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The Diversity and Nitrogen Metabolism of Culturable Nitrate-Utilizing Bacteria Within the Oxygen Minimum Zone of the Changjiang (Yangtze River) Estuary

Wenxuan He[1,2†], Sizhen Liu[3,4†], Zhichen Jiang[1,2], Jinshui Zheng[3,4], Xuegang Li[1,2,5,6] and Dechao Zhang[1,2,5*]*

[1] Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [2] University of Chinese Academy of Sciences, Beijing, China, [3] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China, [4] Hubei Key Laboratory of Agricultural Bioinformatics, College of Informatics, Huazhong Agricultural University, Wuhan, China, [5] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China, [6] Function Laboratory of Marine Ecology and Environmental Sciences, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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***Correspondence:**
Xuegang Li
lixuegang@qdio.ac.cn
Dechao Zhang
zhangdechao@qdio.ac.cn

[†] These authors have contributed equally to this work

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The nitrogen cycle is an indispensable part of the biogeochemical cycle, and the reactions that occur in the ocean oxygen minimum zone (OMZ) mediate much of the loss of nitrogen from oceans worldwide. Here, nitrate-utilizing bacteria were isolated from the water column at 17 stations within the OMZ of the Changjiang (Yangtze River) Estuary using selective media and a culture-dependent method. The microbial diversity, nitrogen metabolism and nitrate reduction test of culturable heterotrophic bacteria were examined. A total of 164 isolates were obtained; they were mostly affiliated with *Proteobacteria* (81.1%), *Actinobacteria* (5.5%), *Bacteroidetes* (12.3%), and *Firmicutes* (0.6%). *Pseudomonas aeruginosa*, *Sphingobium naphthae,* and *Zunongwangia profunda* were found at most stations. Among 24 tested representative strains, 8 were positive for nitrate reduction; they belonged to genera *Aurantimonas*, *Halomonas*, *Marinobacter*, *Pseudomonas*, *Thalassospira*, and *Vibrio*. *Pseudomonas aeruginosa* contained the genes (*napAB*, *norBC*, *nirS,* and *nosZ*) for complete denitrification and may be responsible for mediating denitrification. 66% representative isolates (16/24) contained genes for reducing nitrate to nitrite (*nasA*, *napAB,* or *narGHI*) and 79% representative isolates (19/24) possessed genes for converting nitrite to ammonia (*nirA* or *nirBD*), suggesting that nitrate and nitrite could act as electron acceptors to generate ammonium, subsequently being utilized as a reduced nitrogen source. This study improves our understanding of the microbial diversity within the OMZ of Changjiang Estuary and may facilitate the cultivation and exploitation of bacteria involved in the nitrogen cycle.

Keywords: oxygen minimum zone, the Changjiang Estuary, culturable, nitrogen metabolism, nitrate-utilizing

INTRODUCTION

In the environment, the oxidation state of nitrogen ranges from -3 in ammonium (NH_4^+) to + 5 (NO_3^-), and is governed by several processes. The nitrogen cycle comprises the steps of nitrogen fixation, mineralization, nitrification, anammox, and denitrification (Hayatsu et al., 2008). After nitrogen fixation, nitrification and denitrification carry N return inorganic pool and return it to the atmosphere (Munn, 2011). Some regions experience an imbalance in the input and output of nitrogen: An example of this is the oxygen minimum zones (OMZs) of the world's oceans, which are responsible for approximately 30–50% of the loss of nitrogen from these bodies of water (Codispoti et al., 2001).

Changjiang Estuary is located in the Western Pacific Ocean, East of China. A seasonal OMZ was discovered here at the end of the twentieth Century; its current range is from about 32°−30°N to the southern coastal sea (Chen et al., 2007). The main cause of this hypoxic zone is the inflow of a large amount of fresh water from the Changjiang River coupled with the upward invasion of the Taiwan Warm Current in summer, which leads to a seawater stratification that prevents the vertical transmission of oxygen (Zhang et al., 2007). Under suboxic or anoxic conditions, NO_3^- and NO_2^- are used as terminal electron acceptors by denitrifying bacteria in dissimilatory NO_3^- reduction (denitrification) (Wright et al., 2012). Because of the low oxygen concentration in the OMZ (dissolved oxygen < 2–3 mg/L) (Wang et al., 2012), denitrification that use inorganic nitrogen and release dinitrogen gas (Lam and Kuypers, 2011), are very active (Lam et al., 2009). The accumulation of NO_2^- within oceanic OMZs provides an ideal niche for microbes.

Most previous studies on the prokaryotes responsible for sustaining the nitrogen cycle in the OMZ of the Changjiang Estuary have focused on sedimentary organisms and used culture-independent methods (Dang et al., 2008; Li et al., 2009; Chen et al., 2014; He et al., 2016; Wu et al., 2019). Although important biogeochemical processes take place in the sediment, the metabolic activity and individual characteristics of microorganisms that mediate the loss of N are poorly known. Admittedly, there are limitations of culture method in the ecology exploration, but culture-based studies can allow microorganisms to be isolated from natural samples and enable researchers to perform physiological/experimental assays and elaborate metabolic activities during biogeochemical cycles, and thereby infer the functional and ecological roles of the microorganisms in detail (Mulla et al., 2018; Sanz-Saez et al., 2020). Here, we used a culture-dependent method to investigate microorganisms that participate in the nitrogen cycle in the seawater column of the Changjiang Estuary OMZ, aiming to: (1) explore the diversity of the culturable nitrate-utilizing bacteria; (2) explore nitrogen metabolism based on their genomes data; (3) test nitrate reduction of the isolated strains; (4) investigate the relationship between their distribution and environmental parameters (dissolved oxygen, nitrate, and nitrite).

