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Enrichment and identification of a moderately acidophilic nitrite-oxidizing bacterium

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ABSTRACT

This study enriched a novel nitrite-oxidizing bacterium (NOB, '*Candidatus* Nitrobacter acidophilus') in a laboratory reactor operating at pH 4.5 for treating low-strength ammonia wastewater. Batch experiments showed that '*Ca.* N. acidophilus' oxidized nitrite to nitrate at a rate of 20.7 ± 2.3 μM/h with optimal growth at pH 5, distinguishing it from most previously known NOB strains. Phylogenetic analysis showed that this *Nitrobacter* strain clustered with other *Nitrobacter* strains obtained from acidic environments but was divergent from each other with an average nucleotide identity (ANI) below 85 %. Genomic characteristics revealed that '*Ca.* N. acidophilus' possesses versatile transporter systems. They are different from previously reported *Nitrobacter* strains and indicate acid adaptation mechanisms. Interestingly, the mutualistic interaction with acidophilic ammonia-oxidizing archaea (AOA) *Nitrosotalea* markedly increased the archaeal *amo*A gene expression by 149 times and enhanced ammonia oxidation rates by 5 times, highlighting the NOB's role in alleviating nitrite inhibition on the acidophilic AOA. These findings expand our understanding of bacterial nitrite oxidation and provide valuable insights into an important partnership between acidophilic AOA and NOB in acidic environments.

1. Introduction

Nitrification is a central process in the global nitrogen cycle, as it links ammonia oxidation to nitrate formation, facilitating nitrogen transformation, utilization and removal in natural and engineered ecosystems (Liu et al., 2024). It comprises two major steps, the oxidation of ammonia to nitrite and then to nitrate. Nitrification can be catalyzed by a group of chemoautotrophic microorganisms, including ammonia-oxidizing bacteria (AOB) and archaea (AOA), nitrite-oxidizing bacteria (NOB), and complete nitrifiers (comammox) (Lehtovirta-Morley, 2018; Koch et al., 2019; Su et al., 2023). Nitrification in acidic environments (pH < 5.5) remains elusive (Date et al., 2012), and recent studies have found that nitrification rates in acidic environments are comparable or even higher than those in neutral counterparts (Booth et al., 2005). More recently, a few acidophilic nitrifiers have been identified (Hayatsu et al. 2017; Takahashi et al., 2020; Lehtovirta-Morley et al., 2011). These findings support an overlooked role of nitrification in nitrogen cycling in acidic environments (Li et al., 2018).

For a long time, ammonia oxidation has been considered the rate-limiting step in acidic environments because substrate ammonia is not easily available at low pH (Daims et al., 2016). Extensive studies on acidophilic ammonia oxidation have been conducted (Hayatsu et al. 2017; Lehtovirta-Morley et al., 2011, 2014, 2016; Picone et al., 2021; Wang et al., 2021). Although both AOA and AOB show the capability of performing ammonia oxidation in acidic condition, a recent study found that AOA likely play a more important role than AOB in acidic soils (Zhang et al., 2012). Recently, increasing studies have identified novel acidophilic AOA. For instance, AOA belonging to the class *Nitrososphaeria* in the phylum *Thermoproteota* are widely distributed in a diverse range of ecosystems. Within *Thermoproteota*, members associated with the *Nitrosotalea* genus have the unique ability to optimally

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grow at an acidic pH value of 4.5 (Lehtovirta-Morley et al., 2011, 2014). Indeed, this acidophilic AOA group can functionally predominate among ammonia-oxidizing prokaryotes in acidic soils (Gubry-Rangin et al., 2011). The acidophilic AOA were cultivated and characterized, and the results have repeatedly shown that *Nitrosotalea* is sensitive to nitrite (Lehtovirta-Morley et al., 2011, 2014). For instance, the ammonia oxidation activity of *Nitrosotalea devanaterra* was significantly inhibited by nitrite above 10 μM (Lehtovirta-Morley et al., 2011). This indicates that a mutualist partner that can remove the AOA-produced nitrite at low pH should be vitally important to relieve such nitrite inhibition. Nevertheless, in comparison to extensive studies on acidophilic ammonia oxidation (Hayatsu et al. 2017; Lehtovirta-Morley et al., 2011, 2014, 2016; Picone et al., 2021; Wang et al., 2021), NOB function in acidic environments remain poorly understood (Su et al., 2023; Hankinson and Schmidt, 1988). Acidophilic NOB are essential for mitigating nitrite toxicity and completing nitrification, yet their diversity, physiological adaptations, and ecological roles are underexplored. Based on the aforementioned, we hypothesise the presence of acidophilic NOB, which can form a mutualistic relationship with AOA in acidic environments. To test this hypothesis, an enrichment bioreactor system was used to cultivate NOB in acidic conditions. A series of experiments were conducted to evaluate its physiological relationship with AOA, linking functional validation with metagenomic findings in the co-culture.

