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Insights into the evolutionary and ecological adaption strategies of *nirS*- and *nirK*-type denitrifying communities

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Abstract

Denitrification is a crucial process in the global nitrogen cycle, in which two functionally equivalent genes, *nirS* and *nirK*, catalyse the critical reaction and are usually used as marker genes. The *nirK* gene can function independently, whereas *nirS* requires additional genes to encode nitrite reductase and is more sensitive to environmental factors than *nirK*. However, the ecological differentiation mechanisms of those denitrifying microbial communities and their adaptation strategies to environmental stresses remain unclear. Here, we conducted metagenomic analysis for sediments and bioreactor samples from Lake Donghu, China. We found that *nirS*-type denitrifying communities had a significantly lower horizontal gene transfer frequency than that of *nirK*-type denitrifying communities, and *nirS* gene phylogeny was more congruent with taxonomy than that of *nirK* gene. Metabolic reconstruction of metagenome-assembled genomes further revealed that *nirS*-type denitrifying communities have robust metabolic systems for energy conservation, enabling them to survive under environmental stresses. Nevertheless, *nirK*-type denitrifying communities seemed to adapt to oxygen-limited environments with the ability to utilize various carbon and nitrogen compounds. Thus, this study provides novel insights into the ecological differentiation mechanism of *nirS* and *nirK*-type denitrifying communities, as well as the regulation of the global nitrogen cycle and greenhouse gas emissions.

KEYWORDS
denitrification, ecological differentiation, environmental stresses, metabolic versatility, *nirS* and *nirK*

1 | INTRODUCTION

Denitrifying bacteria contribute significantly to nitrogen removal in diverse aquatic ecosystems, in which two structurally dissimilar nitrite reductases mainly catalyse the conversion of nitrite to nitric oxide, the haeme-cytochrome type NirS and the copper-containing type NirK (Braker et al., 2000; Helen et al., 2016; Priemé et al., 2002). Nitrite reductase encoded by the *nirS* gene reduces nitrite via cytochrome cd_1, a dimer of haeme *c* and haeme d_1 subunits. In canonical denitrification, as observed in *Pseudomonas aeruginosa*, cytochrome cd_1 catalyses the oxidation of a colocalized cytochrome *c*551 to reduce nitrite to nitric oxide at the haeme d_1 site. The *nirS* gene has been reported as a constituent of a larger gene cluster containing genes such as *nirF*, which encode biosynthetic proteins for haeme d_1 (Kawasaki et al., 1997; Philippot, 2002). The gene cluster also includes the nitrite transporter genes such as *nirM* and *nirC* (Hasegawa et al., 2001). On the contrary, NirK can function independently without assistance from other components. Although these two phylogenetically distinct enzymes share similar functions, the two *nir* systems were reported to be incompatible, and denitrifiers were divided into two groups based on whether they possess *nirS* or *nirK* (Jones et al., 2008). Recently, strains with genes containing both reductases have been found, and physiological results indicate that *nirS* and *nirK* show different activities depending on the cultivating conditions (Liu et al., 2020; Wittorf et al., 2018). However, in some cases, these reductases are found to have functional redundancy (Sánchez & Minamisawa, 2018).

There are also differences in the ecological distribution of *nirS* and *nirK* genes in ecosystems. For example, the *nirS*-type denitrifying community has been more active and dominant in soils, freshwater, marine ecosystems and extreme habitats (Graf et al., 2014; Jones & Hallin, 2010; Sun & Jiang, 2022). However, it is more sensitive to environmental changes. In contrast, research indicates that the *nirK*-type denitrifying community is mainly host-associated (Graf et al., 2014) and adaptable to fluctuating environments (Sun & Jiang, 2022). Considering abiotic factors, *nirS* gene abundance responds to nitrate and salinity and shows a higher affinity for nitrite (Goberna et al., 2021; Rinaldo & Cutruzzolà, 2007), whereas *nirK* abundance in soil denitrifiers responds significantly to iron content (Goberna et al., 2021). These observations indicate that two types of denitrifying communities may have different adaptation strategies to the environmental conditions. Another interesting case found that *nirS*- and *nirK*-type denitrifying communities in Lake Donghu showed opposite changing patterns in response to environmental stresses in bioreactors (Zhang et al., 2023). The *nirS*-type denitrifying communities, which exhibit high diversity and abundance in sediments, experienced a notable decrease in taxonomic diversity during cultivation in bioreactors. In contrast, the diversity of the *nirK*-type denitrifying community increased in cultures (Zhang et al., 2023). Therefore, such an opposing phenomenon led us to speculate how different environmental conditions and evolutionary factors influence the adaptation of *nirS* and *nirK* communities.

In this study, we hypothesize that the distinct phylogeny and metabolic characteristics drive the niche differentiation of two types of denitrifying microbial communities. We used metagenome sequencing data from Lake Donghu sediment and corresponding bioreactor samples to test this hypothesis. First, we examined the phylogenetic community structure to understand whether the phylogenetic lineage distribution preference of different *nirS*- and *nirK*-type denitrifying communities contributes to adaptation strategies. Horizontal gene transfer (HGT) is thought to be an essential driving force for microbial evolution and niche adaptation. Therefore, we identified the potential HGT events and predicted the transferred gene functions in microbial communities. We also reconstructed the metabolic potentials for these two groups to reveal their response patterns to environmental stresses. Our study advances the understanding of niche partitioning of *nirS*- and *nirK*-type denitrifying communities in response to environmental changes.

2 | MATERIALS AND METHODS

2.1 | Experimental design, sampling and physicochemical analysis

To investigate the ecological adapting strategies of denitrifying communities in lake ecosystems, we performed nitrogen-removal bioreactors with lake sediments from a typical eutrophic lake, Lake Donghu (Wuhan, China). The subsurface sediment samples with different trophic levels were collected from five sites using a gravity sampler (Zhang et al., 2022). A total of 25 sediment samples were collected for environmental measurements. Four anaerobic bioreactors were performed with subsurface sediments from four sites. Each 5 L bioreactor was seeded with 1.0 kg lake sediments and regularly fed with the inorganic nutrient medium containing NO_2^- and NH_4^+. The bioreactors were maintained for more than 1 year, which could be divided into three phases based on the NH_4^+ and NO_2^- removal efficiencies (Figure S1) (Phase 1: 1–120 days; Phase 2: 121–180 days; Phase 3: 181–371 days) (Zhang et al., 2023). We used lake sediments and bioreactor samples collected at 120, 180 and 371 days for further metagenomic analysis. Corresponding physical and geochemical data were obtained, which showed noticeable environmental changes from natural conditions to bioreactors (Zhang et al., 2023). Briefly, dissolved oxygen (DO) in water contact with sediment, temperature and pH in the sediments were measured in situ by a handheld meter (Extech Instruments, A FLIR Company, USA) (Zhang et al., 2023). The concentrations of NH_4^+, NO_2^- and NO_3^- were measured by an ion chromatography meter (ICS-600; Thermo, USA). The elemental

analyser (Vario TOC; Elemental, Germany) was used to measure the sediments' total carbon (TC).