METHODOLOGY

Sampling Areas and Sampling

Seawater samples were collected from the OMZ of Changjiang Estuary by the "Science III" scientific research ship in August 2020. A total of 61 seawater samples were collected using a CTD (conductivity–temperature–depth) apparatus from 17 stations at varying depths (2–6 depths per station). The stations were designated and located as follows (**Figure 1**): 3000-2 (30.00°N, 123.00°E), 3050-2 (30.49°N, 123.02°E), 3100-1 (30.90°N, 122.45°E), 3100-2 (30.98°N, 122.86°E), 3100-2a (31.01°N, 123.25°E), 3100-3 (31°N, 123.50°E), 3150-2 (31.40°N, 123.00°E), CJ-02 (31.79°N, 123.00°E), DH4-0 (29.63°N, 122.83°E), DH45 (29.36°N, 122.48°E), DH5-0 (29.14°N, 122.48°E), DH5-1a (29.02°N, 122.67°E), DH5-2 (28.90°N, 122.87°E), DH5-2a (28.78°N, 123.07°E), DH5-3 (28.65°N, 123.28°E), DH56 (28.81°N, 122.36°E), and DH6-1 (28.45°N, 122.18°E).

Environmental Parameters

Each sample was placed in a 50-ml brown iodine flask and fixed with manganese sulfate and an alkaline potassium iodide solution, and dissolved oxygen (DO) was measured *in situ* by Winkler iodometry at a relative standard deviation of 2% (Ma et al., 2020). The sample was filtered through a glass-fiber membrane with a pore size of 0.7 μm (Whatman GF/F, United Kingdom), and frozen at −20°C. In the laboratory, the concentrations of nitrate (NO_3-N) and nitrite (NO_2-N) were measured using an automatic nutrient analyzer (SEAL QuAAtro, Germany). Their detection limits were 0.02 and 0.01 μmol/L, respectively (Ma et al., 2019, 2020).

Isolation, Culture, and Purification

On board the boat, the obtained seawater samples were immediately plated on nitrate agar medium (NAM; 0.2% KNO_3, 0.02% $MgSO_4{\cdot}7H_2O$, 0.08% $K_2HPO_4{\cdot}3H_2O$, 2% $NaKC_4H_4O_6$, 1% NaCl, 2% agar) which allowed the growth of nitrate-utilizing bacteria using a clean bench. After incubation at 25°C in constant temperature incubator for 7–14 days, single colonies were selected and subcultured to achieve purity. The pure isolates were stored as a suspension in 20% (w/v) glycerol at -80°C.

DNA Extraction, 16SrDNA Amplification, and Genome Sequencing

Genomic DNA was extracted using a bacterial genomic DNA Mini kit (TaKaRa Bio) following the manufacturer's protocol. The 16S rRNA gene was amplified by PCR with a pair of universal primers (Zhang et al., 2006). The reaction system contained 1 μl of forward and reverse oligonucleotide primers, 1 μl exacted DNA, 12.5 μl 2 × PCR Mix (100 mM KCl, 20 mM Tris-HCl, 3 mM $MgCl_2$, 400 μM dNTPs, 0.1 U/μl Taq polymerase), and ddH_2O to a final volume of 25 μl. The cycling conditions were: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles, with a final extension at 72°C for 10 min. The PCR products was purified using a QIAquick PCR Purification kit (Qiagen). The purified 16S rDNA PCR product was sequenced using dideoxy chain termination/cycle