In this work, a novel NOB strain was enriched in an acidophilic AOA *Nitrosotalea*-dominated culture, along with an exponentially increased ammonia oxidation rate. A near-complete genome of this novel NOB strain was recovered, phylogenetically close to canonical *Nitrobacter*. The genome of this NOB strain has a considerable amount of genes coding for ion transporters, suggesting the potential ability to adapt to acidic environments. Moreover, this NOB strain performs acidophilic nitrite oxidation to nitrate but is susceptible to nitrite accumulation in

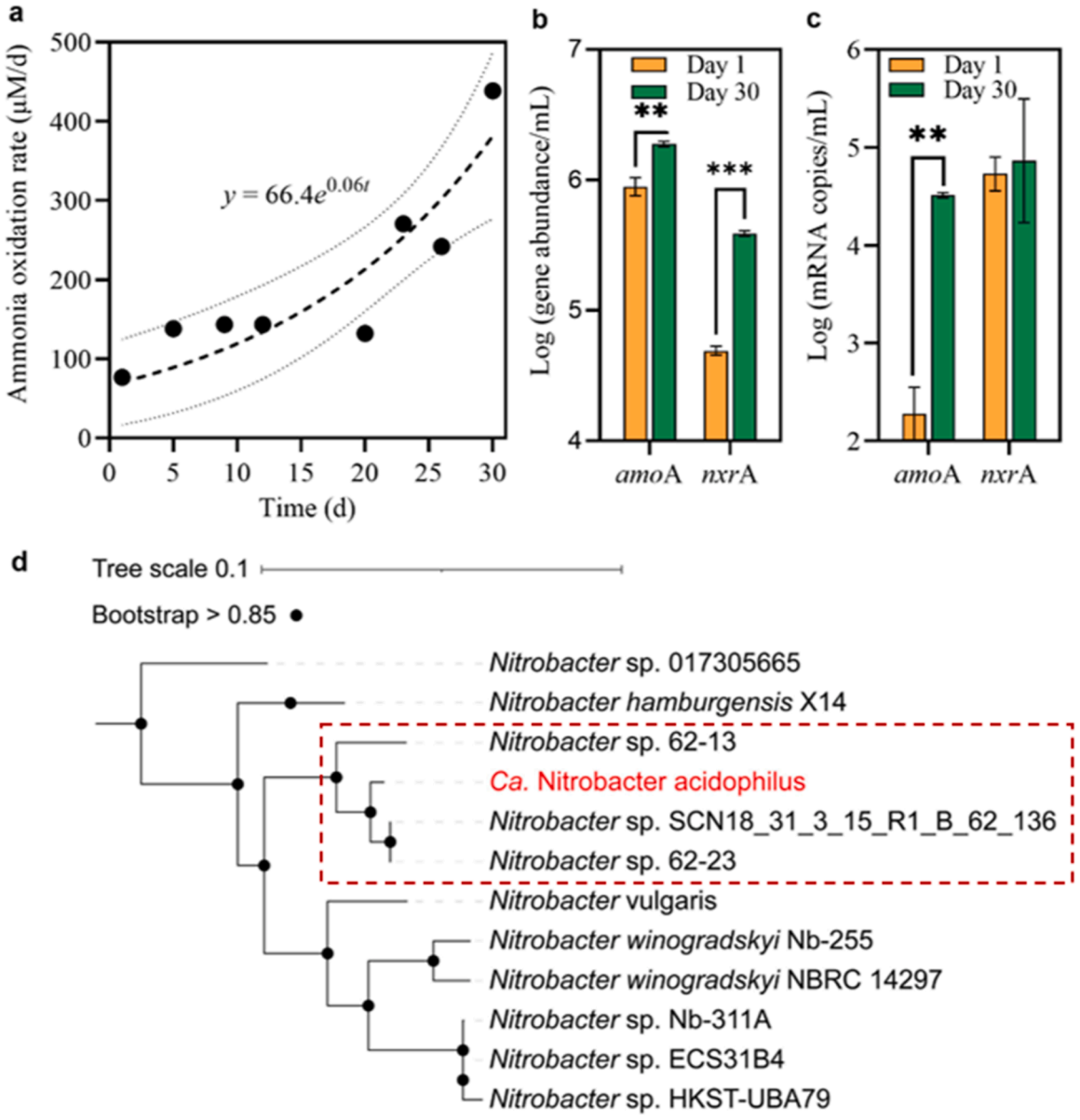

Fig. 1. (a) Profile of the obtained ammonia oxidation rate in the acidic bioreactor with the data fit by the exponential growth model. Two grey dash lines represent a 95 % confident region of model fitting. (b) qPCR and (c) RT-qPCR results for the archaeal *amo*A and *Nitrobacter nxr*A genes: $^{**}p < 0.01$ and $^{***}p < 0.001$. Note: a mismatch was identified close to the 3′ end of the reverse primer in *nxr*A genes from the previous study revealing the diversity of *Nitrobacter* (Poly et al., 2008), which may lead to lower binding and amplification efficiency. (d) Phylogenetic relationship between '*Candidatus* Nitrobacter acidophilus' and other publicly available genomes from the *Nitrobacteraceae* family based on 120 bacterial marker genes. The dark circles indicate bootstrap values > 0.85.

the culture. Based on these features, we designated this new NOB strain as '*Candidatus* Nitrobacter acidophilus' representing its capability to perform nitrite oxidation in acidic environments.

