ARTICLE

Uncultured *Nitrospina*-like species are major nitrite oxidizing bacteria in oxygen minimum zones

Xin Sun¹ · Linnea F. M. Kop² · Maggie C. Y. Lau^{1,3} · Jeroen Frank² · Amal Jayakumar¹ · Sebastian Lücker² · Bess B. Ward¹

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Abstract

Oxygen minimum zones (OMZs) are marine regions where O₂ is undetectable at intermediate depths. Within OMZs, the oxygen-depleted zone (ODZ) induces anaerobic microbial processes that lead to fixed nitrogen loss via denitrification and anammox. Surprisingly, nitrite oxidation is also detected in ODZs, although all known marine nitrite oxidizers (mainly *Nitrospina*) are aerobes. We used metagenomic binning to construct metagenome-assembled genomes (MAGs) of nitrite oxidizers from OMZs. These MAGs represent two novel *Nitrospina*-like species, both of which differed from all known *Nitrospina* species, including cultured species and published MAGs. Relative abundances of different *Nitrospina* genotypes in OMZ and non-OMZ seawaters were estimated by mapping metagenomic reads to newly constructed MAGs and published high-quality genomes of members from the Nitrospinae phylum. The two novel species were present in all major OMZs and were more abundant inside ODZs, which is consistent with the detection of higher nitrite oxidation rates in ODZs than in oxic seawaters and suggests novel adaptations to anoxic environments. The detection of a large number of unclassified nitrite oxidoreductase genes in the dataset implies that the phylogenetic diversity of nitrite oxidizers is greater than previously thought.

Introduction

Fixed nitrogen in large areas of the ocean limits the growth of marine phytoplankton, which are responsible for half of the global photosynthetic carbon fixation [1, 2]. Thus, fixed nitrogen is of vital importance to primary productivity and carbon cycling. Nitrate is the main nitrogen source for phytoplankton and is produced by nitrite oxidizing bacteria

(NOB). All known NOB are obligate aerobes, but Lipschultz et al. [3] and recent studies [4–7] reported the oxidation of nitrite to nitrate within oxygen minimum zones (OMZs). OMZs are characterized by a sharp O₂ gradient (i.e., oxycline) overlying an oxygen depleted zone (ODZ) where the O₂ concentration is low enough to induce anaerobic microbial processes, including denitrification and anaerobic ammonium oxidation (anammox). Since no anaerobic NOB have been identified, it remains unclear how nitrite oxidation occurs in this effectively anoxic environment.

Nitrite oxidation near the top of the ODZ may be fueled by nanomolar levels of O₂ through episodic invasions of oxygenated seawater [8, 9] or by in situ production from photosynthesis in the deep chlorophyll maximum [10]. Nitrite oxidation in the dark core of the ODZ is more difficult to explain. Nitrite oxidation either occurred under nearly anoxic conditions (nanomolar oxygen levels; [11]) or was inhibited when oxygen increased to the micromolar range [5], implying the reaction was carried out by NOB specialists adapted to low O₂ or anoxia.

Species affiliated with the phylum Nitrospinae are the dominant NOB in the marine environment [12–14]. The

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✉ Xin Sun
xins@princeton.edu

¹ Department of Geosciences, Guyot Hall, Princeton University, Princeton, NJ 08544, USA

² Department of Microbiology, Radboud University, Nijmegen, AJ 6525, the Netherlands

³ Present address: Laboratory of Extraterrestrial Ocean Systems, Institute of Deep-Sea Science and Engineering, Chinese Academic of Sciences, Sanya, Hainan 572000, China

only two species in culture, *Nitrospina gracilis* and *Nitrospina watsonii*, were isolated under high (atmospheric level) O₂ concentrations [15, 16]. The genome of *N. gracilis* has been published [14], and the 16S ribosomal RNA (rRNA) gene sequence of *N. watsonii* is more than 97% identical to that of *N. gracilis* [15]. Their physiological characteristics suggest that these two *Nitrospina* species are unlikely to be responsible for apparently anaerobic nitrite oxidation in ODZs.

NOB diversity has been explored by sequencing *Nitrospina*-like 16S rRNA genes in seawater [13, 17] or nitrite oxidoreductase (*nrx*) genes in marine sediments [18]. These approaches, however, do not allow linking the 16S rRNA-inferred phylogeny to a functional gene (e.g., *nrx*)-derived physiological potential. Single cell genomics and metagenomic binning allow whole-genome reconstruction, which links taxonomic identities of uncultured species to their functional potentials. A recent study recovered novel *Nitrospina*-like single-cell amplified genomes (SAGs) from the ocean, but only two incomplete SAGs (13 and 21% estimated genome completeness) originated from an OMZ [19].