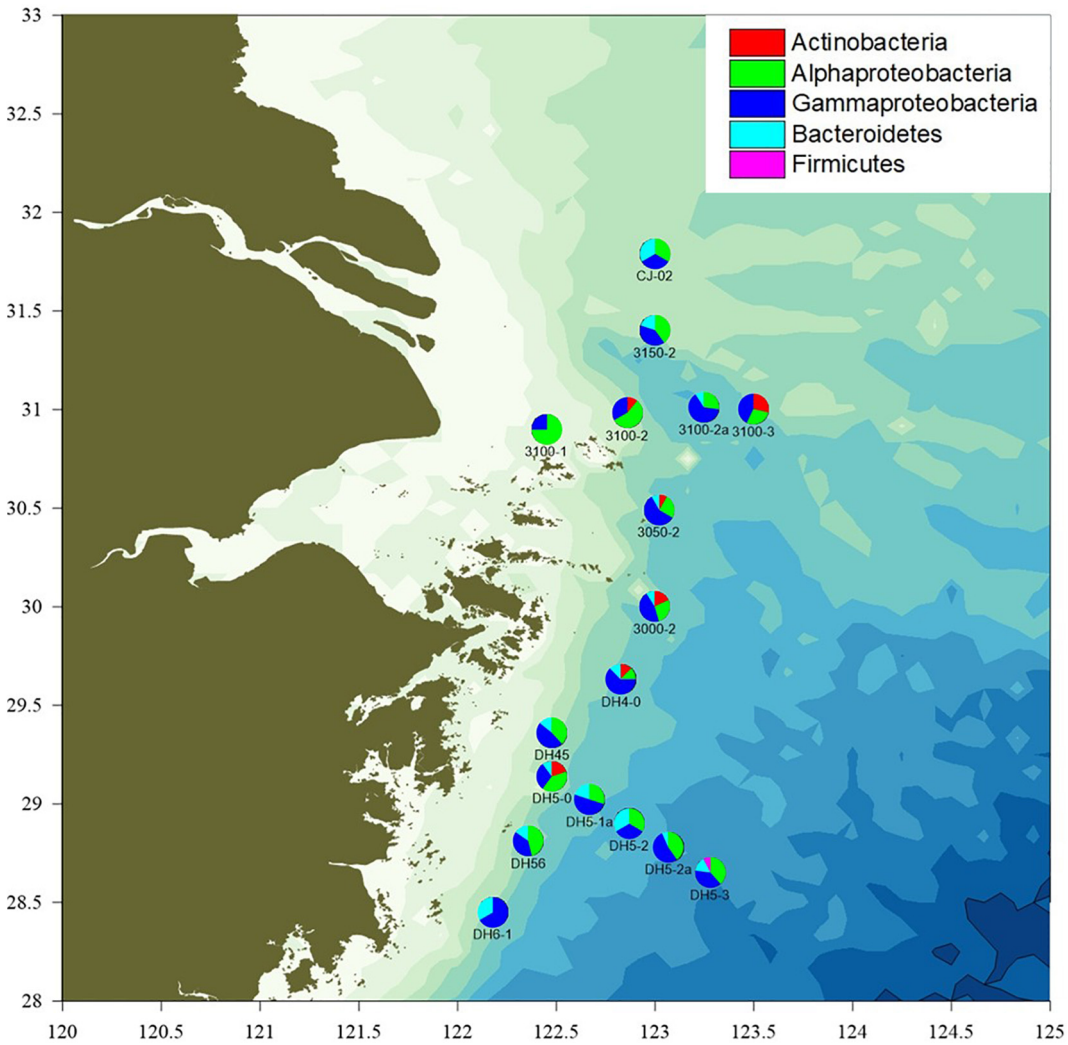

FIGURE 1 | The sampling locations and proportion of isolates classified to each phylum and (partly) class in the Changjiang River Estuary OMZ.

sequencing, as applied with an ABI 3730XL sequencer (Applied Biosystems) and version 3.1 of the ABI Big Dye Terminator kit (Applied Biosystems). We compared the produced 16S rDNA sequences with those in the Ezbiocloud database to identify the isolates (Yoon et al., 2017). Strains having > 98% 16S rRNA gene sequence similarity to a given species and matching with the same species in a GenBank search were assigned to that species (Zhang et al., 2012).

Genome Sequencing, Assembly and Annotation

A paired-end library with an insert size of 350 bp was constructed for each genome, and sequenced using Illumina NovaSeq 6000 platform. The resulting 150 bp paired-end reads with about 200X, were quality checked and assembled using FastQC (v0.11.9) and SPAdes genome assembler v3.15.2 (Prjibelski et al., 2020). De Braiju graph-based assembly was tested with K-mers between 21 and 77, and the quality of the assemblies were evaluated by BUSCO (5.0.0) (Manni et al., 2021). Genes prediction and annotation of all genomes were generated using Prokka v1.14.6 (Seemann, 2014).

Phylogenetic Tree Construction

To clarify the taxonomic status and evolutionary relationship among strains, proteins in 24 genomes were divided into Orthologous groups using OrthoFinder version 2.5.2 (Emms and Kelly, 2017, 2018) and 197 single-copy genes were shared in all genomes. Briefly, an all-to-all Blast was set as E-value $< 1 \times 10^{-3}$, and the BLAST BIT scores were standardized based on gene length and phylogenetic distance. For each of these single-copy genes, all protein sequences were aligned using MAFFT v7.480 (Katoh and Standley, 2013). Each lineal homologous genome was selected to construct a gene tree by FastTree (Letunic and Bork, 2021) and constructed Species Tree using STRIDE algorithm. ITOL (Letunic and Bork, 2021) was used to visualize the phylogenetic tree.

Analysis of Nitrogen Metabolism

To identify genes for nitrogen metabolism, all of the annotated genes were searched against the KEEG database using KAAS BBH BLAST (GENES data set: prokaryotes) (Moriya et al., 2007). Genes belonging to different nitrogen metabolism types were classified by manual selection according to the results of KAAS.