2. Results and discussion

2.1. Enhanced acidophilic archaeal ammonia oxidation in the presence of bacterial nitrite oxidation

With inoculation of AOA *Nitrosotalea* and NOB *Nitrospira*-dominating co-culture, the laboratory-scale MBR initially performed stable conversion of ammonium to nitrate at an acidic pH of 4.5. Nitrite concentration in the bioreactor was always under the detection limit (i.e., $\leq$ 14 μM) and ammonium was fully converted to nitrate (Figure S1). It was serendipitously observed that the ammonia oxidation rate exponentially increased and became surprisingly 5 times higher within a month of operation (Fig. 1a). To verify what nitrifying microorganisms were in the biomass, metagenomic sequencing was applied to two subsamples collected in this period to examine functional *amo*A and *nxr*A genes. The results revealed the dominance of *Nitrosotalea*-like *amo*A, and surprisingly, *Nitrobacter*-like *nxr*A genes (Figure S2), both accounting for > 99 % of total known *amo*A and *nxr*A sequences. The quantitative polymerase chain reaction (qPCR) analysis showed that archaeal *amo*A and *Nitrobacter nxr*A genes concurrently increased in one month, by 1.1 and 6.9 times, respectively (Fig. 1b). Assessed by reverse transcription-qPCR (RT-qPCR), the archaeal *amo*A gene expression increased by 149 times (Fig. 1c), confirming the dramatically enhanced activity of *Nitrosotalea*. Moreover, the absolute abundance of the archaeal *amo*A gene in the present work was quantified to be 6.1 ± 0.2 × 10^6 copies per mL-biomass (n = 6). Although the gene abundance in this work is close to that in a previous culture of *Nitrosotalea devanaterra* (Lehtovirta-Morley et al., 2011), the observed ammonia oxidation rate is about five times higher. This high archaeal ammonia oxidation rate was likely attributed to the co-cultured *Nitrobacter*, acting as a nitrite scavenger, which will be further discussed below.

2.2. Genome recovery of the new NOB strain

Assembly and binning of metagenomic sequences were used to retrieve high-quality metagenomic assembled genomes (MAGs) (> 90 % completeness and < 5 % contamination), which consisted of a *Nitrosotalea* MAG and a *Nitrobacter* MAG as the only identifiable nitrifiers, suggesting that these two strains played vital roles in the acidic nitrification observed. Phylogenetic analysis of 122 single-copy proteins placed the *Nitrosotalea* MAG within the *Thaumarchaeal* 1.1a-associated group (Pu, 2020). The *Nitrobacter* MAG was identified as a novel species because the average nucleotide identity (ANI) to known *Nitrobacter* ranges between 83.4 %−86.2 % and average amino acid identity (AAI) ranges between 81.5 %−83.6 %, below the cut-off of 95 % for species delimitation (Jain et al., 2018; Goris et al., 2007). As such, we designated this new NOB strain as '*Ca.* N. acidophilus', representing its capability to perform aerobic nitrite oxidation in acidic environments.

The genome of '*Ca.* N. acidophilus' has a GC content of 62.26 % and a size of 3.96 Mb, comparable to other *Nitrobacter* species (Table 1). '*Ca.* N. acidophilus' encodes *nxr*AB for nitrite oxidation. It also employs a complete Calvin-Benson-Bassham (CBB) cycle genes cluster for carbon fixation, harbouring type I RuBisCO (*cbb*L, *cbb*S) and an aerobic carbon-monoxide dehydrogenase (*cox*SML). Although multiple copies of *nxr*A and *nxr*B were contained, only one of the *nxr*AB central clusters encodes the putative accessory proteins, similar to *Nitrobacter hamburgensis* (Starkenburg et al., 2008). A single gamma subunit of nitrate reductase or *nar*I/*nar*V-like protein was found, suggesting that '*Ca.* N. acidophilus' may also catalyze the reverse reaction of nitrite oxidoreductase, i.e., reducing nitrate to nitrite. Such anaerobic growth relying on dissimilatory nitrate reduction was reported for some *Nitrobacter* species (Freitag and Bock, 1990). Genes for assimilatory nitrate reduction (*nas*AB) were not detected, which aligns with prior studies on *Nitrobacter* (Starkenburg et al., 2006). Also, '*Ca.* N. acidophilus' does not contain genes encoding assimilatory nitrite reductase (*nir*BD) for ammonia production from nitrite.