2.2 | Metagenome sequencing, assembly and analysis

Total genomic DNA extraction was performed according to our previous study (Zhang et al., 2023). Raw sequencing data were trimmed using Trimmomatic v0.38 (Bolger et al., 2014). The filtered reads with an average quality score >20 were further assembled into contigs by MEGAHIT (v1.2.9). All resulting contigs were binned using Metabat2 and MaxBin2 and improved using the Bin_refinement and Reassemble_bins module in metaWRAP (Uritskiy et al., 2018). Metagenome-assembled genomes (MAGs) with completeness >50% and contamination <10% were kept for further analyses based on the estimation of CheckM (v1.0.12) (Parks et al., 2015). GTDB-tk was used to perform taxonomic annotation for MAGs based on the Genome Taxonomy Database GTDB-r202 (Chaumeil et al., 2020). Putative protein-coding sequences (CDSs) of each genome were predicted using Prodigal v2.6.3 with the '-p meta' parameter (Hyatt et al., 2010), and the CDSs were annotated against the Cluster of Orthologous Groups of proteins (COG) and the Kyoto Encyclopaedia of Genes and Genomes Orthology (KEGG) databases using DIAMOND by applying *e*-values $<10^{-5}$ (Buchfink et al., 2015). The relative abundance of MAG representatives in the metagenomes was calculated by 'coverm genome' module in CoverM v0.7.0 (https://github.com/wwood/CoverM) with default settings.

2.3 | Phylogenetic analysis

The genomes with estimated completeness >50% and contamination <10% were kept for phylogenetic analysis. The phylogeny of MAGs from the sediments of Lake Donghu and bioreactor samples were analysed according to previous studies (Jiao et al., 2022). Multiple sequence alignment (MSAs) of 120 bacterial marker genes were generated by GTDB-Tk and used to construct a phylogenetic tree using IQ-Tree (v.1.6.12) with parameters (-m MFP -nt 20 -bb 1000) (Nguyen et al., 2015). LG+F+R10 was selected as the best-fit model for subsequent phylogenetic estimates according to the Akaike Information Criterion (AIC), Corrected Akaike Information Criterion (Corrected AIC) and Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al., 2017). Reference genomes were downloaded from the NCBI datasets for the phylogeny of *nirS* or *nirK* genes. Then, sequences for each gene were identified and translated, as mentioned above. Protein sequences were further aligned using MAFFT (Katoh & Standley, 2013), and maximum likelihood phylogenetic trees were constructed using IQ-Tree with parameters (-m MFP -nt AUTO -bb 1000 -alrt 1000). All phylogenetic trees were visualized and annotated using iTOL (Letunic & Bork, 2016).

2.4 | Identification of horizontal gene transfer and visualization

To identify HGT events within the Lake Donghu and bioreactor microbial community, we used MetaCHIP (v.1.9.0) to detect HGT events among all MAGs (Song et al., 2019). Briefly, each predicted gene aligned using the best-match method was compared among taxa based on the defined phylogenetic tree. Then, the gene was identified as a candidate gene for HGT if the best match came from the non-self-group. The putative HGT was then refined by the phylogenetic approach, which allowed us to identify the direction of gene flow. The gene flow networks within the community were visualized at phylum and class levels using the 'Circlize' package in R (Hu et al., 2014). Horizontally transferred functions were predicted by analysing protein sequences on the KEGG Automatic Annotation Server (KAAS) with 'single-directional best hit' and 'for prokaryotes' parameters (Moriya et al., 2007) and eggNOG-mapper concerning the eggNOG 5 database (Cantalapiedra et al., 2021).

3 | RESULTS

3.1 | Phylogenetic composition and genomic analysis of the recovered MAGs

A total of 284 MAGs (completeness >50%, contamination <10%) were obtained after quality filtering and dereplication (Table S1). Taxonomic classification showed that only 25 (8.8%) and 4 (1.4%) MAGs could be classified as known genera and species, respectively (Table S2). The most abundant phylum (17.2%) among MAGs was *Proteobacteria* (49), of which the most abundant group belongs to the order *Burkholderiales* (20). The second most abundant phylum (15.4%) among MAGs was *Chloroflexota* (44), and more than half belong to *Anaerolineales*. In addition, 22 Archaeal MAGs were recovered, most belonging to *Thermoproteota* (8) and *Halobacteriota* (7). Twenty MAGs were retrieved from bioreactor samples. They were from *Chloroflexota* (5), *Patescibactria* (4), *Bacteroidota* (2), *Proteobacteria* (2), *Zixibacteria* (2), *Acidobacteria* (1), *Gammatimonadetes* (1), *Planctomycetota* (1), *Elusimicrobiota* (1) and one phylum-unsigned genome.

Based on the genes of denitrification pathways present in each genome, a total of 152 MAGs were identified as putative denitrifying bacteria, with 26 encoding *nirS* and 32 encoding *nirK* (Figure 1). The *nirS*-type denitrifying genomes retrieved from Lake Donghu sediment were mainly distributed in five classes, namely *Gammaproteobacteria* (20), *Anaerolineae* (2), *Alphaproteobacteria* (1), *Actinomycetia* (1). However, the taxonomy of *nirK*-type denitrifying community was more diverse, including *Anaerolineae* (8), *Limnocylindria* (5), *Nitrospiria* (5), *Acidimicrobiia* (4), *Gemmatimonadetes* (2), *Alphaproteobacteria* (1), *Bathyarchaeia* (1), and two class unsigned genomes. After cultivation, only two genomes belonging to class *Anaerolineae* and *Baterodia* encoded *nirS*.