Nitrate Reduction Test

Representative strains were tested for their ability to utilize nitrate. Each pure isolate was transferred to nitrate liquid medium (1 g/L KNO_3, 10 g/L peptone, 1 L sterile seawater) and incubated at 25°C for 7 days. The presence of nitrite was measured in culture media using Griess reagents as previously described (Dong and Cai, 2001). Briefly, 2 drops of Griess A and Griess B reagents (Haibo Reagent, China) were sequentially added to the liquid medium. After incubation for 2 min, the test sample was compared with the control. If the test sample was red, pink, or orange, there was nitrite in the tube and the isolate was scored as positive for nitrate reduction and marked as " + " If no color appeared, zinc dust was added. The lack of any color change at this point was taken as indicating that there was no residual nitrate in the liquid medium, meaning that the strain could reduce nitrate completely; this was marked as " ++ " If the liquid medium changed from colorless to red, pink, or orange after the addition of the zinc dust (as did the control), indicating the continued lack of nitrite, the strain was considered to be unable to reduce nitrate, and was marked as"−"

RESULTS AND DISCUSSION

Diversity of the Culturable Nitrate-Utilizing Bacteria

A total of 164 isolates were obtained from the different seawater layers sampled at 17 different stations. Most (63.4%) of the NAM-derived isolates belonged to *Pseudomonas* (57), *Sphingobium* (43), followed by *Alteromonas* (10), and *Zunongwangia* (21) (**Supplementary Figure 1**). Based on phenotypic characteristics (colony morphology and pigmentation) and 16S rRNA gene sequencing, 24/164 isolates were identified as potentially unique (<98% sequence similarity) and further studied (**Supplementary Table 1**). The majority of the potentially unique strains (81.1%) belonged to phylum *Proteobacteria*, with a predominance of *Gammaproteobacteria* (45.7%) and *Alphaproteobacteria* (35.4%). The other strains belonged to phyla *Actinobacteria* (5.5%), *Bacteroidetes* (12.3%), and *Firmicutes* (0.6%). These four phyla were previously isolated from surface seawater samples obtained from Changjiang Estuary and adjacent areas, as assessed using a culture-independent molecular approach (Wang et al., 2017). The *Proteobacteria* represent a major group (phylum) among the well-known and readily cultivable marine microorganisms, and hence are of biological significance (Rusch et al., 2007; Sunagawa et al., 2015; Fernandes et al., 2019). Studies on surface sediments of the hypoxic zone near the Changjiang Estuary and in the East China Sea also revealed that the bacterial community composition mainly comprised *Gammaproteobacteria* (Ye et al., 2016).

Vibrio spp. are distributed worldwide in coastal and ocean waters and sediments. In this study, we isolated some *Vibrio* spp. that are reported to be pathogenic, such as fish pathogenic *Vibrio* (*Vibrio anguillarum*) and crustacean pathogenic *Vibrio campbellii* (Faruque and Nair, 2006). A previous study found that *Vibrio campbellii* was dominant in Changjiang Estuary (Wang et al., 2020). The appearance of these *Vibrio* spp. in Changjiang Estuary is notable given that their abundance was previously found to relate to the plankton community and human disease (Vezzulli et al., 2016). *Sphingobium soli* appeared in a surface seawater sample (site 3100-2; 0 m); as this strain was originally isolated from soil (Du et al., 2015), we speculate that it may have been washed into sea by the Changjiang River. Six genera found in the OMZ water column of Changjiang Estuary were previously reported in other OMZs: *Halomonas* (Arabian Sea, South Pacific), *Marinobacter* (Arabian Sea, South Pacific), *Pseudomonas* (South Pacific), *Vibrio* (Arabian Sea, South Pacific, North Pacific), *Marinomonas* (South Pacific), and *Marinobacter* (Arabian Sea) (Stevens and Ulloa, 2008; Beman and Carolan, 2013; Mulla et al., 2018; Fernandes et al., 2020).

Numerous strains of *Pseudomonas aeruginosa* (52/164) were isolated in the present study, suggesting that *Pseudomonas aeruginosa* may be widespread in the seawater of Changjiang Estuary. As a well-known ubiquitous environmental bacterium, *Pseudomonas aeruginosa* is one of the top three causes of opportunistic human infections (Stover et al., 2000). Its presence in rivers and seawater is commonly attributed to contamination from domestic sewage (Devicente et al., 1991). Considering that Changjiang Estuary is adjacent to densely populated metropolises, such as Shanghai City and Hangzhou City, it is not surprising that *Pseudomonas aeruginosa* was found to be abundant in the water column. In recent years, the continuous expansion of the Changjiang Estuary's summer OMZ has shown a close relationship with the input of nutrients from runoff of Changjiang River (Chi et al., 2017). These nutrients also lead to environmental problems, such as reduced water quality and red blooms, keeping the ecosystem of Changjiang Estuary in a sub-healthy state.

Ecology-Focused Statistical Analysis

The number of species (S), the number of isolates (N), the species richness (Margalef index, d), the species evenness (Pielou's evenness, J'), and the species diversity (Shannon index, H') are shown in **Supplementary Table 2**. The culturable bacteria diversity (H') was the highest at station 3100_2a and lowest atstation 3100-1, where only two different species were isolated.