In this study, we used metagenomics to investigate NOB in all three major OMZs, including the Eastern Tropical North (ETNP) and South Pacific (ETSP) and the Arabian Sea. We recovered metagenome-assembled genomes (MAGs) of two uncultured *Nitrospina*-like species (estimated completeness up to 93.1%) from the ETNP and ETSP, which allowed us to analyze their genomic potentials and determine their relative abundance in all three major OMZs and global oxygenated seawaters.

Materials and methods

Experimental site and sample collection

Seawater samples were collected from a coastal station in the OMZ of the ETSP (20.50°S, 70.70°W; Fig. S1) in 2013 on the R/V Nathaniel B. Palmer (NBP 1305). The vertical minimum O₂ concentration was consistently below the STOX sensor detection limit (10 nM; [20]). Particulate material from 8 L of seawater was collected on 0.22-μm Sterivex filters using a peristaltic pump. Sterivex filters were flash-frozen in liquid nitrogen onboard and stored at −80 °C until DNA extraction. Based on published profiles of O₂, nutrients (Fig. S2; [21]) and nitrite oxidation rates [22], samples from four depths (45, 80, 200, and 300 m) were selected for metagenomic sequencing.

DNA extraction and sequencing

DNA was extracted using the plant tissue protocol (All Prep DNA/RNA Mini Kit, Qiagen, Valencia, CA, USA) from

Sterivex filters. The quality of extracted DNA (length and concentration) was verified by the Agilent 2100 Bioanalyzer System. Randomly amplified DNA fragments were paired-end sequenced on an Illumina MiSeq to generate a total of 28 million read pairs (average length 350 bp) for four samples. DNA quality checking, random amplification, and sequencing were conducted by the Genomics Core Facility of Lewis-Sigler Institute for Integrative Genomics at Princeton University.

Metagenomic analyses

Raw reads were deposited on the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) server [23] under the accession numbers mgm4842562.3, mgm4842563.3, mgm4842564.3, and mgm4842565.3. MG-RAST read annotation was performed to gain an overview of microbial community composition. The RefSeq database was chosen to classify all reads into different species based on best hits, and the Subsystem database to assign reads into different functional categories. α-diversity of microbial species and functional genes was calculated based on classification results against the RefSeq database and the Subsystem database, respectively. Analyses targeting NOB are described below.

The four ETSP metagenomes from individual depths were combined, and the metagenome co-assembly and binning were performed using a pipeline developed in-house. Briefly, the quality of the reads was assessed using FastQC (Babraham Bioinformatics, Babraham Institute, Cambridge, UK). Reads were trimmed and quality-filtered using BBDuk from BBTools (DOE Joint Genome Institute, Walnut Creek, CA, USA) and assembled into metagenomic contigs using metaSPAdes [24]. Subsequently, the filtered reads were mapped to the assembled contigs using the Burrows-Wheeler Aligner [25] and SAMtools (v. 1.6, [26]) to obtain coverage information. Binning was performed using BinSanity [27], COCACOLA [28], CONCOCT [29], MaxBin 2.0 [30], and MetaBAT 2 [31]. Bins were dereplicated and aggregated using the DAS Tool [32], and their quality was assessed by checkM [33]. Two *Nitrospina*-like MAGs from ETSP metagenomes were submitted to NCBI under BioSample accession numbers SAMN10411459 (MAG-1) and SAMN10411419 (MAG-2). We also constructed one *Nitrospina*-like MAG from a published ETNP metagenome [34] using MetaBAT 2, but no *Nitrospina*-like MAG could be constructed from ETNP metagenomes from Glass et al. [35] or ETSP metagenomes from Stewart et al. [36].

Protein-coding sequences in the ETSP *Nitrospina*-like MAGs were predicted by Prodigal v.2.6.3 [37], and annotated using Prokka 1.12-beta [38] with the RefSeq bacterial nr protein database as a reference. Key genes (Table S3)

were identified using a combination of the prokka annotation, best bi-directional BLAST hits with *N. gracilis*, and BLASTp searches. First, best bi-directional BLAST hits (e-value cutoff: 10^{-9}) were determined between the amino acid sequences of *N. gracilis* and the two ETSP MAGs. Subsequently, the prokka annotation was searched for the remaining key genes. Presence or absence of genes in one or both MAGs was determined with BLASTp searches (e-value cutoff: 10^{-10}). BLASTp hits with a bitscore higher than 59 were individually evaluated. No *nrxB* genes were binned into either MAG, but we identified the best *nrxB* candidates for the MAGs as follows: we assembled reads from each metagenome separately and got a MAG (MAG-80m) almost identical to ETSP MAG-1 (average nucleotide identity = 99.5%), which contained a *nrxB* sequence. We used this *nrxB* sequence to search against all contigs from the co-assembly, and extracted the best hit (NODE_69234, 99.1% identical). In addition, we calculated the corrected relative abundance of all *nrxB* genes found in the ETSP metagenomes and compared them with the ETSP *Nitrospina*-like MAGs.