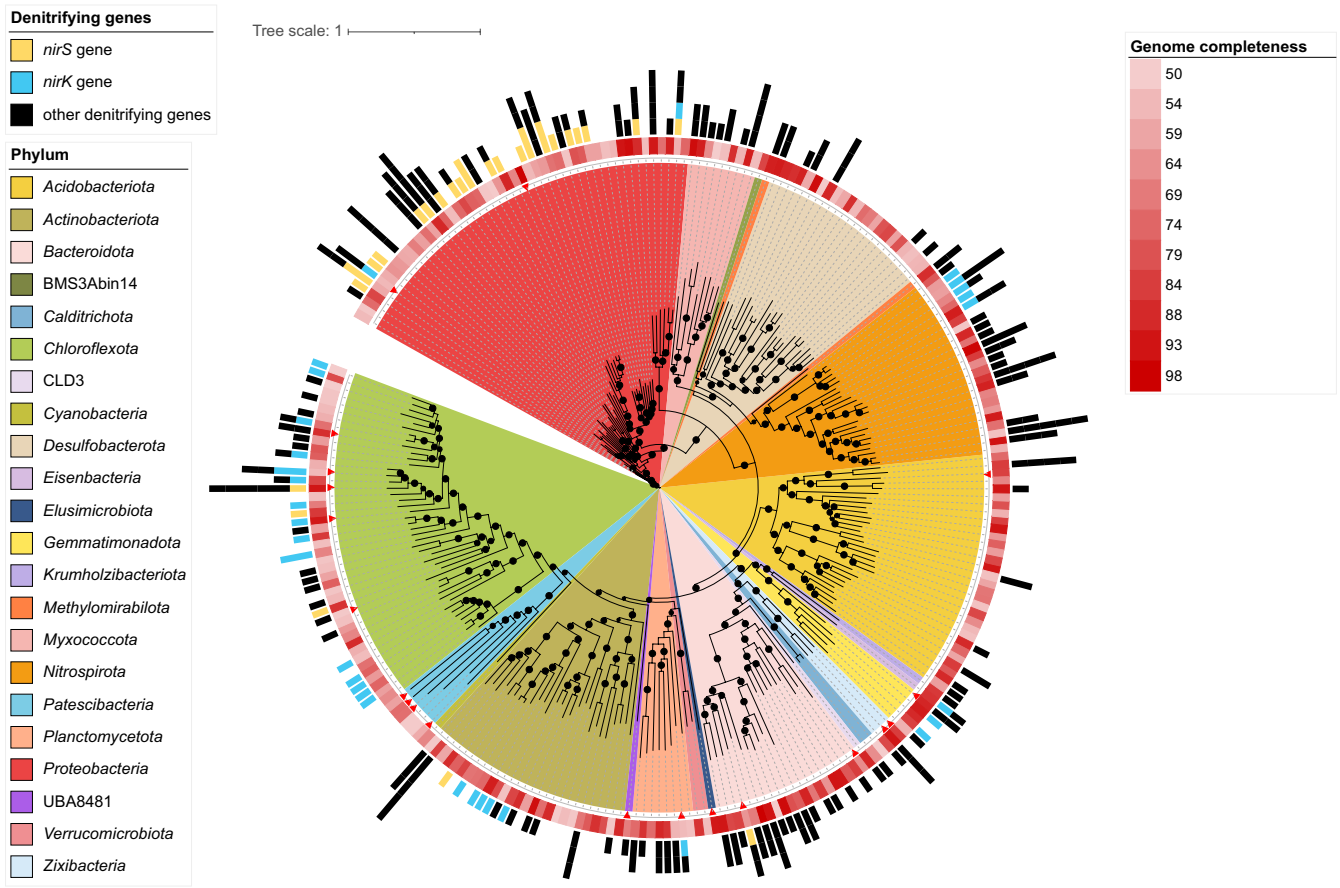

FIGURE 1 Phylogenetic relationships of 264 bacterial MAGs (completeness >50%, contamination <10%) based on 120 bacterial marker genes (Parks et al., 2017). The nodes with a bootstrap value >80% are indicated as black solid dots. Internal branches of the tree are coloured by phylum. The red triangles represent the genomes retrieved from bioreactor samples. The outer heatmap shows the estimated genome quality. The bar plot displays the number of denitrifying genes (*narG*, *narH*, *narI*, *napA*, *napB*, *nirS*, *nirK*, *norB*, *norC*, *nosZ*) present in each genome, among which *nirS* and *nirK* genes denoted by different colours.

Moreover, four genomes encoding *nirK* belonged to *Anaerolineae* (2), *Brocadiae* (1) and *Gammaproteobacteria* (1). Notably, we also found that one genome, DH_sed_bin.72 from *Alphaproteobacteria*, encoded both *nirS* and *nirK* genes. Then, we showed that *the nirK-type denitrifying community was more adaptable to environmental changes*, and most of their relative abundance increased in the first two stages (Phase 1 and Phase 2) (Figure S1). In contrast, the *nirS*-type denitrifying community grew well mainly in the middle stage (Phase 2).

3.2 | Functional analysis of horizontal gene transfer

To study whether HGT contributes to denitrifying microbial communities adapting to the environment, we calculated the potential HGT events using the MetaCHIP algorithm. We detected 1423 transfer events between 30 different taxonomic phyla, where 239 HGT events showed identity >80% (Figure 2a, Table S3). Inter-phylum HGT frequently happened except for *Nitrospira* members. *Proteobacteria* and *Acidobacteriota* were the prominent gene donors in the communities. Besides, the phyla that encounter fewer HGT events tend to be the primary gene recipients, such as *Verrucomicrobiata*, *Zixibacteria*, *Bacteroidota*, *Halobacteriota* and *Krumholzibacteria*. A similar pattern was also observed at the class level. To measure the frequency of HGT events in each MAG, we first classified genomes according to their denitrifying abilities into four categories: *nirS*-type denitrifiers, *nirK*-type denitrifiers, other potential denitrifiers (genomes encoding other denitrifying genes except *nir* genes, i.e., *narG*, *narH*, *narI*, *napA*, *napB*, *norB*, *norC*, *nosZ*), and non-denitrifiers (genomes not encoding any denitrifying gene). The result showed that the frequency of receiving genes by *nirS*-type denitrifiers was lower than the other three groups (Figure 2b), and there was no significant difference between the other three groups.

To identify the functions of HGT genes involved in microbial adaption to environmental stress, we annotated genes transferred between sediment MAGs to 530 unique COG functions. In comparison, 987 genes could be assigned to 580 unique KEGG functions (Figure S1a). Functional annotation against the COG database revealed that the functions belonging to lipid (I) and inorganic ion (P) transport and metabolism were preferentially subject to HGT between *nirS*-type MAGs. Accordingly, carbohydrate transport and