Denitrification Potential of Strains

Using a culture-dependent method has the advantage of enabling researchers to investigate the physiological and biochemical properties of microorganisms that are rare (even down to a single cell) in a sample. Here, we used the Griess test to analyze 24 representative strains from NAM for their ability to reduce nitrate. Of them, 8 strains were found to reduce nitrate: 4 strains (*Vibrio natriegens*, *Vibrio campbellii*, *Halomonas meridiana,* and

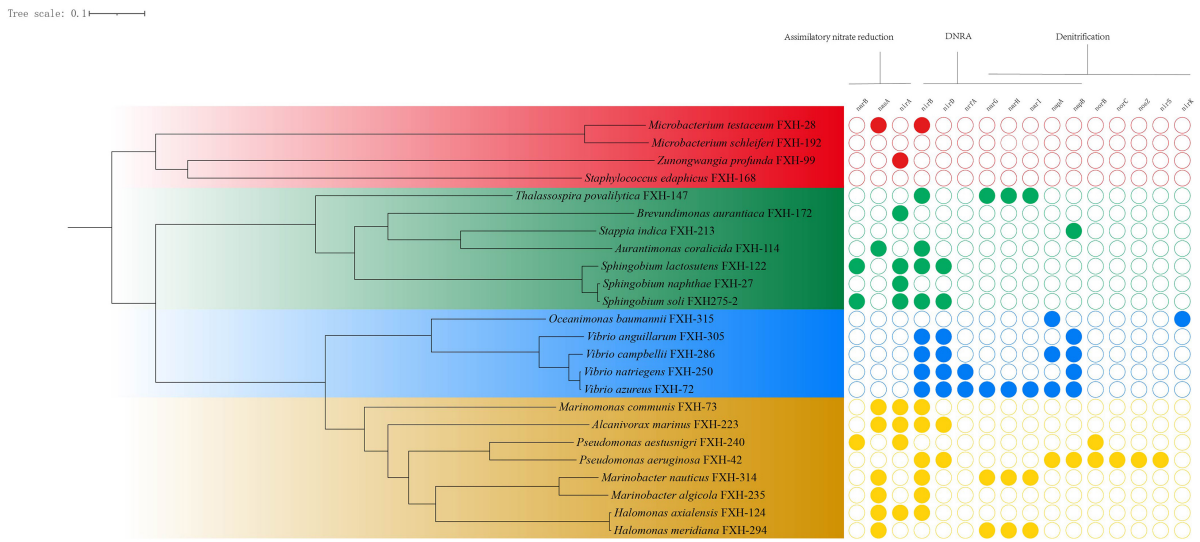

FIGURE 2 | The genomic phylogenetic tree of representative isolates and corresponding genes involved in nitrogen metabolism.

Marinobacter nauticus) exhibited a strong positive reaction and could reduce nitrate completely during a 7-day incubation (**Supplementary Table 3**); other strains positive for nitrate reduction included *Pseudomonas aeruginosa, Vibrio azureus, Aurantimonas coralicida,* and *Thalassospira povalilytica*.

Most of the nitrate reduction-positive strains were not widely distributed in the OMZ. An exception was *Pseudomonas aeruginosa*, which could reduce nitrate and was found at almost all of the test stations (16/17); it was present in 37 water samples and represented 31.7% of the strains isolated from NAM. Although the representatives of *Pseudomonas aeruginosa* isolated in the present study did not reduce nitrate completely under the utilized aerobic conditions, the wide distribution of this microbe as a dominant isolate and its ability to utilize a denitrification mechanism under anaerobic conditions prompt us to speculate that *Pseudomonas aeruginosa* may be the mediator of the loss of nitrogen from the OMZ of Changjiang Estuary.

Nitrogen Metabolism of Representative Isolates

The genomes of 24 representative isolates were annotated for understanding the metabolic potentials based on the key genes of metabolic pathways of nitrogen. In the environment, nitrate levels are influenced by the microbially driven processes of nitrate reduction, whereby nitrate is reduced initially to nitrite, which subsequently can be reduced by denitrification or *via* assimilatory or dissimilatory nitrate reduction to ammonia (ANR or DNRA) (Smith et al., 2007). As shown in **Figure 2**, most representative isolates possessed genes involved in ANR or DNRA, and were lack of genes involved in final three steps in denitrification pathway (*nirKS*, *norBC,* and *nosZ*).

Sixty six percent representative isolates (16/24) contained genes for reducing nitrate to nitrite (*nasA*, *napAB,* or *narGHI*) and 79% representative isolates (19/24) possesses genes for converting nitrite to ammonia (*nirA* or *nirBD*), suggesting that nitrate and nitrite could act as electron acceptors to generate ammonium, subsequently being utilized as a reduced nitrogen source. For example, all 4 *Vibrio* isolates contained the genes (*nirBD* and *napB*) for DNRA, suggesting that *Vibrio* isolates may be involved in nitrate reduction to ammonia.