dRep [39] was used to select high-quality (completeness > 75%), non-redundant genomes from all published genomes of members of the phylum Nitrospinae for further analyses. The selected genomes can be found in the NCBI database under the accession numbers GCA_000341545.2, GCA_002453875.1, GCA_001542995.1, GCA_001803525.1, GCA_002238965.1, GCA_002377645.1, GCA_002500605.1, GCA_002786505.1, GCA_002787235.1, GCA_002796165.1, and at IMG/M under the accession numbers 2681812871, 2651870060, 2651870063, and 2651870059. A phylogenetic tree of these genomes was constructed based on concatenated alignments of 92 core genes using the UBCG pipeline [40] with default settings. The genomes of the following four *Nitrospira* species served as outgroup: *Nitrospira moscoviensis* NSP M-1 (GCF_001273775.1), *N. inopinata* ENR4 (GCF_001458695.1), *N. japonica* NJ11 (GCF_900169565.1), and *N. defluvii* (GCF_000196815.1). The final tree was constructed from the concatenated UBCG alignment using RAxML v. 8.2.11 [41] with the “GTRCAT” protein model and 100 bootstraps replications.

The average nucleotide identity (ANI) between all available Nitrospinae genomes and the recovered *Nitrospina*-like MAGs was calculated using orthoANI [42]. Genome comparison among dereplicated published Nitrospinae genomes and newly constructed *Nitrospina*-like MAGs was performed with the anvi'o v.4 pangenomics workflow [43], using BLASTp to calculate similarities between amino acid sequences and with an MCL inflation parameter of 10 [44]. Unique genes in each OMZ MAG (Table S4A, B) and genes found in both OMZ MAGs, but not any of the other Nitrospinae genomes (Table S4C) were determined using the anvi'o interactive view of the

pangenome. Amino acid sequences were annotated using BLASTp searches (e-value cutoff: 10^{-10}) against the Swissprot and the NCBI nr databases. Hits shorter than 100 bp and eukaryotic hits were removed.

Contigs in *Nitrospina*-like MAGs were searched against the SILVA-119 database using BLASTn in order to identify *Nitrospina*-like rRNA genes (ETSP MAG-1: 427 bp, ETSP MAG-2: 882 bp). A 16S rRNA gene reference set was constructed by blasting (BLASTn) the *N. gracilis* 16S rRNA gene sequence (NR_104821.1) against the NCBI nt database. The 16S rRNA gene sequences of the top 250 hits were then clustered with usearch [45] using an identity threshold of 0.99. In addition, 16S rRNA sequences from published Nitrospinae genomes were extracted by blasting the genome sequence against the SILVA-132 database. All 16S rRNA gene sequences were aligned using MUSCLE v. 3.8.425 (alignment length 1644 bp; [46]) and a phylogenetic tree was calculated without use of conservation filters in RAxML v. 8.2.11 [41] with the “GTRCAT” protein model and 100 bootstraps replications.

nrxB genes in the metagenome were identified by blasting (tBLASTn) the *N. gracilis* *nrxB* sequence (AGF29467.1) against all contigs. Only hits with identity >50% to the *N. gracilis* *nrxB* and a minimum length of 390 bp were used for the subsequent analysis. The *nrxB* tree was constructed from a MUSCLE alignment (v. 3.8.425, alignment length 512 bp; [46]) without use of conservation filters in RAxML v. 8.2.11 [41] with the rapid bootstrapping algorithm (100 bootstraps) using automatic protein model selection (PROTGAMMAAUTO). Selenate reductase sequences served as outgroup. Trees were visualized in iTOL v4.2.3 [47].