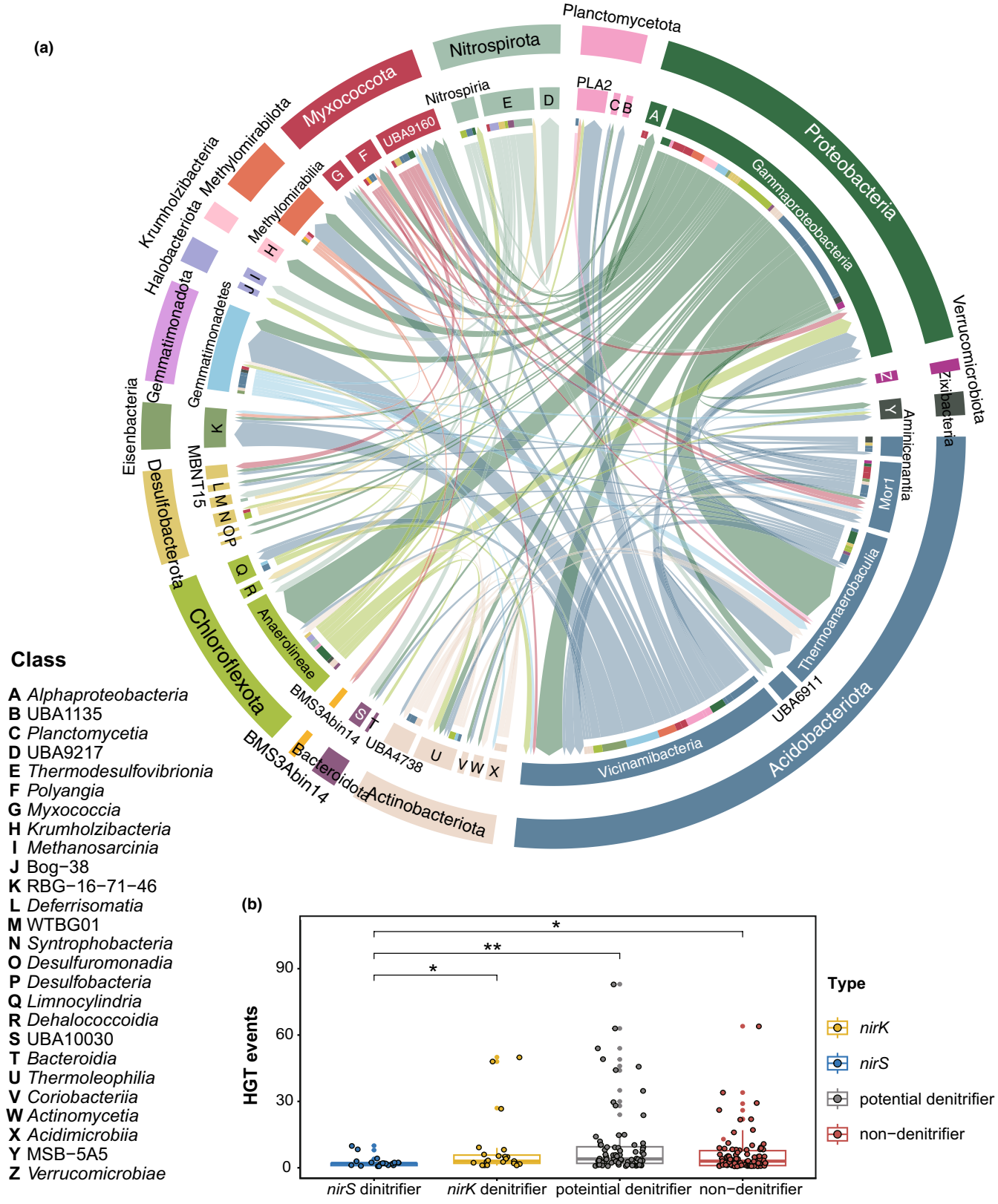

FIGURE 2 Identification and functional prediction of horizontal gene transfer (HGT) events in microbial communities. (a) HGT with high identity (>80%) between metagenome-assembled genomes (MAGs) of different phyla was visualized. The two rings are coloured by MAG taxonomic affiliation, going from phylum to class and from outer to inner rings. The bands connect the various taxa, with the width of the bands correlating to the number of HGT events. The band arrows direct the HGT gene flows from donors to recipients. (b) HGT frequencies among different groups in microbial communities were compared. Each dot represents a recipient genome. Significant differences in HGT frequency between the two groups are denoted with asterisks (Wilcoxon test, $^{*}p < .05$; $^{**}p < .01$).

metabolism (G) and cell wall/membrane/envelope biogenesis (M) were enriched for HGT in *nirK*-type MAGs. We further found differences in the functions of shared genes obtained between *nirS*- and *nirK*-type denitrifying communities (Figure S2). Briefly, the *nirS*-type denitrifying community obtained more genes involved in lipid (I) and inorganic ion (P) transport and metabolism. In comparison, *nirK*-type denitrifying community obtained more genes involved in carbohydrate transport and metabolism (G) and cell wall/membrane/envelop biogenesis (M). These results indicated that HGT may contribute to two types of denitrifying communities adapting to diverse environments through different metabolic strategies.

3.3 | Different evolutionary patterns between *nirS* and *nirK* genes

To investigate complete evolutionary histories, we constructed the maximum-likelihood phylogenies for *nirS* and *nirK* genes with reference sequences in the public database. The NirK protein phylogeny includes three main clades and sequences from Lake Donghu microbial communities were present in all three clades (Figure 3). The NirK sequences of the phylum *Chloroflexota*, *Nitropspira* and *Proteobacteria* were assigned to two clades, suggesting diverse evolutionary origins. The protein sequence from DH_sed_bin.244 was the only *Nitrospira* NirK placed within a cluster of *Gemmatimonadota* and *Eisenbacteria*, suggesting potential HGT events from diverse phyla to *Nitrospira* members. The NirK from *Planctomycetota* enrich_bin97 was identified within the *Chloroflexota* group, indicating its potential acquisition from this group. One NirK from *Actinobacteria* DH_sed_bin.108 was identified as a sister branch to the lineage of *Nitrospira*. Additionally, the NirK of *Gemmatimonadota* and *Actinobacteriota* remained monophyletic, supporting that they may have obtained the *nirK* gene through vertical inheritance but with multiple gene loss events. These results suggested a complex evolutionary history of NirK with diverse donors.

The maximum-likelihood phylogeny of NirS identified that *Bacteroidota* enrich_bin13 was monophyletic with *Bacteroidate*

