^T and its four closely related species were presented in a Venn diagram using OrthoVenn3 (Fig. S9). Strain JX-1^T consisted of a total of 2089 gene clusters, while the other four bacteria demonstrated a total of 2267, 1829, 2109, and 2032 gene clusters, respectively. It was observed that strain JX-1^T and *D. caeni* HY-14^T had the highest degree of cluster similarity, sharing a total of 1899 gene clusters, surpassing the 1673, 1700, and 1651 gene clusters shared between strain JX-1^T and the other four strains, respectively. Analysis of the five bacteria shows a “core” genome consisting of 1375 clusters of orthologous genes, most of which encode proteins with functions associated with cellular metabolism, motility, colonization and specialized membrane exchange systems. We identified 41 gene clusters unique to strain JX-1^T, whereas its closest relatives, *D. caeni* Y-14^T and *T. alkaliphila* B4199^T, contained 19 and 18 gene clusters, respectively. In the pairwise orthology analysis heatmap of the five genomes, strain JX-1^T and *D. caeni* HY-14^T display the deepest coloration, indicating a close relationship (Fig. S10). Through the above analysis, strain JX-1^T and *D. caeni* HY-14^T were more closely related than the other four strains.

The genome sequences of the five strains were annotated for analysis using the RAST pipeline and the results are presented in Table S11. The strain JX-1^T had a total of 1171 subsystem features. The number of subsystem features for each of the five bacteria was approximately 1100, with little significant variation overall. The number of subsystem features

for carbohydrate and aromatic metabolism in strain JX-1^T was found to be significantly higher than in the other bacterial strains. The comparative analysis will focus primarily on nitrogen metabolism, sulfur metabolism, and phosphorus metabolism within the subsystem features.

Using the GhostKOALA tool for annotated functional gene analysis of nitrogen metabolism in five strains, we discovered differences in their nitrogen metabolic pathways (Fig. S11). Strains JX-1^T and *T. alkaliphila* B4199^T both lacked genes associated with denitrification and dissimilatory nitrate reduction to ammonium, but possessed only the gene for ammonia assimilation. Conversely, *D. caeni* HY-14^T, “*T. acetoxidans* CY1220” and *T. denitrificans* X2^T all possessed a plethora of genes associated with denitrification, with *T. denitrificans* X2^T having the highest abundance. Only *D. caeni* HY-14^T had genes associated with dissimilatory nitrate reduction to ammonium.

The analysis of sulfur metabolism (Table S11) showed that the number of genes related to sulfur metabolism was limited in *D. caeni* HY-14^T and *T. alkaliphila* B4199^T, whereas strain JX-1^T, *T. denitrificans* X2^T, and “*T. acetoxidans* CY1220” had a higher abundance of such genes. Analysis of phosphorus metabolism revealed that the five strains had little difference in phosphorus metabolism and shared 17 genes related to phosphorus metabolism (Table S12). Strain JX-1^T had three unique genes: the 2-aminoethyl phosphonate ABC transporter ATP-binding protein (TC3.A.1.9.1), the 2-aminoethyl phosphonate: pyruvate aminotransferase (EC 2.6.1.37), and the phosphonoacetaldehyde hydrolase (EC 3.11.1.1). These genes were not present in other strains, as shown in Table S12.

We also identified genes related to salt and alkali tolerance in the gene annotation results of strain JX-1^T, including the Na⁺/H⁺ antiporters NhaD and NhaB, and a Na⁺-driven multidrug efflux pump. The results were also consistent with previous physiological experiments examining the bacterium’s tolerance to salt and alkali conditions.

Analysis of the genome sequences of strain JX-1^T and four additional strains via the dbCAN3 meta-server, revealing strain JX-1^T harbors a total of 91 genes encoding different CAZymes. These include 6 genes for auxiliary activities (AA), 4 genes for carbohydrate-binding module (CBM), 3 genes

for carbohydrate esterase (CE), 32 genes for glycosyl hydrolases (GH), and 46 genes for glycosyl transferases (GT) (Table S13). In addition, 11 signal peptides were identified. When comparing the CAZymes in strain JX-1^T and other bacterial strains, it was observed that despite minor differences in the total number of CAZymes among these five bacterial strains, GTs and GHs consistently predominate, while CE, CBM, and AA classes are less abundant (Table S13).

The capacity of strain JX-1^T to produce secondary metabolites was assessed using antiSMASH software, revealing three potential gene clusters that could be responsible for the synthesis of ectoine, a RiPP-like compound, and a redox cofactor related metabolite (Table S14). Comparing the secondary metabolite-producing gene clusters among five strains, the ectoine, RiPP-like, and redox cofactor gene clusters were present in all strains except *T. alkaliphila* B4199^T (lacking ectoine), "*T. acetoxidans* CY1220" (lacking redox cofactor). Among them, ectoine had the highest similarity at 66% and 75%. Ectoine, a ubiquitous compatible solute in halophilic and halotolerant bacteria, serves as a critical osmoprotectant enabling these organisms to withstand saline environments (Hu et al 2024; Feng et al 2024). Of the five strains studied, all demonstrated salt tolerance and, with the exception of *T. alkaliphila* B4199^T, were highly likely to produce ectoine through their secondary metabolic pathways. This finding supports the idea that genes responsible for ectoine biosynthesis are indeed commonly found in bacterial species adapted to high salinity conditions.