The occurrence of the two newly constructed *Nitrospina*-like MAGs in all three major OMZs was determined by mapping reads from this study and published metagenomic data from the ETNP [35], the ETSP [36] and Arabian Sea [48] to the MAGs using Bowtie2 (v. 2.3.1; [49]) and SAMtools (v. 1.6; [26]). In order to determine the presence of the OMZ *Nitrospina*-like MAGs in the surface ocean, 12 co-assemblies of TARA Oceans metagenomes [50] were screened. To limit the use of computational resources, unique genes of the MAGs determined with the anvi'o pangenomics workflow were blasted (tBLASTn) against the co-assemblies first. If there were hits with more than 98% identity for a certain co-assembly, unique genes were blasted (tBLASTn) against all individual metagenomes generating this co-assembly. Subsequently, read mapping was performed for those individual metagenomes that had hits with more than 98% identity. The presence of other Nitrospinae genomes in the ETSP OMZ was determined by mapping metagenomic reads from the ETSP OMZ to these genomes as described above. All Bowtie2 mappings were conducted without trimming (“end-to-end”) and using the

“very-sensitive” preset mode. Only reads with a mapping quality above 20 were included. Relative abundances were calculated by dividing the number of reads mapped to the MAG by the total number of reads in the sample.

Results and discussion

Microbial community composition varies with depth in the ETSP OMZ

The presence of an ODZ at the sampling station in ETSP was indicated by the local nitrite maximum, nitrate deficit and undetectable O₂ concentration at 75–400 m (Fig. S2). The 45 m sample was from oxic surface seawater ([O₂] = 85 µM, [NO₂⁻] = 0.3 µM), the 80 m sample was from the top of the ODZ ([O₂] < 10 nM, [NO₂⁻] = 0.3 µM), and both 200 m and 300 m samples were from the anoxic core of the ODZ (undetectable O₂, [NO₂⁻] > 6 µM).

Variations in chemical and physical conditions with depth in the ETSP OMZ corresponded to a shift in microbial communities. Microbial species α-diversity was highest at the top of the ODZ (80 m; Fig. S3). The α-diversity at 45 m was similar to those at 200 m and 300 m, but the microbial communities in oxic and anoxic environments differed in composition (Fig. S4). For example, Proteobacteria (about half of which are *Pelagibacter* spp.) were relatively more abundant in samples from the core of the ODZ (200 m, 300 m). The prevalence of *Pelagibacter* spp. in the anoxic ODZ core is consistent with the nitrate reducing capacity of some *Pelagibacter* clades, previously reported in the ETNP [34]. Thaumarchaeota (ammonia-oxidizing archaea, AOA) had high relative abundances at 45 m and 80 m, but were undetectable in the core of the ODZ. Consistently, the AOA *Nitrosopumilus maritimus* was identified as one of the most abundant species only in the surface of the ETSP OMZ [36]. This observed confinement of AOA to oxygenated water layers was likely driven by O₂ availability, since ammonia oxidation in AOA is an obligate aerobic process.

The shift in microbial communities was also observed in functional gene-based analyses, where the α-diversity decreased with increasing depth (Fig. S3). One example of functional gene variation among communities from different depths was the absence of photosynthesis genes in the dark core of the ODZ (Fig. S5).

Two novel *Nitrospina*-like species were discovered in ODZs

Four *Nitrospina*-like MAGs were constructed from the new ETSP metagenomes and one MAG from a previously published ETNP metagenome ([34]; Table S1). Based on

single-copy gene counts [33], the ETSP MAG-1 and MAG-2 both had an estimated completeness of 93.1%, and the ETNP MAG of 77.1%. Due to the low completion of ETSP MAG-3 and MAG-4 (completeness < 30%), these were excluded from later analyses. The fact that multiple *Nitrospina*-like MAGs were recovered, however, suggests that distinct *Nitrospina*-like NOB populations co-exist within OMZs. The GC content (45.6% for ETSP MAGs and 45.7% for the ETNP MAG, Table S1) of OMZ MAGs was consistently lower than that of *Candidatus Nitromaritima* RS (~50.0%; [51]), *N. gracilis* (57.3%; [14]), and *N. watsonii* (55.6%; [15]), but similar to that of a *Nitrospina*-like MAG recovered from the Northern Gulf of Mexico (45.5%; [52]).

