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Metagenome-assembled genomes for oligotrophic nitrifiers from a mountainous gravelbed floodplain

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ABSTRACT

Riparian floodplains are important regions for biogeochemical cycling, including nitrogen. Here, we present MAGs from nitrifying microorganisms, including ammonia-oxidizing archaea (AOA) and comammox bacteria from Slate River (SR) floodplain sediments (Crested Butte, CO, US). Additionally, we explore MAGs from potential nitrite-oxidizing bacteria (NOB) from the *Nitrospirales*. AOA diversity in SR is lower than observed in other western US floodplain sediments and *Nitrosotalea*-like lineages such as the genus TA-20 are the dominant AOA. No ammonia-oxidizing bacteria (AOB) MAGs were recovered. Microorganisms from the Palsa-1315 genus (clade B comammox) are the most abundant ammonia-oxidizers in SR floodplain sediments. Established NOB are conspicuously absent; however, we recovered MAGs from uncultured lineages of the NS-4 family (*Nitrospirales*) and *Nitrospiraceae* that we propose as putative NOB. Nitrite oxidation may be carried out by organisms sister to established *Nitrospira* NOB lineages based on the genomic content of uncultured *Nitrospirales* clades. Nitrifier MAGs recovered from SR floodplain sediments harbor genes for using alternative sources of ammonia, such as urea, cyanate, biuret, triuret, and nitriles. The SR floodplain therefore appears to be a low ammonia flux environment that selects for oligotrophic nitrifiers.

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INTRODUCTION

Nitrification, the oxidation of ammonia to nitrate via nitrite, links nitrogenous organic matter remineralization to nitrogen (N) loss processes in the microbial N-cycle. Nitrification is carried out by phylogenetically constrained guilds of microorganisms, including: ammonia-oxidizing archaea (AOA) or bacteria (AOB), responsible for the oxidation of ammonia to nitrite; nitrite-oxidizing bacteria (NOB), responsible for the oxidation of nitrite to nitrate; and complete ammonia-oxidizing bacteria (comammox), capable of carrying out both steps. A host of environmental factors impact the distribution of these different ammonia oxidizers (AOA, AOB, and comammox) and their relative contribution to nitrification in terrestrial environments, including but not limited to pH, temperature, and ammonia concentrations (see Lu *et al.*, 2020 and references therein). For example, AOB are generally predominant in higher ammonia flux environments, such as fertilized soils, while AOA thrive in lower ammonia and acidic environments (Gubry-Rangin *et al.*, 2010; Verhamme *et al.*, 2011; Hink *et al.*, 2018). Comammox strains *Nitrospira inopinata* and *Candidatus* Nitrospira kreftii have a very high affinity for ammonia (Kits *et al.*, 2017; Sakoula *et al.*, 2021) and comammox organisms appear to be adapted to low substrate flux environments (Costa *et al.*, 2006; Pinto *et al.*, 2016; Kits *et al.*, 2017; Sakoula *et al.*, 2021). Although comammox are generally adapted to oligotrophic conditions and can even be inhibited by high ammonia concentrations (Sakoula *et al.*, 2021), recent studies have investigated possible niche-partitioning between the two clades of comammox bacteria (clade A and clade B) and found that some terrestrial clade A comammox may be adapted to survive in copiotrophic environments (Palomo *et al.*, 2018; C. Li *et al.*, 2021; Li *et al.*, 2023; Gao *et al.*, 2023). Alternative sources of ammonia, such as urea, may also play an

important role in selecting for certain nitrifiers (Koch *et al.*, 2015; Palatinszky *et al.*, 2015; Hink *et al.*, 2018; Vilardi *et al.*, 2024). NOB are found in several phyla, including the *Proteobacteria*, *Nitrospirota* (formerly *Nitrospirae*), *Nitrospinota* (formerly *Nitrospinae*), and *Chloroflexi,* and many encode metabolic flexibility (Daims *et al.*, 2016). NOB across and within different lineages display niche partitioning shaped by a host of environmental factors, including temperature, pH, oxygen, and nitrite concentrations (Nowka *et al.*, 2015; Daims *et al.*, 2016; Koch *et al.*, 2019; Spieck *et al.*, 2020; Keuter *et al.*, 2022). Of the NOB, *Nitrospira* are the most diverse and are ubiquitous in terrestrial environments, including soils and freshwater systems (Altmann *et al.*, 2003; Daims *et al.*, 2016; Vijayan *et al.*, 2021).

Floodplains are important sites for both organic matter cycling and nitrification. As nitrogenous organic matter is broken down, ammonia is either recycled and assimilated for biomass production or transformed to oxidized N compounds (i.e., nitrite and nitrate) through nitrification. Floodplain sediments can host a great diversity of AOA (Reji *et al.*, 2022) and AOB (Cardarelli *et al.*, 2020), though it appears that AOA are numerically dominant over AOB in several distinct floodplains, including in the Wind River and Upper Colorado River Basin (Cardarelli *et al.*, 2020) as well as the Amazon River Basin (Monteiro *et al.*, 2022). Diverse AOA MAGs from both the terrestrial *Nitrososphaerales* and marine *Nitrosopumilales* orders were recovered from riparian floodplain sediments from the Wind River Basin near Riverton, WY, USA and showed distinct distribution patterns structured by depth (Reji *et al.*, 2022). Not only are AOA abundant and diverse, but both AOA and comammox bacteria can be highly active within floodplain sediments, especially those with low organic carbon, as observed in East River, CO

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floodplain sediments where nitrification genes were the most highly expressed genes in metatranscriptomes (Matheus Carnevali *et al.*, 2021). Nitrification may also support anaerobic methanotrophy in the Amazonian floodplains, where AOA, *Candidatus* Methanoperedens, and *Candidatus* Methylomirabilaceae are all abundant community members and co-occur during flood season (Bento *et al.*, 2021; Gontijo *et al.*, 2021; Monteiro *et al.*, 2022).