Analysis of the genome sequence revealed *Pseudomonas aeruginosa* FXH-42 had a complete set of the denitrification enzymes (NapAB, NorBC, NirS, and NosZ) that reduce nitrate to molecular nitrogen *via* nitrite, nitric oxide (NO), and nitrous oxygen (NO_2), which is consistent with the previous reports (Ye et al., 1995; Arai, 2011). *Oceanimonas baumannii* FXH-315 possessed the genes for nitrate reductase (*napA*) and nitrite reductase (*nirK*), but lack of the genes for nitrous oxide reductase (*norBC*) and nitrous oxide reductase (*nosZ*, functional for yielding N_2), suggesting that it had the potential to release NO in the OMZ of the Changjiang Estuary.

The Relationship Between Bacterial Distribution and Dissolved Oxygen, NO_2^--N, and NO_3-N

By testing the DO, NO_2^--N, and NO_3^--N levels of different sites, we found that DO consistently decrease with increasing depth, whereas the levels of NO_2^--N and NO_3^--N varied with location. Across the sampled sites, NO_2^--N ranged from 1.029 μmol/L (3150-2, 10 m) to 0 (DH5-3, 0 m) and NO_3^--N from 27.96 μmol/L (3100-1, 3 m) to 0 μmol/L (DH5-2a, 3 m) (**Figure 3**). Water depth also had important effects on the composition of microorganisms. Especially in the OMZ of Changjiang Estuary, hypoxia is caused by water stratification, which didn't affect creatures of the surface seawater, but causes the microbial structure in the lower (hypoxic) strata to be completely different from that at the water surface (Levin, 2003). The literature lacks a strict definition of hypoxic zone; 2 mg/L DO

has been regarded as the limit of hypoxia in Changjiang Estuary (Guo et al., 2019), but a DO below 4∼6 mg/L will reportedly impact the survival of aquatic organisms (Gray et al., 2002).

A previous study used pyrosequencing of 16S rRNA genes to show that bacterial richness decreased with DO, and that it was maximal at the edge of the OMZ and decreased within it (Beman and Carolan, 2013). *Pseudomonas aeruginosa*, *Sphingobium naphthae,* and *Zunongwangia profunda* were found at most stations with different DO, NO_3-N and NO_2-N values. *Pseudomonas aeruginosa*, *Sphingobium naphthae*, *Zunongwangia profunda*, *Microbacterium testaceum,* and *Halomonas axialensis* were found in zones with DO values lower than 3 mg/L. *Pseudomonas aeruginosa* was unique at hypoxia zone (DO < 2 mg/L), while *Marinobacter algicola*, *Sphingobium soli*, *Vibrio natriegens*, *Vibrio anguillarum*, and

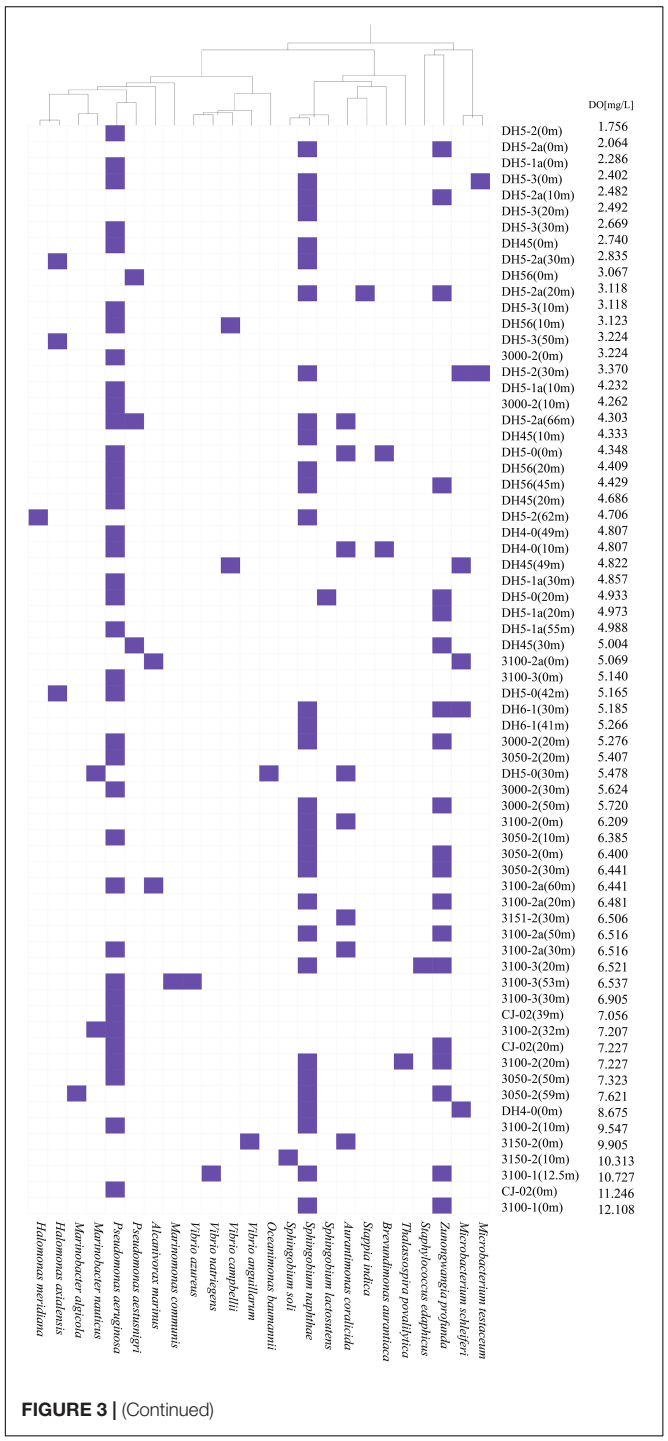

FIGURE 3 | (Continued)