Based on the established genus and species cutoff thresholds (ANI < 70% and < 95%, respectively; [53]), pairwise genome comparisons indicated that the three MAGs represent two novel species without cultured representatives (Fig. 1). Although from two different geographic regions, ETSP MAG-1 and ETNP MAG were highly similar to each other (ANI = 99.6%) and likely represent the same species, which is distinct from ETSP MAG-2 (ANI = 83.6%; Fig. 1). Due to this high genomic identity and the higher completeness of ETSP MAG-1 based on the dRep analysis, ETNP MAG was excluded from later analysis. Both remaining MAGs differed from all publicly available *Nitrospina*-like species at the species level (ANI < 75%) and from *N. gracilis* (the only cultured representative for which a genome is available) at the genus level (ANI < 67%). The novelty of the OMZ MAGs was also confirmed by phylogenetic analyses based on concatenated bacterial core protein alignments encompassing a dereplicated subset of all published high-quality (genome completeness > 75%) *Nitrospina*-like genome representatives (Fig. 1). In the resulting tree, the two ETSP MAGs formed a separate clade with two draft genomes from the North Atlantic Gyre (*Nitrospina* sp. AG-538-K21 and AB-629-B18) as closest relatives. This phylogenetic divergence of the two ETSP MAGs was also indicated by 16S rRNA gene-based phylogenetic analyses (Fig. 2). In the tree containing 16S rRNA gene sequences of the two ETSP MAGs, *Nitrospina* 16S rRNA gene sequences, and long sequences (>1000 bp) from all publicly available *Nitrospina*-like genomes, sequences of ETSP MAGs are closest to sequences recovered from the northeast subarctic Pacific Ocean OMZ, indicating that *Nitrospina*-like organisms found in OMZs are phylogenetically distinct from other marine NOB, including the two cultured representatives, *N. gracilis* and *N. watsonii*. This genome-level divergence implies greater functional diversity among NOB, as previously suggested [54].

The observed differences of the novel *Nitrospina*-like OMZ MAGs from cultured representatives and all MAGs derived from non-OMZ environments indicate niche

Fig. 1 Phylogenetic placement of the two *Nitrospina*-like MAGs from the ETSP OMZ metagenomes (in bold) based on concatenated alignments of 92 core protein sequences. The tree contains all available non-redundant *Nitrospinae* genomes with an estimated completeness >75%. Open and closed circles represent bootstrap support $\geq 90\%$ and

=100%, respectively. The arrow indicates the location of the outgroup (*Nitrospira*). The scale bar represents 10% estimated sequence divergence. In the right panel, the average nucleotide identities (%) between the *Nitrospinae* genomes are shown

differentiation of *Nitrospina* species in the ocean. O_2 and NO_2^- might be the primary drivers of this differentiation, since non-OMZ marine environments usually have much higher O_2 but much lower NO_2^- concentrations. In addition, O_2 and NO_2^- concentrations used to enrich *N. gracilis* [16] were at least 100-fold higher than those found in the OMZ.

OMZ NOB are more diverse than previously thought

Functional genes often are conserved across phylogenetically distant species. Since the organisms responsible for nitrite oxidation in OMZs remain unknown, using the *nxr* gene as a proxy for nitrite oxidizing capacity may identify novel microorganisms mediating this reaction. We used the *N. gracilis* *nxrB* gene sequence to probe for related genes in the metagenomic assemblies. We also estimated their relative abundance in the ETSP OMZ (Fig. S6). None of the 17 *nxrB*-like genes present in the metagenome could unequivocally be linked to either of the two MAGs, but based on consistent relative abundance distribution of genes versus MAGs (Fig. S6) and the association of a *nxrB* gene in the

ETSP MAG-80m, we have identified likely candidates. We propose that NODE_69234 and NODE_114897 contain the best *nxrB* candidates for MAG-1 and MAG-2, respectively. Both of these two *nxrB* genes and another *nxrB* gene were highly similar to the *nxrB* of *N. gracilis* (NODE_102582, 96.9%; NODE_69234, 96.7%; and NODE_114897, 96.7% identity) and clustered with *Nitrospina* *nxrB* genes in the tree (Fig. 3). This analysis implies that the two novel OMZ MAGs represent *Nitrospina*-like species mediating nitrite oxidation in the ETSP OMZ. Two other *nxrB*-like sequences (NODE_1303 and NODE_9134) cluster together with anammox (Fig. 3), which is consistent with the detection of anammox activity at the same time in this OMZ [22] and the ability of anammox bacteria to oxidize nitrite to nitrate.

Moreover, a group of *nxrB*-like sequences formed a novel sequence cluster between the anammox and *Nitrospina* *nxrB* clusters. This group is most closely related to MAGs recovered from a suboxic/anoxic aquifer near Rifle (CO, USA; [55]). It is tempting to speculate that these putative *nxrB* sequences represent novel, yet unidentified nitrite oxidizers, as was also implied by a recent gene-centric *nxrA*-based analysis performed in the Arabian Sea [48].