A new cluster was formed within the *Nitrobacteraceae* family, containing '*Ca.* N. acidophilus' and other three MAGs retrieved from tailings effluent with thiocyanate and ammonium sulfate (Fig. 1d) (Kantor et al., 2015; Huddy et al., 2021). The four MAGs (as a new cluster in the tree) share common accessory proteins with the genomes of five neutrophilic *Nitrobacter*. In total, 33,353 coding sequences were identified from the nine *Nitrobacter* genomes and could be further grouped into 8664 protein clusters. Pangenome analysis revealed that 1879 out of 8664 protein clusters constituted the core genome of *Nitrobacter*, among which 265 accessory protein clusters (220 identified CDS) were exclusively shared by the new cluster. Most of these shared accessory protein clusters were related to enzymes and transporters, suggesting potential acidophilic tolerance mechanisms for acidophiles (Table 1). For example, different from neutrophilic *Nitrobacter* (Starkenburg et al., 2008), '*Ca.* N. acidophilus' genome contains a considerable amount of genes coding for transporters, with a total of 179 open reading frames (ORFs), and 12.4 % of all orthologs could play a role in transport (Fig. 2). The encoded transporters are very versatile, including Na^+/H^+ antiporter (*mnh*), Ca^{2+}/H^+ antiporter (*cha*A/CAX), cation/H^+ antiporter (*yrb*G), monovalent cation/H^+ antiporter (*nha*K), K^+/H^+ antiporter (*cvr*A/*nha*P2), ATP binding cassette transporters (ABC) for Fe^{2+}, NH_4^+, Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, SO_4^{2-}, PO_4^{3-}, and a K^+-stimulated pyrophosphate-energized sodium pump (*hpp*A) that can maintain transport activity over a wide range of H^+ and Na^+ concentrations. These transporters were expected to facilitate the exchange of positive ions with protons, helping '*Ca.* N. acidophilus' maintain ion balance and resist ion flux (Baykov et al., 2013; Krulwich et al., 2011; Kanjee and Houry, 2013) and enabling cellular integrity and function under acidic pH. Although the functional activity of these transporters was not directly validated, their presence aligns with the organism's ability to

Table 1
Genome characteristics of Candidatus 'N. *acidophilus*' in comparison with known *Nitrobacter*.

Parameters	'*Candidatus* N. acidophilus'	N. *winogradskyi* Nb-255	N. *hamburgensis* X14	N. sp. Nb-311A	N. *vulgaris* strain Ab1	N. *winogradskyi* strain NBRC 14,297
Size (bp)	3962,137	3402,093	4406,967	4104,352	3900,476	3960,943
GC content	62.26	62.05	61.71	60.37	59.83	61.3
Scaffold N50 (bp)	104,710	3402,093	4406,967	235,022	130,999	48,653
Number of scaffolds	74	1	1	44	95	172
Number of coding sequences	3833	3143	4113	4256	3901	3715
Number of tRNAs	52	49	55	53	51	ND
Completeness (CheckM)	97.97	99.8	99.89	98.87	99.63	99.58
Redundancy (CheckM)	0.20	0	0.49	0	0.34	0.39
Copies of *nxr*A / *nxr*B	3 / 2	2 / 2	3 / 2	2 / 3	2 / 3	6 / 1
Reference	This study	Starkenburg et al. (2006)	Starkenburg et al. (2008)	GOLD collection	Mellbye et al. (2017)	NBRC collection