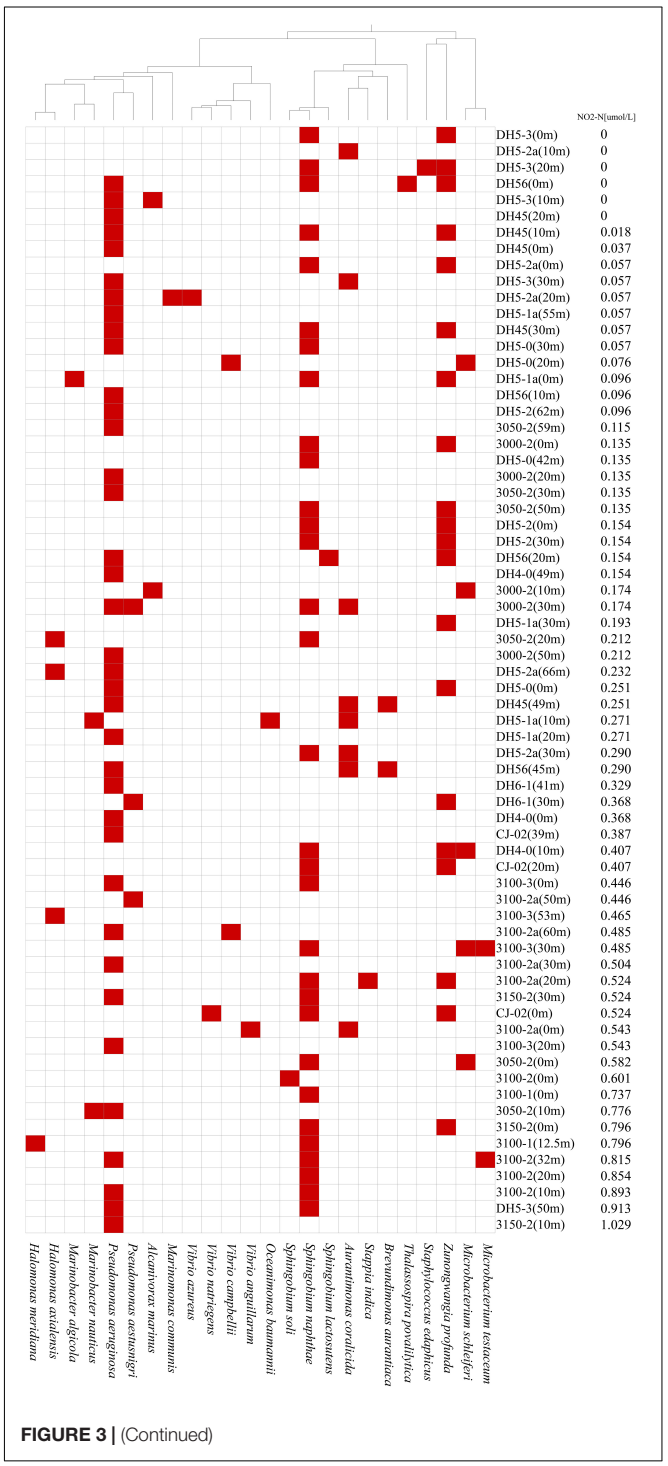

FIGURE 3 | (Continued)