Fig. 2 16S rRNA gene-based phylogenetic analysis of selected members of the genus *Nitrospina*. Sequences of the two ETSP OMZ MAGs from this study are indicated in bold. Open and closed circles represent bootstrap values $\geq 70\%$ and $\geq 90\%$, respectively. The arrow indicates the position of the outgroup (*Nitrospira*). The scale bar

corresponds to 10% estimated sequence divergence. The tree was calculated using the rapid bootstrapping algorithm (100 bootstrap iterations). No conservation filter or weighting mask was used, resulting in 1644 valid alignment positions

Fig. 3 Phylogenetic analysis of selected *nxrB*-like sequences. Sequences from the ETSP OMZ metagenomes are indicated in bold. Open and closed circles represent bootstrap values $\geq 70\%$ and $\geq 90\%$, respectively. The arrow indicates the position of the outgroup (selenate

reductase sequences). The scale bar corresponds to 10% estimated sequence divergence. The tree was calculated with a rapid bootstrapping algorithm (100 bootstrap iterations). No conservation filter was used, resulting in 512 alignment positions

Lastly, there are two *nxrB* sequences (NODE_16314 and NODE_85900) only distantly related to classified *nxrB* genes. Their low identities to sequences represented in the NCBI nr database (NODE_16314, 55% identity to *nxrB* from *Candidatus Scalindua brodae* [KHE93153.1]; NODE_85900, 57% identity to hypothetical protein RBG1_1C00001G0009 from the candidate division Zixibacteria bacterium RBG-1 [EQB62430.1]), their distinct clustering in the phylogenetic tree (Fig. 3), and the fact that they could not be binned into any MAG leave their exact function as well as their phylogenetic affiliation unclear.

However, a role in either nitrite oxidation or nitrate reduction is highly probable.

Distribution of *Nitrospina*-like MAGs and implications for nitrite oxidation in OMZs

The relative abundances of ETSP MAG-1 and MAG-2 were determined in order to estimate their distributions in three major OMZs (Fig. 4). Both species were very rare in oxygenated surface seawaters. MAG-1 was more abundant than MAG-2 at the top of the ODZ, but the relative abundance of

Fig. 4 Relative abundance of OMZ MAG-1 and MAG-2 in ETSP, ETNP, and Arabian Sea OMZ metagenomes from this and other studies, and relative abundance of non-OMZ *Nitrospina*-like MAGs in ETSP metagenomes from this study. Shaded areas in the plots indicate

the location of the anoxic ODZ. Dashed lines between dots are used as a visual guide to differentiate different NOB, they do not indicate linear changes

MAG-2 usually exceeded that of MAG-1 in the core of the ODZ. Niche differentiation within the OMZ (ODZ-top versus ODZ-core) might result from different O_2 and NO_2^- concentrations, as well as other environmental factors (such as temperature, light, and nitrate). In the Arabian Sea, the two MAGs were more abundant at 600 m than at 170 m, and they had similar relative abundances at both depths. The O_2 concentration at 600 m ($2.8 \mu M$) in the Arabian Sea

was slightly above detection limit ($2.5 \mu M$), and the NO_2^- concentration was only $0.5 \mu M$ [56]. Conditions at 600 m of the Arabian Sea are more similar to the environments at the top of the ODZ in the ETSP and ETNP than to the ODZ core, where O_2 is below $10 nM$ and NO_2^- is at μM levels. These contrasting distributions imply different O_2 preferences, although both MAGs are present at all depths. Conditions at the top of the ODZ apparently allow the

coexistence of different NOB adapted to either low O₂ (nM-level) or anoxic conditions. Nitrite oxidation rates in OMZs either increased with only nM levels of O₂ addition [11] or decreased when O₂ addition reached μ M levels [5, 57]. One possible explanation for this paradox is that samples from different sites were dominated by NOB adapted to different O₂ conditions.

The relative abundance of *Nitrococcus mobilis* is comparable with *Nitrospina*-like MAGs only in the Arabian Sea OMZ (0.27% at 170 m and 0.43% at 600 m). In all other OMZs (ETNP, ETSP-Stewart, ETSP–this study), *Nitrococcus* was not detected. The other Nitrospinae species could only be found at very low abundances in this dataset of the ETSP OMZ (Fig. 4). In addition, OMZ *Nitrospina*-like species are the only NOB species detected by binning from ETSP and ETNP metagenomes. These results suggest that OMZ *Nitrospina*-like NOB, rather than *Nitrococcus* or other *Nitrospina* species (i.e., *N. gracilis* or *Ca. Nitromaritima* RS), are the dominant NOB in the OMZ. At the same time that our metagenomic samples were collected, Babbitt et al. [22] detected nitrite oxidation in incubation bottles in which both anaerobic nitrogen-loss processes, anammox and denitrification, were also detected. The distribution of nitrite oxidation rates with depth was consistent with that of the estimated relative abundance of the two MAGs recovered here (Fig. 4), suggesting these novel species were indeed responsible for the measured rates.