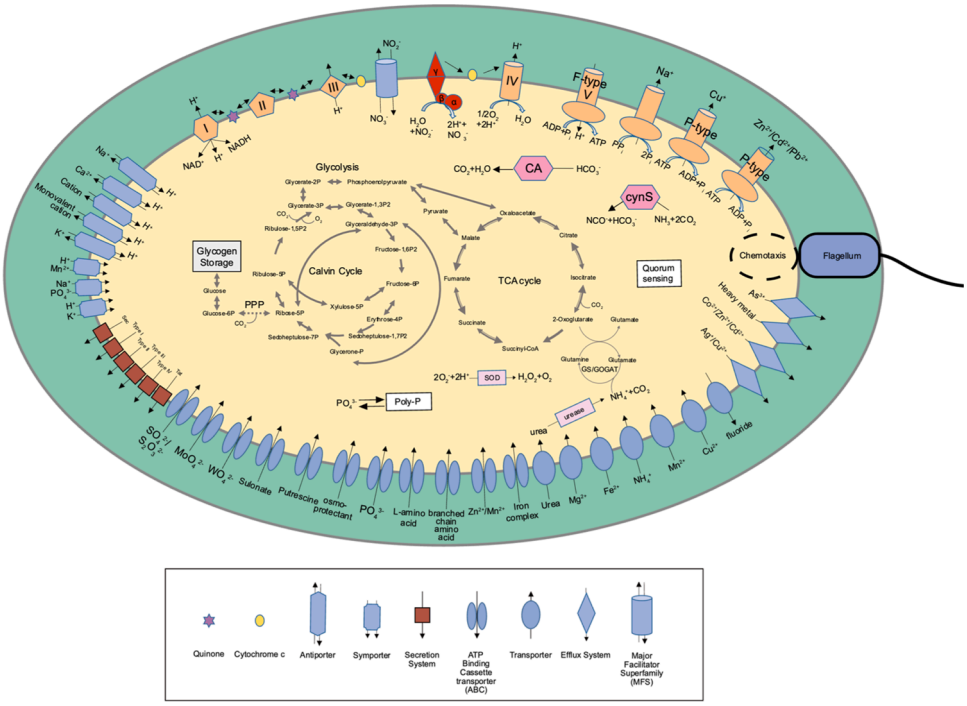

Fig. 2. Schematic prediction on metabolic features of '*Candidatus* Nitrobacter acidophilus'. The light-yellow area represents cytoplasm, and the green area represents periplasm. NXR: nitrite oxidoreductase, CA: carbonic anhydrase, cynS: cyanate lyase, SOD: superoxide dismutase. Roman numerals label enzyme complexes of the electron transport chain.

thrive in acidic conditions and oxidize nitrite efficiently (see the results below).

2.3. Acidophilic oxidation of nitrite to nitrate

While metagenomic analyses provided insights into the genomic potential of the enriched culture, functional validation was performed to confirm nitrite oxidation activity through batch experiments and gene expression analyses. We cultivated '*Ca.* N. acidophilus' by providing nitrite as the sole nitrogen source. Biological conversion of nitrite to nitrate was obtained at pH 4.5 without nitrogen loss (Fig. 3a), compared to marginal chemical conversion as a control (Figure S3). Serial dilution-to-extinction was applied to obtain a highly enriched culture, as indicated by the Fluorescence *in-situ* Hybridization (FISH) analysis with Cy-3 labelled Nit 3 and FITC labelled EUB mix. Cells appeared to be coccus-shaped with aggregations under acidic conditions (Figure S4). qPCR analysis showed that the *Nitrobacter*-like *nxr*A gene copies increased significantly along with the oxidation of nitrite to nitrate in four days of batch-wise incubation (Fig. 3b). Calculation based on the exponential increase of *nxr*A gene copies versus time suggests a net growth rate of 1.5 per day (equal to a doubling time of 11 h) at pH 5 for the enrichment.

Nitrite oxidation to nitrate of the enriched culture was observed within a wide pH range from 4.5 to 8.0 (Fig. 3c). The maximum nitrite oxidation rate of 20.7 $\pm$ 2.3 μM/h was observed at acidic pH 5. At the optimal pH of 5, the nitrite oxidation rate increased when nitrite concentration increased to 110 μM and decreased at a higher nitrite concentration (Fig. 3d), showing that the present microbial nitrite oxidation was susceptible to nitrite inhibition, consistent with studies on NOB *Nitrobacter* (De Boer et al., 1991; Hankinson and Schmidt, 1988). At acidic pH, nitrite is protonated and forms free nitrous acid, an inhibitor to most known nitrifying microorganisms (Duan et al., 2020; Zheng et al., 2021). As characterized, both '*Ca.* N. acidophilus' and AOA *Nitrosotalea* were susceptible to nitrite (Lehtovirta-Morley et al., 2011, 2014), indicating a mutualistic lifestyle of the acidophilic nitrifiers in the acidic nitrifying bioreactor. Specifically, NOB '*Ca.* N. acidophilus' acts as a scavenger to remove nitrite produced by AOA *Nitrosotalea*, to protect both from the inhibition by nitrite and free nitrous acid.

Overall, metagenomic analysis identified '*Ca.* N. acidophilus' as the dominant NOB in the enrichment culture. Batch experiments demonstrated nitrite oxidation to nitrate at pH 5, which was strongly correlated with an increase in *Nitrobacter*-like *nxr*A gene copies as detected by qPCR. Phylogenetic analysis confirmed that the dominant NOB accounted for >99 % of *nxr*A sequences, minimizing the contribution of minor populations. These findings indicate that '*Ca.* N. acidophilus' is responsible for the observed nitrite oxidation activity, supporting its role as the primary nitrite oxidizer in the enrichment culture.

2.4. Functional interdependence between acidophilic AOA and NOB in acidic environments

In this work, the biochemical functions of the enriched NOB and AOA were validated through wet-lab experiments. Batch experiments confirmed nitrite oxidation by the enriched NOB at pH 4.5, while reactor operation showed a significant increase in ammonia oxidation rates in the presence of NOB, highlighting their mutualistic interaction. The concurrent exponential increase in archaeal *amo*A and *Nitrobacter*-like *nxr*A gene copies further supported the functional roles of AOA and NOB in the nitrification process. While metagenomics provided insights into potential adaptations, the observed physiological activities validate the biochemical functions of the enriched microorganisms under acidic conditions.