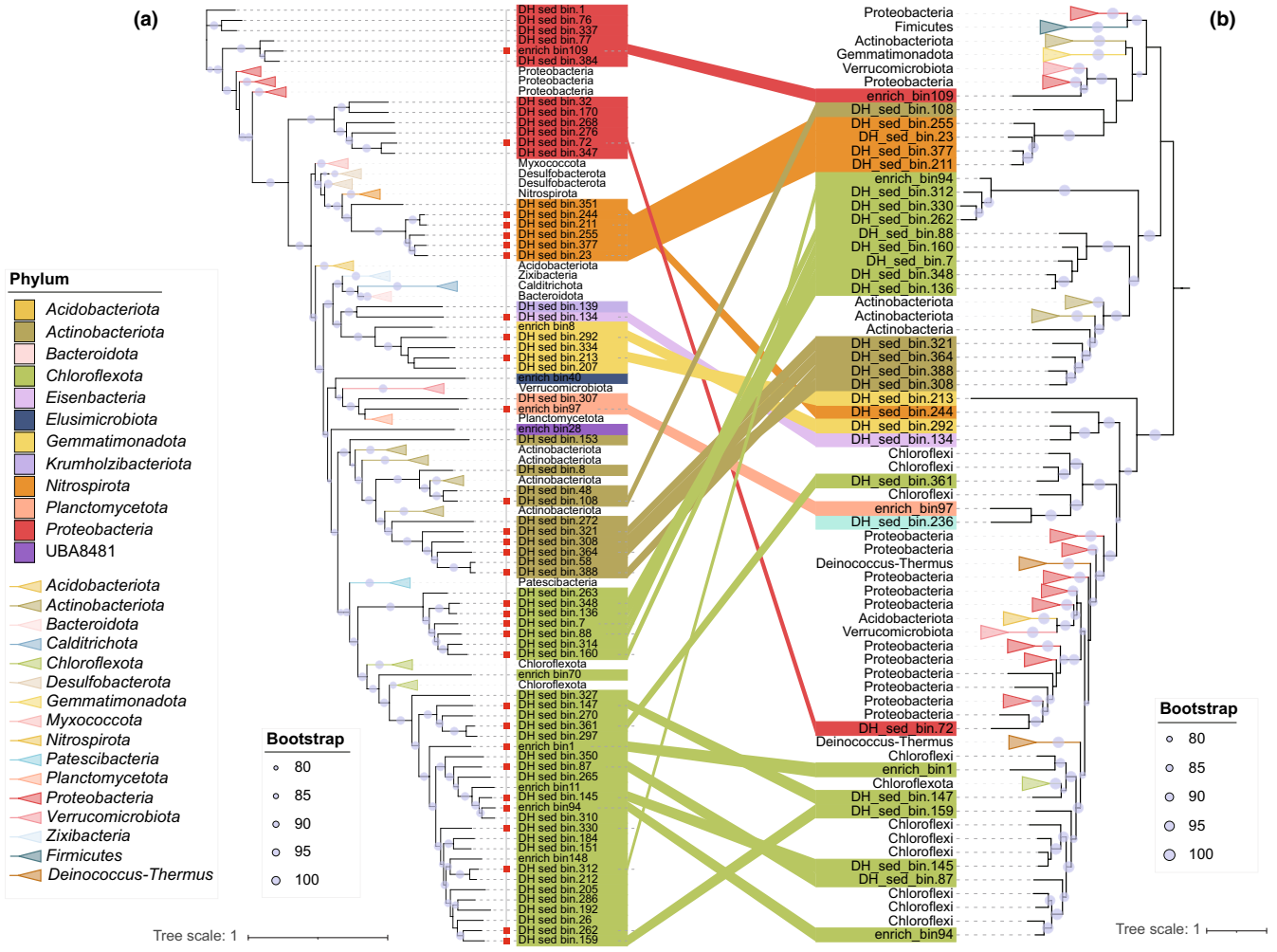

FIGURE 3 Consistency between the phylogenomic trees of the whole communities and the phylogeny of NirK. (a) The phylogenetic tree of Lake Donghu microbial communities was constructed as in Figure 1. The red square represents the MAGs with *nirK* genes. (b) The maximum likelihood tree of the NirK protein. Collapsed groups are labelled with taxonomic group names and are coloured according to the legends.

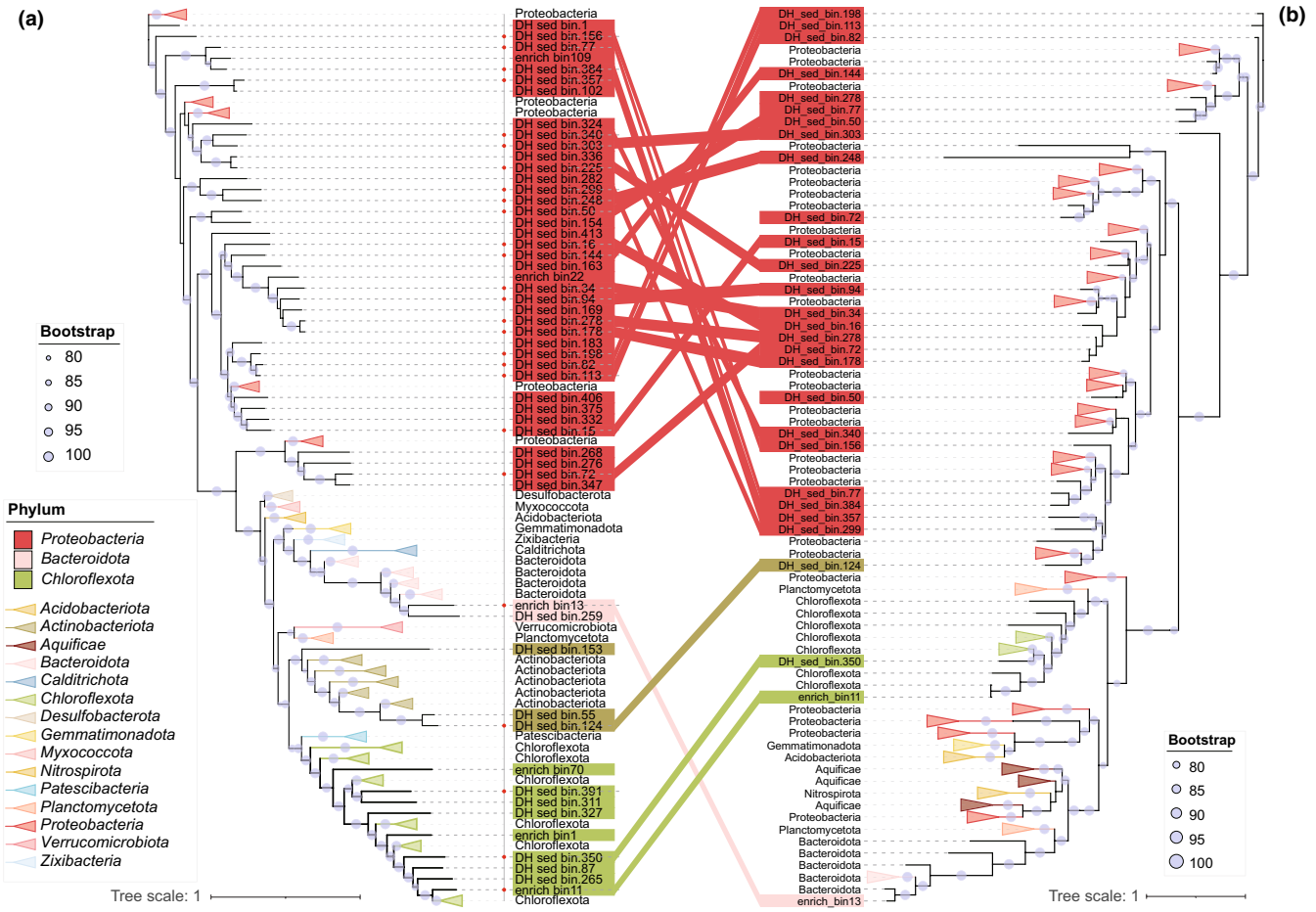

FIGURE 4 Consistency between the phylogenomic tree of the whole communities and the phylogeny of NirS. (a) The phylogenetic tree of Lake Donghu microbial communities was constructed as in Figure 1. The red circle represents the MAGs with the *nirS* gene. (b) The maximum likelihood tree of the NirS protein. Collapsed groups are labelled with taxonomic group names and are coloured according to the legend.

groups in both trees (Figure 4). A similar result was also found in *Chloroflexota* DH_sed_bin.350 and enrich_bin11, which were placed within the *Chloroflexota* clusters. These suggested that the synteny of *nirS* genes within *Bacteroidota* and *Chloroflexota* were conserved. The phylogeny of NirS identified *Proteobacteria* as the most deeply branching group. The sequences from *Proteobacteria* comprised the most basal groups of two trees and were identified in separate clusters of *Proteobacteria* in this polyphyletic region. *Actinobacteriota* DH_sed_bin.124 resided on a deep branch sister to a *Proteobacteria* lineage, potentially suggesting the gene transfer from *Proteobacteria* to this *Actinobacteriota* genome. The maximum-likelihood phylogeny of NirS recovered the monophyly of *Bacteroidota* and *Chloroflexota* sequences except for one *Actinobacteria* sequence, indicating *nirS* genes were mainly inherited vertically.

3.4 | The metabolic potential for denitrifying communities to adapt to environmental stresses

From natural Lake Donghu to bioreactor systems, many environmental factors were changed as selective pressures on microbial growth. For example, the incubation temperature in bioreactors increased by about 5°C compared to the water temperature in the lake. Although the NO_2^- showed considerable variations in Lake Donghu sediment, ranging from 2.65 to 12.43 mg·N/kg, its concentration in bioreactors was maintained at 30 mg·N/L. However, the concentration of NH_4^+ decreased during the transition from the eutrophic lake (15.7–66.81 mg·N/kg) to bioreactors (10 mg·N/kg). The total carbon (TC) in Lake Donghu ranged from 28.34 to 48.82 mg/g. At the same time, bioreactors were continuously supplemented with inorganic synthetic wastewater to eliminate the organic matter in the systems, which means the organic carbon was rapidly depleted at the very first stage (Zhang et al., 2023). Therefore, we hypothesized that two denitrifying groups may have particular physiological patterns contributing to their survival in response to environmental stress, such as warming, oxidative stress and nitrite toxicity.