Contrastingly, in non-OMZ metagenomes from the surface ocean, the OMZ *Nitrospina*-like MAGs were not found, or were detected only at very low relative abundances (Table S2). Together with the phylogenetic distance between OMZ and non-OMZ NOB (Figs. 1 and 2), these results indicate that the *Nitrospina*-like MAGs found in this study are unique to OMZ environments. The detection of novel unclassified *nrxB* genes (Fig. 3 and Fig. S6), however, indicates that other species may also contribute to nitrite oxidation in OMZs. Since it is usually easier to recover (draft) genomes from more abundant species, other less abundant species may have been captured only partly, and the total relative abundance of NOB in OMZs thus was likely underestimated by our approach.

Metabolic pathways of OMZ MAGs

Although neither of the two *Nitrospina*-like MAGs is 100% complete, it is useful to evaluate presence and absence of metabolic pathways to assess the biogeochemical role and metabolic potential of the new organisms, and to motivate further research on key pathways. Some of the genes identified as “missing” here might be present in the complete genome, and thus targeted efforts should be made to verify their absence. Adaptations in their respiratory pathways might allow the OMZ *Nitrospina*-like species to

occupy low O₂ or anoxic niches. ETSP MAG-1 encodes a b(a/o)3-type (type B) cytochrome *c* oxidase, which could function as terminal oxidase in this organism (Tables S3 and S4). This contrasts with *N. gracilis*, which encodes a *cbb*₃-type terminal oxidase (C-type). Both B- and C-type oxidases have higher affinities than those belonging to type A, which indicates that *Nitrospina* generally are well adapted to low-oxygen environments [58]. Thus, NOB from ODZs with high-affinity terminal oxidases might be able to start oxidizing nitrite when trace amounts of O₂ are supplied. These NOB might be the major contributors to the nitrite oxidation rates measured [5] and modeled [8] at both upper and lower boundaries, where O₂ diffuses into the ODZ. However, a cytochrome *c* oxidase could not be found in ETSP MAG-2 (Tables S3 and S4). If this lack of high O₂-affinity terminal oxidases in MAG-2 is not due to the incompleteness of the draft genome, it could explain why MAG-2 is less abundant than MAG-1 at the ODZ top where nM levels of O₂ are available (Fig. 4). Moreover, this might indicate the presence of novel unidentified cytochrome *c* oxidases, or the use of alternative pathways to oxidize nitrite anaerobically. Similar to *N. gracilis*, both MAGs encode proteins with limited similarity to cytochrome *bd*-type quinol oxidases, which are also conserved in all genome-sequenced *Nitrospira* and anammox species [59]. However, based on the lack of quinol-binding sites, they are unlikely to function as canonical cytochrome *bd* oxidases [14], and the possible role of these cytochrome *bd*-like oxidases in nitrite oxidation remains unknown.

Most genes involved in oxidative phosphorylation are present in both ETSP MAGs (Table S3). Consistent with known *Nitrospina* species [14, 19], the novel *Nitrospina*-like NOB fix CO₂ via the reductive tricarboxylic acid cycle (rTCA; Table S3). While two of the three key genes for the rTCA cycle, ATP-dependent citrate lyase (ACL), and 2-oxoglutarate:ferredoxin oxidoreductase (OGOR) were detected in both MAGs, not all subunits of the pyruvate:ferredoxin oxidoreductase (POR) were identified. The apparent absence of two of the subunits of POR is likely due to genome incompleteness and insufficient sequencing depth, as some subunits of the complex were found (Table S3). In addition, there was no indication of other CO₂ fixation pathways in these *Nitrospina*-like organisms, such as Calvin–Benson–Bassham cycle used by *Nitrococcus mobilis* [57]. The use of the rTCA cycle by the novel *Nitrospina*-like species is consistent with their apparent preference for low energy, low-oxygen environments [14, 19].