We obtained a novel nitrite oxidizer '*Ca.* N. acidophilus' that was partnered with AOA *Nitrosotalea* in a bioreactor operating at pH 4.5. Along with the enrichment of this NOB, the activity of AOA *Nitrosotalea* dramatically increased. The enrichment of AOA (Nitrosotalea) and their high ammonium oxidation rates in acidic environments can be attributed to several factors. First, the lack of competition from acid-sensitive AOB allows AOA to dominate ammonia oxidation. Second, Nitrosotalea species exhibit physiological adaptations, such as acid-tolerant enzymes and efficient proton transport mechanisms, enabling their activity at low pH. Finally, the presence of '*Ca.* N. acidophilus' alleviates nitrite accumulation, reducing nitrite stress on AOA and allowing for exponential

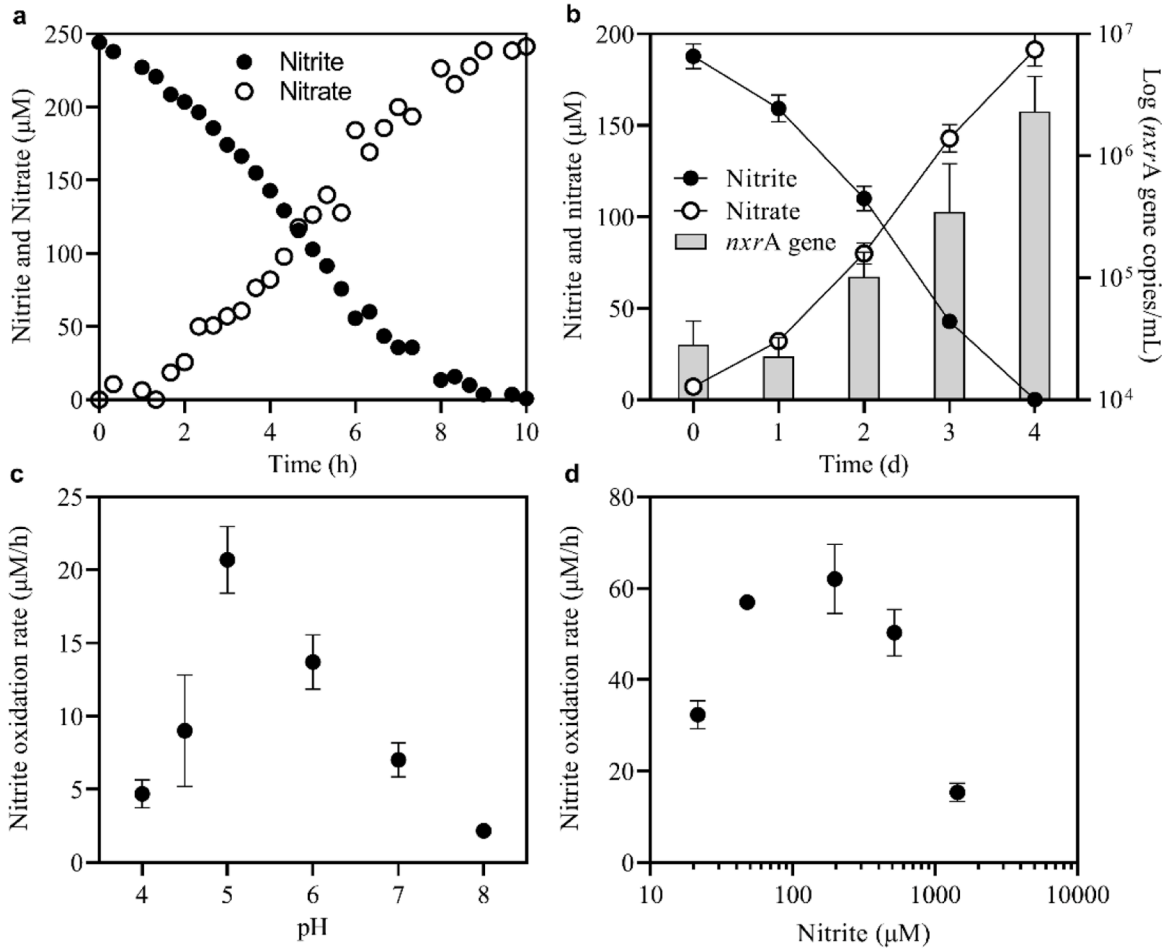

Fig. 3. (a) Biological nitrite oxidation to nitrate at pH 4.5. (b) Nitrite oxidation to nitrate with the increase in *nxr*A gene copies in a flask test at pH 5. (c) Nitrite oxidation and nitrate production rates measured for the enriched culture using batch tests under different pH conditions. (d) Measured nitrite oxidation rates of the enrichment with elevated nitrite concentrations. Results (a-d) are obtained from Experiments (1–4), respectively, as detailed in SI.

increases in ammonia oxidation rates. These findings highlight the ecological importance of AOA-NOB interactions in acidic environments. With the characterization, the optimal pH for '*Ca*. N. acidophilus' was around 5 and a nitrite concentration above 110 μM significantly inhibited its activity. These results indicated an important and necessary role for this NOB in the elimination of toxic nitrite in the acidic bioreactor. In specific, '*Ca.* N. acidophilus' oxidizes nitrite to less inhibitory nitrate, creating a conducive environment for *Nitrosotalea*. In turn, the ammonia oxidation activity of *Nitrosotalea* provides a steady supply of nitrite, enabling the proliferation and activity of NOB. This interaction highlights a functional interdependence between acidophilic NOB and AOA in acidic environments. This tightly coupled partnership exemplifies a mutualistic ecological relationship essential for sustaining nitrification in acidic environments where conventional nitrifiers are ineffective. Future studies incorporating molecular biological techniques and multi-omics will help elucidate the underlying mechanisms.