To test our hypothesis, we examined the metabolic characteristics of *nirS*- and *nirK*-type denitrifying communities (Figure 5, Table S4). The cytochrome bc_1 complex, also known as complex III, is an essential segment of the electron transfer chain of the respiratory process, which catalyses electron transfer from ubiquinol or menaquinol to c-type cytochrome, providing a proton gradient and

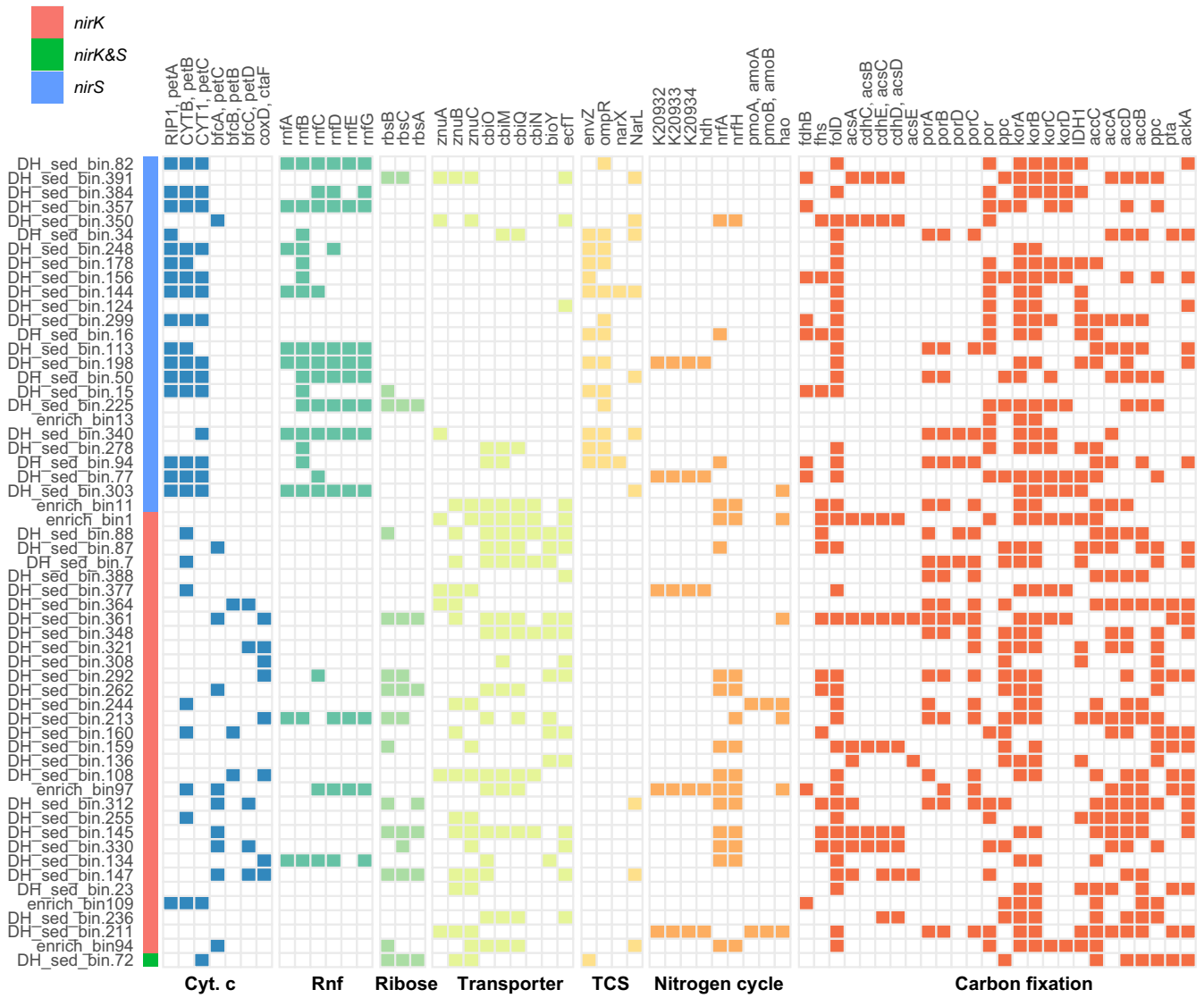

FIGURE 5 Heat map showing the presence of the functional genes in each *nirS*- and *nirK*-type metagenome-assembled genomes (MAGs). The presence of a gene is denoted by a box, coloured by pathway assignment. The absence of a gene is represented with a white box. Cyt. *c*, cytochrome *c* oxidoreductase; Ribose, Ribose transport system; Rnf, Rhodobacter nitrogen fixation complex; TCS, two-component system.

membrane potential for ATP synthesis. The cytochrome reductase encoded in *nirS*-type MAGs is predominantly ubiquinol-cytochrome *c* reductase. We identified 13 of 26 *nirS*-type denitrifiers encoded genes for complete ubiquinol-cytochrome *c* reductase (UQCRFS1, CYTB, CYC1), while only one *nirK*-type genomes enrich_bin97 contained all three genes for ubiquinol-cytochrome *c* reductase (Figure 5). On the contrary, *nirK*-type genomes encoded more genes of menaquinol-cytochrome *c* reductase (MQCRA, MQCRB, MQCRC) (Figure 6), which were seldom detected in *nirS*-type genomes. Thus, these two denitrifying communities prefer different quinone pools, which may reflect their oxygen tolerance (Braasch-Turi et al., 2022). *Rhodobacter* nitrogen fixation (Rnf) complex is a multifunctional respiratory enzyme catalysing the oxidation of reduced ferredoxin to the reduction of NAD^+ (Figure 6a). The genes responsible for the Rnf complex were predominantly encoded in *nirS*-type microbes. These results reflected that *nirS*-type denitrifying communities exhibited higher oxygen tolerance than *nirK*-type denitrifying.

The *nirK*-type denitrifying community widely encoded transporter for ribose and metallic ions (*znuABC*, *cbiOMQN*) and biotin (*bioY*, *ecfT*) (Figure 5), which are critical cofactors for multiple catalytic reactions. On the contrary, more *nirS*-type denitrifiers than *nirK*-type denitrifiers encoded genes of nitrate- and nitrite-responsive sensors (*narX*, *narL*, *narQ*, *narP*) (Figure 5), which control gene expression in response to nitrate or nitrite regulation (Figure 6a). The gene *envZ* encodes a histidine kinase/phosphatase that regulates the phosphorylation state of the transcription factor encoded by *ompR* to respond to the osmolarity changes in the medium. These two genes show an apparent preference for the *nirS*-type denitrifying community (Figure 5), indicating that they have better adaptability to osmotic pressure changes (Figure 6a).