Although O₂ diffusion could influence waters just below the oxycline, nitrite oxidation occurs in the core of the ODZ where such O₂ supply is not possible. While OMZ NOB were speculated to couple nitrite oxidation to iodate reduction [22], this is unlikely to be the explanation of

apparently anaerobic nitrite oxidation NOB in ODZs because iodate, similar to O_2 , is also depleted in ODZ cores [60]. Anaerobic nitrite oxidation can be catalyzed by anoxygenic photosynthesis [61], which, however, is also unlikely in the dark ODZ core. It should be noted that although anammox bacteria also oxidize nitrite anaerobically to nitrate, the low anammox rates at this site could not explain the measured nitrite oxidation rates. One possible explanation for this observation is the production of nitrate through nitrite dismutation [62]. To date, only one study directly explored the possibility for nitrite dismutation in the Namibian OMZ, but failed to detect this process [57]. However, further studies in other OMZs are needed to test this possibility. Anaerobic nitrite oxidation may be mediated by novel *Nitrospina*-like species discovered in this study, or by other yet unidentified species as indicated by the detection of unclassified putative *nrrB* genes (Fig. 3). One possibility we cannot rule out is that the in situ nitrite oxidation rates were zero, especially at the ODZ core, and that measured nitrite oxidation rates were fueled by trace O_2 contamination during sampling. The detection of *Nitrospina*-like species by both marker gene analyses and metagenomic binning, however, implies that these bacteria have the potential to oxidize nitrite with terminal electron acceptors other than O_2 , or conserve energy by alternative anaerobic pathways for survival in anoxic environments. The potential of the two MAGs to reduce nitrite is indicated by the presence of nitrite reductase genes, but no nitric oxide reductase gene or nitrous oxide reductase gene was recognized in the MAGs, as is the case for all NOB genomes. Therefore, it is unlikely that the OMZ NOB play a role in complete denitrification. The possibility of conducting nitrate respiration when conditions do not favor nitrite oxidation has been shown for the NOB *Nitrococcus* [57] and *Nitrospira* [63]. Thus, OMZ NOB might survive in ODZs by alternative anaerobic metabolisms and turn on the conventional nitrite oxidation pathway when minute amounts of O_2 become available.

Although perhaps not central to the nitrite oxidation metabolism, the draft genomes contained additional features that provide insights into the ecology of the OMZ NOB. In contrast to *N. gracilis* [14], the two ETSP MAGs do not encode a sulfhydrogenase or a sulfite:cytochrome *c* oxidoreductase, making it unlikely that these organisms use sulfur compounds for energy conservation. It is known that some nitrifiers can use urea or cyanate as nitrogen source for assimilation or as indirect energy source in a reciprocal feeding mechanism in symbiosis with ammonia-oxidizing microorganisms (AOM; [63, 64]). While neither of the ETSP MAGs possesses a *cynS* gene, the presence of genes encoding a urease and urea transporters in both MAGs indicates their potential to use urea. In reciprocal feeding, they would provide ammonia from urea degradation to the

AOM and would receive their energy source nitrite in return. Chlorite dismutase precursor genes were found in both ETSP MAGs. Since the *Nxr* can catalyze chlorate reduction to chlorite [65], these novel species have the potential to reduce chlorate. Chlorate is not abundant in anoxic marine environments, so the chlorite dismutase genes might support some unidentified alternative anaerobic pathway. Lastly, both OMZ MAGs encoded a siderophore transport systems, but neither of them has a siderophore biosynthesis pathway (Table S3). Many microbes produce siderophores to scavenge essential metals, mainly iron, from the environment [66]. The presence of siderophore transporters indicates that these *Nitrospina*-like organisms may take advantage of siderophores produced by other organisms in the OMZ, as has been suggested for the ammonia-oxidizing bacterium *Nitrosomonas europaea* [67].

Conclusions

Three novel *Nitrospina*-like MAGs from ETSP and ETNP OMZs, representing two novel NOB species, were reconstructed from newly obtained and previously published metagenomic data. They represent nearly complete genomes of two novel species, which lack cultured representatives and are distinct from *N. gracilis* and *N. watsonii* at the genus level. The relative abundance patterns of these novel NOB were consistent with the distribution of nitrite oxidation rates in OMZ environments. The OMZ MAGs were rarely observed in metagenomic datasets from oxygenated surface seawaters, and the NOB recovered from oxygenated regions are rarely present in OMZs, implying niche partitioning of marine NOB species. These novel *Nitrospina*-like OMZ NOB, along with additional unidentified putative nitrite oxidizers indicated by the presence of unclassified *nrrB*-like sequences, may contribute to the apparently anaerobic nitrite oxidation detected in OMZs, or may use alternative pathways to survive in ODZs when conditions are not favorable for nitrite oxidation. The discovery of novel NOB species in an environment thought to be unsuitable for NOB suggests that the versatility and diversity of NOB thus may be even larger than previously recognized.

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Author contributions XS and BBW planned the study. XS and LFMK performed all metagenomic analyses. JF and MCYL set up pipelines

for metagenomic analyses. AJ provided DNA samples. XS, LFMK, SL, and BBW analyzed data and wrote the paper. All authors approved the final paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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