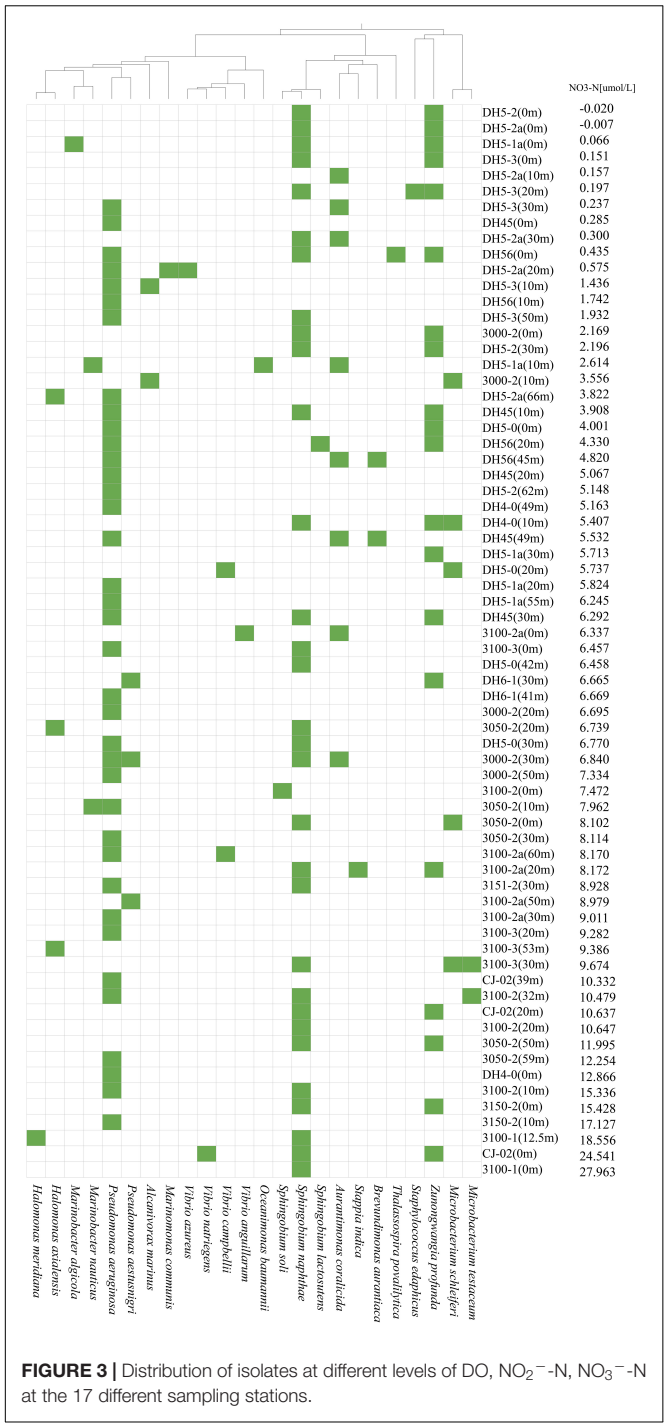

FIGURE 3 | Distribution of isolates at different levels of DO, NO_2^--N, NO_3^--N at the 17 different sampling stations.

Thalassospira povalilytica could only exist at oxygen-rich zones (DO > 7 mg/L) on the surface of the ocean. The numbers of species increased significantly in mid-oxygen zone (4 mg/L < DO < 6 mg/L).

Vibrio natriegens and *Halomonas meridian* which showed strong positive for nitrate reduction and contained genes for DNRA, were isolated from sites with high levels of nitrate (24.5 and 18.6 μmol/L) and nitrite (0.5 and 0.8 μmol/L), respectively. *Microbacterium testaceum* was only found in seawater where DO was lower than 4 mg/L, but the values of NO_2^--N and NO_3^--N were relatively high. Similarly, *Stappia indica* was located in seawater with low DO and high NO_2^--N and NO_3^--N values while *Thalassospira povalilytica* which contained genes (*nirB* and *narGHI*) for DNRA was distributed in seawater with high DO and low NO_2^--N and NO_3^--N values.

CONCLUSION

In the present research, a total of 24 representative strains were isolated. The majority of these strains belonged to phylum *Proteobacteria*, with a predominance of *Gammaproteobacteria* and *Alphaproteobacteria*. Of 24 representative isolates from NAM, 8 had the ability to reduce nitrate. *Pseudomonas aeruginosa*, *Sphingobium naphthae,* and *Zunongwangia profunda* were found at most stations. Genome analysis indicated that 66% representative isolates contained genes for reducing nitrate to nitrite (*nasA*, *napAB,* or *narGHI*) and 79% representative isolates possessed genes for converting nitrite to ammonia (*nirA* or *nirBD*), suggesting that nitrate and nitrite could act as electron acceptors to generate ammonium, subsequently being utilized as a reduced nitrogen source. *Pseudomonas aeruginosa* contained the genes (*napAB*, *norBC*, *nirS,* and *nosZ*) for complete denitrification and may be a mediator of denitrification within the OMZ of Changjiang Estuary. This study improves our understanding of the microbial diversity within the OMZ of Changjiang Estuary from the perspective of the cultivation and potential exploitation of culturable nitrate-utilizing bacteria involved in the nitrogen cycle.

DATA AVAILABILITY STATEMENT

All sequences generated in this study have been deposited to GenBank. Representative isolates correspond to accession numbers MZ127529, MZ127532, MZ127533; MZ127538, MZ127545, MZ127546, MZ127551, MZ127556, MZ127560, MZ127562, MZ127564, MZ127565, MZ127566, MZ127567, MZ127569, MZ127571, MZ127572, MZ127573, MZ127576, MZ127578, MZ127579, MZ127580, MZ127582, and MZ127584. The genomes supporting the reported results have been deposited to GenBank under the BioProjectID PRJNA770179.

AUTHOR CONTRIBUTIONS

XL and DZ designed this research. XL collected the seawater samples and tested environmental parameters. WH and ZJ isolated the strains and performed 16SrDNA amplification and DNA sequencing. SL and JZ performed the genome sequencing, assembly and annotation and analysis of the geomes and nitrogen metabolism. WH tested nitrate reduction of strains. WH and SL drafted the manuscript. XL and DZ supervised the study and contributed to text preparation and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.720413/full#supplementary-material

Supplementary Figure 1 | The phylum **(A)** and genus-level **(B)** composition of bacteria isolated from NAM.

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