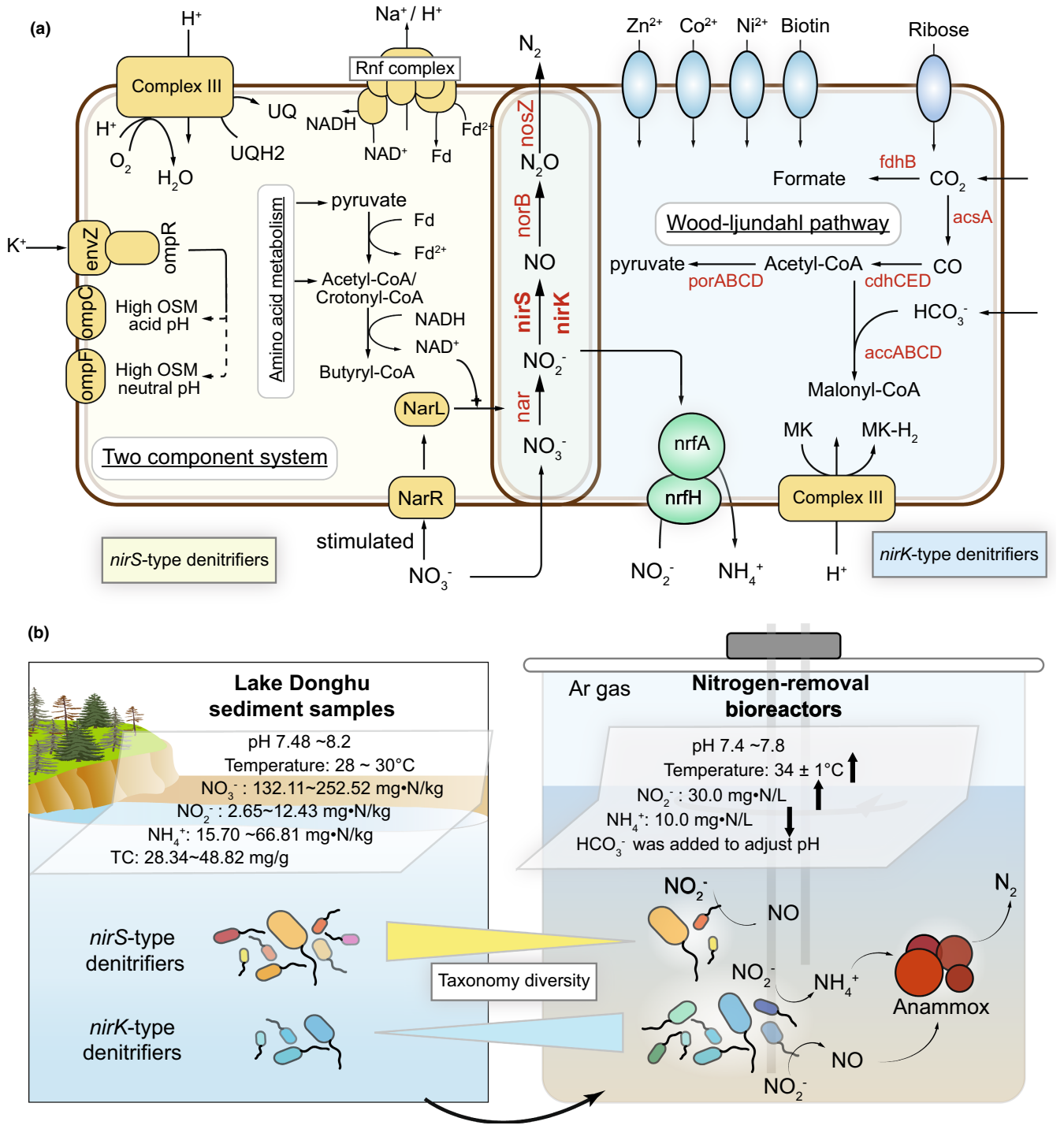

FIGURE 6 The metabolic characteristics of two denitrifying groups (a), and proposed interactions between two types of denitrifying communities and anammox bacteria in bioreactor systems (b). First, physicochemical properties differed during the transitions from Lake Donghu sediments to bioreactors, especially the nitrite and ammonium. Then, the possible coupling mechanisms were also proposed. Since the diverse *nirK*-type denitrifying community also harboured *nrfA* and *nrfH* genes, they could produce both nitrite and ammonium through different nitrogen-transformation processes, which could be further utilized as substrates by anammox bacteria. Thus, *nirK*-denitrifiers show stronger interactions with other members in microbial communities than *nirS*-type denitrifying community. MK, menaquinone; OSM, osmolality; UQ, ubiquinone.

We searched for other nitrogen transformation processes to further explore the nitrogen metabolic potential of two types of denitrifying communities. A noteworthy result was that denitrifying communities harbour versatile nitrogen resource utilizers. Two *nirS*-type genomes (DH_sed_bin.198, DH_sed_bin.77) and three *nirK*-type genomes (DH_sed_bin.377, DH_sed_bin.211 and enrich_bin97) contained the complete anammox pathway (Figure 6b). Similarly, two *nirS*-type genomes (DH_sed_bin.350, enrich_bin11)

and 10 *nirK*-type genomes encode the genes (*nrfA*, *nrfH*) responsible for cytochrome *c* nitrite reductase, which catalysed nitrite reduction into ammonia. These results recovered more versatile metabolic potential for nitrogen resources in the *nirK*-type denitrifying community compared to the *nirS*-type denitrifying community, to some extent explaining their well-adapting status in bioreactor conditions.

We also investigated the bacterial carbon fixation of MAGs in communities since there was little accessible organic carbon in bioreactors. There were two *nirS*-type MAGs and five *nirK*-type MAGs encoded key genes (*acsABCD*) for the Wood–Ljungdahl pathway (Figure 6a), indicating a potential to reduce CO_2 to acetyl-CoA. The essential genes for oxoglutarate ferredoxin oxidoreductase (*korABCD*) were widely encoded in eight *nirS*-type genomes, which catalyse succinyl-CoA to 2-oxoglutarate in reductive tricarboxylic acid (rTCA) cycle. The enzyme acetyl-CoA carboxylase (*accABCD*) in 3-hydroxypropionate bicycle catalyses the acetyl-CoA to malonyl-CoA and bicarbonate fixation, which were widely encoded in eight *nirK*-type genomes. The results indicated that denitrifying communities have diverse pathways to utilize the inorganic carbon in the systems to support their growth.

4 | DISCUSSION

Denitrification is one of the essential processes to remove excess nitrogen from ecosystems, while two functionally equivalent *nirK*- and *nirS*-type denitrifying communities are key players. A previous study found that the *nirS* gene has a higher frequency of co-occurrence with *nor* and *nosZ* and is more likely to be involved in the complete denitrification than the *nirK*-type denitrifying community (Graf et al., 2014). Our results also showed that *nirS* always co-occurred with other denitrifying genes, suggesting that the *nirS*-type denitrifying community could be involved in the complete denitrification process and contribute more to N_2O emissions than the *nirK*-type denitrifying community. Thus, revealing their adaptation mechanisms in response to environmental stresses has important ecological implications for developing novel technologies for nitrogen removal and N_2O reduction. In this study, we analysed the evolution and metabolic landscape of *nirK*- and *nirS*-type denitrifying communities from Lake Donghu sediments and bioreactor samples, and our results generally support our hypothesis that *nirK*- and *nirS*-type denitrifying communities have different phylogenetic diversity and metabolic versatility, enhancing their adaptation.