Conclusions

The obtained results, including phylogenetic analysis using 16S rRNA and VBCG genes, phylogenomic tree construction based on protein-coding gene sequences, genome sequence relatedness and annotations, respiratory quinone and fatty acid methyl ester analysis, and comprehensive phenotypic characterizations, collectively demonstrate that the strain analyzed in this study represents a novel species within the genus *Denitrificimonas*. Furthermore, several genes within the bacterium were identified that are linked to the bacterium's significant tolerance to high salt concentrations. This discovery is consistent

with the observed physiological traits. Therefore, we propose the name *Denitrificimonas halotolerans* sp. nov. Although the bacterium belongs to the genus *Denitrificimonas*, strain JX-1^T was found to lack the genes associated with denitrification and dissimilatory nitrate reduction to ammonium (DNRA) compared to its closest relative *D. caeni* HY-14^T. However, it has a greater number of genes related to sulfur metabolism. The strain JX-1^T harbours a total of 2089 gene clusters and shares 1899 of these clusters with its closest relative *D. caeni* HY-14^T. It possesses 32 genes for GH and 46 genes for GT, along with 11 signal peptides. Moreover, it also demonstrates potential to produce 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 salinity, alkalinity, and ammonia nitrogen remain elusive.

Description of *Denitrificimonas halotolerans* sp. nov.

Denitrificimonas halotolerans sp. nov.

(ha.lo.to'le.rans. Gr. gen. masc. n. *halos*, salt; L. pres. part. *tolerans*, tolerating, enduring; N.L. part. adj. *halotolerans*, salt-tolerating).

Cells are Gram stain negative, facultatively anaerobic and motile, rod-shaped by electron microscopy, and a flagellum is observed. Cell size is about 0.9–1.0 μm wide and 2–3.3 μm long with single, paired or stacked rod cells. Colonies are slightly yellow, circular with regular edges, smooth, shiny, convex and reach a diameter of about 1.5 mm after 24 h of cultivation on LB at 28 °C. Optimal growth conditions for strain JX-1^T are 20–30 °C (4–42 °C), pH 7.0–8.0 (6.0–11.0), 5% (w/v) NaCl (0.0–8.0%), and ammonia nitrogen concentration of 500 mg/L (200–5000 mg/L).

Positive reactions are observed for Tween 20, 60, 80, catalase, cytochrome c oxidase, while negative reactions are observed for casein and gelatin. The API ZYM kit gives positive reactions for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, while cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphatase give

weak reactions. Negative results are obtained with trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The API 20NE kit gives positive reactions for aesculin hydrolysis, malic acid and weak reactions for D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium gluconate, adipic acid, phenylacetic acid. Negative reactions are 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 show weak reactions for mannitol, rhamnose, amygdalin, and negative reactions for β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase, indole production, acetoin production (Voges Proskauer), gelatinase, glucose, inositol, sorbitol, saccharose, arabinose. The Biolog GEN III MicroPlate test results are positive for 1% (w/v) NaCl, 1% (w/v) sodium lactate, rifamycin SV, guanidine HCl, L-malic acid, lithium chloride. It shows weak reaction for pH 6, N-acetyl-D-galactosamine, 4% NaCl, 8% (w/v) NaCl, D-galactose, L-rhamnose, inosine, 1% sodium lactate, D-serine, D-glucose-6-PO₄, 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-acetylneuraminic acid, α -D-glucose, D-mannose, D-fructose, 3-methylglucose, D-fucose, L-fucose, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-fructose-6-PO₄, D-aspartic acid, D-serine, troleandomycin, minocycline, gelatin, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid, serine, pectin, mucic acid, D-saccharic acid, p-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.

The respiratory quinones of strain JX-1^T are predominantly Q9 with a minor presence of Q8. The major fatty acids are summed feature 8 (C_{18:1}ω7c/C_{18:1}ω6c), iso-C_{15:0}, and anteiso-C_{15:0}. Major polar lipids include aminolipid, aminophospholipid, diphosphatidylglycerol, glycolipid, phosphatidylethanolamine, phosphatidylglycerol, and phospholipid. The genome size is 2.78 Mb, with a G+C content of 46.12 mol %. It includes 2761 genes (2710 coding), 44 tRNAs, 3 rRNAs, 4 ncRNAs, and also has 1 gene each for 5S rRNA, 16S rRNA, and 23S rRNA. The organism was found to possess several genes linked with tolerance to high salinity, alkaline conditions, and elevated ammonia nitrogen levels. It also demonstrates potential of producing secondary metabolites including ectoine, RiPP-like compounds, and redox cofactors. The type strain, JX-1^T (=MCCC 1K08958^T=KCTC 8395^T), was isolated from UASB sludge treating landfill leachate in Wuhan, China. The 16S rRNA gene sequence of strain JX-1^T has the accession number OR878460. The accession number of the assembly genome is JAXIVU000000000.

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Author contributions DRQ designed the research. CY, CBX, DTL, and HZ carried out the isolation, storage and polyphasic taxonomy. SHW, YRQ, and QL conducted the growth experiment on nitrogen substrates. SHW, JD, and LY performed the genome analysis. SHW, JW, and XW drafted the manuscript. All authors read and approved the final version of the manuscript.

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Data availability The 16S rRNA gene sequence obtained in this study has been assigned GenBank accession numbers, including OR878460 for strain JX-1^T. The whole genome shotgun projects have been deposited as JAXIVU 000000000 for strain JX-1^T.

Declarations

Competing interests The authors declare no competing interests.

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