The implications of this partnership extend beyond bioreactor systems to broader environmental contexts, such as in acidic soils. Increased soil acidity is occurring globally, which usually relates to human activities with extensive use of nitrogenous fertilizers. If fertilization rates are over plant requirements, nitrate can leach away from the root zone leaving behind hydrogen ions. In such environments, studies have shown that acidophilic AOA *Nitrosotalea* members are globally distributed ammonia oxidizers in acidic soils (He et al., 2012), albeit at the cost of potentially exacerbating soil acidification through continuous nitrification processes. This study reports that the partnership between acidophilic AOA such as *Nitrosotalea* and NOB like '*Ca.* N. acidophilus' represents a crucial ecological relationship in acidic environments. These microorganisms cooperate to drive nitrification under conditions where most known nitrifiers are inhibited, forming a mutualistic alliance essential for maintaining nitrogen cycling in acidic natural environments and bioreactors. These partnerships likely influence the structure and functionality of microbial communities in acidified soils, contributing to nutrient cycling while potentially driving further soil acidification (Booth et al., 2005). Moreover, the mutualistic relationship between acidophilic AOA (Nitrosotalea) and NOB ('*Ca.* N. acidophilus') is likely shaped by environmental factors such as pH, nitrite accumulation, and substrate availability. In acidic environments with limited nitrite-oxidizing competitors, '*Ca.* N. acidophilus' dominates. However, in more diverse communities, other NOB genera or comammox bacteria could compete, potentially altering the partnership. Similarly, the presence of AOB may influence ammonia oxidation dynamics, depending on their tolerance to acidic conditions. Long-term studies under varying environmental conditions are needed to understand how this partnership evolves and adapts to ecological pressures. Nevertheless, the mutualistic relationship between acidophilic AOA and NOB offers a model for understanding how microorganisms adapt to and thrive in extreme conditions, potentially uncovering new pathways for biogeochemical cycling. Further research into their metabolic strategies, ecological roles, and long-term impacts on soil acidification could provide insights into managing nitrogen use in agriculture and mitigating the adverse effects of soil acidification.

After obtaining a high-quality draft genome of '*Ca.* N. acidophilus', this study also identified that this NOB strain has versatile transporters

that may enable it to live in acidic environments. Such resilience of these acidophilic nitrifiers highlights their potential utility in engineered systems. For instance, acidic nitrifying bioreactors leveraging this partnership could provide a sustainable solution for treating nitrogen-rich acidic wastewater, reducing toxic nitrite accumulation while enhancing nitrogen removal efficiency. By exploring their genomic adaptations and metabolic strategies, future research could unlock new approaches to enhance nitrogen cycling and address the environmental challenges associated with acidification.

3. Conclusions

This study identified '*Ca.* N. acidophilus' as a novel NOB capable of thriving in acidic conditions (optimal pH 5). Genomic analysis revealed acid adaptation mechanisms, while co-culture experiments demonstrated its role in alleviating nitrite inhibition for AOA, enhancing ammonia oxidation rates by 5 times. These findings provide insights into nitrification in acidic environments and highlight the potential of biotechnological applications in wastewater treatment.

4. Methods and materials

4.1. Operation of an acidic nitrifying bioreactor

A laboratory-scale membrane bioreactor (MBR) with a working volume of 2 L was inoculated with a nitrifying culture, which was sourced from a freshwater reservoir sediment (Pu, 2020). After long-term cultivation, the biomass was dominated by AOA *Nitrosotalea* and NOB *Nitrospira*, with abundances of 51.2 % and 3.1 % of the microbial community by 16S rRNA gene amplicon sequencing, respectively (Pu, 2020). The bioreactor was placed in the dark, operated at 22 ± 1 °C, and fed with ammonium of 13.5 ± 1.5 mg NH_4^+-N/L (as NH_4Cl) in continuous flow mode. The medium also consisted of (per liter) KH_2PO_4, 0.075 g, $MgCl_2{\cdot}7H_2O$, 0.2 g, $CaCl_2{\cdot}2H_2O$, 0.3 g, 0.2 mL of alkaline trace element solution and 0.5 mL of acidic trace element solution (Ettwig et al., 2009). Hydraulic retention time (HRT) was initially set at 13.6 d and was gradually shortened to 1.7 d along with the continuous operation. Oxygen was supplied using an air pump, which gave a high dissolved oxygen (DO) concentration of above 6 mg O_2/L in the bioreactor. pH was measured by a pH meter (Oakton, USA), and controlled at 4.5 ± 0.1 by a programmable logic controller (PLC) system dosing 1 M HCl/1 M NaOH. Cooperation of NOB and AOA was evaluated by monitoring ammonia consumption, nitrite accumulation, and nitrate production, and the numbers of NOB and AOA using qPCR and RT-qPCR. At each sampling interval (twice per week), 3 mL of liquid sample was filtered for the analysis of ammonium, nitrite and nitrate concentrations. The qPCR and RT-qPCR were carried out with details in Supplementary Materials.