The evolutionary history of *nirS*- and *nirK*-type denitrifying communities is reflected in their phylogenetic diversity (Jones et al., 2008). In this study, metagenomic sequencing exhibited diverse phylogenetic diversity in putative denitrifying bacteria. The genes *nirS* and *nirK* are widely used as marker genes to study the ecological behaviour of denitrifiers in environments. Some organisms contain more than one gene copy of *nirS* or *nirK* (Etchebehere & Tiedje, 2005). Besides, *nirS* and *nirK* have been found within one organism (Graf et al., 2014; Liu et al., 2020; Wittorf et al., 2018). These phenomena were all detected in our study, although the activity of these two genes needs further confirmation. Similarly, we found that the *nirK*-type denitrifying community exhibited greater taxonomic diversity than the *nirS*-type (Helen et al., 2016; Wei et al., 2015). Consistent with previous research (Braker et al., 2000; Hallin et al., 2018), we found that *nirK* presented more versatile evolutionary origins than *nirS* based on phylogenetic analysis. A possible explanation is gene transfer, which could be derived from HGT (Etchebehere & Tiedje, 2005).

Evolutionary adaption is vital to successful survival in microbial communities in response to changing environmental conditions (Dmitrijeva et al., 2024; Hallin et al., 2018). Bacteria acquire foreign DNA, referred to as mobile genetic elements (MGEs), through transformation, conjugation and transduction (Brito, 2021). The functions conferred by MGEs may contain elements that facilitate organism niche adaptation and survival in response to environmental stress, driving microbial evolution (Mishra et al., 2012; Skoog et al., 2023). For example, bacteria have evolved and adapted to antibiotic pressure by acquiring antibiotic resistance genes (ARG) via HGT due to the massive antibiotic usage in farming (Alderman & Hastings, 1998; Mishra et al., 2012). We found frequent inter-phylum HGT, which is important for the recipient organism to acquire metabolic capabilities and occupy a novel ecological niche (Caro-Quintero & Konstantinidis, 2015). Previous phylogenetic analysis also found that NirK sequences from the same habitats tend to cluster more than sequences retrieved from highly related taxa (Enwall et al., 2010; Heylen et al., 2006; Wei et al., 2015; Yuan et al., 2012), confirming the possible HGT of this type denitrifying gene. Nevertheless, the tree topology of the NirS sequences agreed more with the phylogenetic tree based on 120 bacterial marker genes, indicating that the *nirS* gene tended to be inherited vertically and cannot be generalized in environmental communities. Therefore, *nirK* could have a higher transfer propensity than *nirS*. In this way, the *nirS* gene may encounter more gene losses in the communities if the hosts cannot survive the selection pressure. In contrast, *nirK* tends to act as MGEs and persist in microbial communities, providing the microbial community with a competitive edge against other organisms within the fluctuating environment (Brito, 2021). In this study, the transfer of *nirS* or *nirK* genes was not observed, but we found that *nirS*-type denitrifiers have significantly lower HGT frequency than other denitrifiers. It is confirmed that contacting rates and shared environmental conditions favour HGT and selection for specific MGEs (Brito, 2021; Groussin et al., 2021). Thus, the low abundance of the *nirS*-type denitrifying community may reduce the chance of cell-to-cell contact or access to free DNA in the environment, which in turn affects their resistance to environmental stresses.

The physiological and metabolic characteristics influence the ecological niche differentiation of *nirS*- and *nirK*-type denitrifying communities. Microbial communities residing in bioreactors experienced specific selection stresses, such as oxygen limitation, nitrite concentration upshift, increasing temperature and ammonia limitation in an inorganic medium (Zhang et al., 2023). Here, we found that the *nirS*-type denitrifying community exhibited a high tolerance for oxygen and osmotic pressure. Consistent with previous studies,

their denitrifying activity was more sensitive to the nitrate and nitrite concentration in the environment (Goberna et al., 2021). So, the oxygen, nitrate and nitrite changes mainly explained their abundance. Nevertheless, the *nirS*-type denitrifying community were found to widely encode Rnf complex systems, which could act as versatile metabolic exchange centres, including N_2-fixation (Schmehl et al., 1993), carbon dioxide-fixation (Biegel et al., 2009) and utilization of low-energy substrates (e.g., ethanol and lactate) (Neumann-Schaal et al., 2019; Seedorf et al., 2008). These indicated that the *nirS*-type denitrifying community has a powerful metabolic system for energy conservation, enabling them to exhibit a relatively stable growing status (Heylen et al., 2006). On the contrary, the *nirK*-type denitrifying community was more adaptable to anaerobic environments. They have versatile transporters for ribose, an essential component of nucleic acids, suggesting that they may utilize extracellular nucleic acids as nutrients. Additionally, more transporter genes for metallic ions and biotins are present in the *nirK*-type denitrifying community compared to the *nirS*-type. As microbial communities were cultivated oligotrophically using inorganic carbon, the diverse transporters encoded in the *nirK*-type denitrifying community facilitated their growth by improving sensitivities to nutrients in the environments. Furthermore, we found that they could be involved in multiple nitrogen-metabolism pathways, including anammox and dissimilatory nitrate reduction to ammonium (DNRA), which may strengthen the interaction within microbial communities through the coupling of multiple metabolic processes (Figure 6b). Specifically, the *nirK*-type denitrifying community could either reduce nitrite into nitric oxide through the denitrification process or reduce it into ammonium through the DNRA process. The product from these two processes could be utilized as substrate by anammox bacteria, which were proven to exhibit high activity in bioreactors. Together, our results explained to some extent why the *nirK*-type denitrifying community could grow well in bioreactors.

5 | CONCLUSION

Our metagenome sequencing analysis unveiled different evolutionary and metabolic adaptation strategies of *nirS*- and *nirK*-type denitrifying communities in response to environmental changes. We found that the *nirS*-type denitrifying community did not receive as many genes as the *nirK*-type denitrifying community through HGT, which may explain their decreased diversity in response to changing environmental conditions. The phylogeny of two marker genes indicated that the *nirS* gene was more evolutionarily conservative, with a higher propensity to be lost than the *nirK* gene in response to environmental changes. On the contrary, the *nirK* gene exhibited diverse evolutionary origins, further supporting the idea that they could be generalized within the communities through HGT and develop more strategies for adapting to environmental changes. Also, the metabolic analysis revealed that the *nirS*-type denitrifying community had a relatively stable metabolic system for energy conservation to help them survive in bioreactor systems. In contrast, *nirK*-type denitrifying community tended to adapt to oxygen-limited environments, such as the bioreactor systems. Nevertheless, their involvement in the multiple nitrogen-metabolism pathways may contribute to interacting with other microbial community members through coupling multiple metabolic processes. These results explained why the *nirK*-type denitrifying community could grow well in bioreactors. Therefore, our study provides novel insights into the ecological differentiation mechanism of *nirS*- and *nirK*-type denitrifying communities and contributes to more precisely estimating their roles in nitrogen cycling networks in this pressing global climate change and anthropogenic pollution.

AUTHOR CONTRIBUTIONS

YZM and QYY conceived this study. Field collection was conducted by DDZ, WGZ, HPL, KW, and MYN. YZM, MAA and DDZ performed experiments and analysed the data. YZM drafted the manuscript. YZM, LLC, XLY, ZLH, QLZ, and QYY edited and revised the manuscript. All authors reviewed and approved the results and the revisions made to the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are open available in SRA database under the accession numbers PRJNA828340 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA828340) and PRJNA906637 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA906637).

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SUPPORTING INFORMATION

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