4.2. Metagenome sequencing, library preparation, and taxonomy assignment

Metagenomic sequencing data was generated on the Illumina NextSeq500 (Illumina Inc., San Diego, California USA) with the PE150 platform following the in-house pipeline of the Australian Centre for Ecogenomics. The genomic sequencing library was prepared using the Nextera XT Kit (Illumina Inc., San Diego, California USA) following the manufacturer's instructions, using 1 ng of input DNA normalised to a 0.2 ng/μL concentration. The NextSeq High Output kit (Illumina Inc., San Diego, California USA) was used for sequencing according to the manufacturer's recommendations. Paired-end Illumina reads in FASTQ format were trimmed with Cutadapt v1.8 (Martin, 2011), using a minimum phred score of 20 and a minimum length of 50 bp. All trimmed reads were *de novo* assembled by metaSPAdes v 3.13.0with kmers 21, 33, 55, 63, 77, 99 and 127 (Nurk et al., 2017). Trimmed reads were mapped to the assembled scaffolds and scaffolds were binned using MaxBin2 (V 2.2.6) (Wu et al., 2016), MetaBAT 2 (v2.12.1) (Kang et al., 2019) and CONCOCT (Alneberg et al., 2014) respectively with default parameters. Completeness and contamination of the recovered MAGs were estimated using CheckM v1.2.1 lineage workflow (Parks et al., 2015).

Taxonomy assignment was performed on MAGs using bacterial and archaeal marker genes from representatives as implemented in the Genome Taxonomy Database Toolkit v2.4.0 (GTDB-tk) (Parks et al., 2022). The results were manually checked, and 2 MAGs of nitrifiers were obtained. Gene prediction for these MAGs was carried out using Prodigal v2.6.7 (Hyatt et al., 2010). Predicted protein-coding sequences (CDSs) of these MAGs were subsequently searched against the KEGG GENES database using BlastKoala (Kanehisa et al., 2016).

4.3. Phylogenetic analysis

To reveal phylogenetic placement of the enriched NOB, a detailed selection of phylogenetic neighborhoods was used to construct a phylogenetic tree. Eleven published *Nitrobacter* genomes were retrieved from the NCBI-RefSeq database. Taxonomic assignment of the MAGs and genomes was determined using GTDB-tk as mentioned above. 120 marker genes were identified and retrieved using Prodigal v2.6.7 (Hyatt et al., 2010) and hidden Markov models (HMMs) by HMMER v3.3.2 package, and the aligned gene sets were concatenated, resulting in multiple sequence alignments (MSA) of 5037 amino acids. A maximum-likelihood tree was constructed on this collection of sequences using IQ-TREE2 (Minh et al., 2020), with the best-fit model of "LG+F + R3″ and 1000 iterations by the ultrafast bootstrap option. The phylogenetic tree was visualized with iTOL v6 (https://itol.embl.de/) (Letunic and Bork, 2024).

4.4. Cultivation and characterization experiments

100 mL biomass from the acidic nitrifying bioreactor was inoculated into a 2 L glass bottle, containing 900 mL fresh medium (the same as the medium used for bioreactor operation) with sodium nitrite (2 mg N/L). The culture was incubated at pH 4.5 ± 0.1 and at room temperature, using magnetic stirring at 200 rpm for aeration. The enrichment culture was obtained by regular serial dilution (dilute once nitrite accumulation was detected), and biomass was transferred from the 2 L glass bottle to a 250 mL flask. Nitrite consumption was measured by MQuant test stripes (Merck KGaA, Darmstadt, Germany). Once nitrite was depleted, it was replenished to 2 mg N/L. After selectively enriching the NOB culture, batch tests were conducted for the characterization as detailed in the SI.

CRediT authorship contribution statement

Jun Xia: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Zicheng Su:** Writing – original draft, Software, Investigation, Data curation. **Chen Cai:** Writing – review & editing, Methodology. **Tao Liu:** Writing – review & editing, Validation, Methodology. **Zhiguo Yuan:** Writing – review & editing. **Min Zheng:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All sequencing data were saved in the NCBI Sequence Read Archive (SRA) database under the Bioproject PRJNA1020623 (MAG Accession number: JAWDCU000000000).

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Supplementary materials

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