Here we present nitrifier MAGs generated from Slate River, CO floodplain sediments collected in June 2018. The Slate River is a high elevation, mountainous river with a gravelbed floodplain where the water table is influenced by snowmelt, precipitation, and evapotranspiration, and reaches its peak height in late spring (May/June). Generally, the fine grain sediments transition from unsaturated and oxic to saturated and anoxic below ~110 cm in the floodplain sites sampled (Dewey *et al.*, 2021).

EXPERIMENTAL PROCEDURES

Study site and environmental data

The Slate River is located in Gunnison County, CO, USA. We sampled 2 floodplain locations (OBJ1 and OBJ2) below the confluence of Oh-Be-Joyful Creek and Slate River (38°54'34.59'' N, 107°1'43.40'' W) every 20 cm from 50-150 cm below the ground surface. Samples were collected on June 18, 2018 (OBJ2) and June 20, 2018 (OBJ1) after the peak water table height in early June. Aqueous phase concentrations of dissolved inorganic nitrogen (DIN) were measured via water extraction by suspending 1 g of sediment in 10 mL of ultrapure water, shaking at room temperature for 2 h, centrifuging at 4,000 rpm for 10-15 min, then filtering the aqueous phase

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through a 0.22 µm disc filter (Fisher Scientific). DIN (ammonia and nitrate) concentrations were measured on a SmartChem 200 Discrete Analyzer (WestCo Scientific Instruments, Brookfield, CT) by spectroscopy.

DNA extraction, sequencing, and metagenomic processing

DNA was extracted from 4.76-5.29 g of frozen soil using the DNeasy PowerMax Soil Kit (Qiagen) with an added incubation step at 80 °C for 40 min immediately after bead beating to improve yields; DNA was eluted in 5 mL solution C6, and concentrated down to ~1 mL with a VivaSpin Turbo 15 column (Sartorius). Metagenomic sequencing was performed by JGI via a FICUS project (Proposal ID 504298) on an Illumina NovaSeq S4. Quality Controlled Filtered Raw metagenome data (JGI Project Ids 1359525 to 1359536) was downloaded from JGI Genome Portal for assembly, binning, and refining using the metaWRAP (v1.3.2) pipeline (Uritskiy *et al.*, 2018). Assemblies and co-assemblies were made using MEGAHIT (v1.3.3) and binned using contigs > 2000 nt and > 2500 nt, respectively, using MetaBAT2 (v2.12.1) (Kang *et al.*, 2019), MaxBin 2.0 (v2.2.6) (Wu *et al.*, 2016), and CONCOCT (v1.0.0) (Alneberg *et al.*, 2013) with multiple fastq files grouped by surface (50cm depth), middle depth (70-110 cm), and anoxic depths (130-150 cm). Bins were consolidated and filtered using *metawrap bin_refinement* to be above > 50% completeness and have < 10% contamination as calculated via CheckM (v1.1.3) (Parks *et al.*, 2015). All MAGs were dereplicated using dRep (v3.1.1) (Olm *et al.*, 2017) at 98% average nucleotide identity (ANI). Taxonomic classification for each representative MAG was made using the Genome Taxonomy Database toolkit (GTDB-tk) (Chaumeil *et al.*, 2020) with the database release 07-RS207. Reads were competitively recruited to dereplicated MAG library

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using Bowtie2 (v2.4.2) (Langmead and Salzberg, 2012) and the default parameters. Abundances are displayed as unpaired reads recruited per genome size of MAG in kilobases of MAG divided by gigabase of metagenome (RPKG) and coverage values calculated using inStrain (Olm *et al.*, 2021). Genes were called using Prodigal (v2.6.3) (Hyatt *et al.*, 2010) and translated gene annotations were performed via KEGG using GhostKOALA (Kanehisa *et al.*, 2016) and eggnog-mapper (v2.1.12) using the eggnog 5 database (Huerta-Cepas *et al.*, 2019; Cantalapiedra *et al.*, 2021).

Phylogenomic and pangenomic analysis

Pangenomic and phylogenetic analyses were carried out using *anvi'o* (v8) (Eren *et al.*, 2015). For *Thaumarchaeota* analyses, *Nitrososphaeria* (n = 553) species representatives from the Genome Taxonomy Database (GTDB; RS220) and *Thaumarchaeota* MAGs generated from the Wind River Basin (Reji *et al.*, 2022), East River Basin (Matheus Carnevali *et al.*, 2021), and additional freshwater environments such as the Amazon River (Santos-Júnior *et al.*, 2017; Pinto *et al.*, 2020), Lake Lugu (Ren and Wang, 2022), the Yangtze River (Liu *et al.*, 2020), and Jiulong River estuary (Zou *et al.*) were downloaded. For *Nitrospirales* analyses, *Nitrospirales* (n = 254) species representatives from GTDB (RS220) and MAGs generated from the Wind River Basin (Reji *et al.*, 2022), East River Basin (Matheus Carnevali *et al.*, 2021), and Antarctic soils and lake sediments (Han *et al.*, 2024) were downloaded, with only genomes > 70% complete (n = 256) used for making pangenomes. Pangenomes were annotated using *anvi-run-hmms* for ribsomal and tRNA (tRNAscan-SE v2.0.12) genes as well as COG, Kegg KO, and Pfams. Concatenated ribosomal trees were made using *anvi-get-sequences-for-hmm-hits* using *Nitrospirales* genomes > 70% complete

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(n = 256) and *Nitrososphaeria* genomes > 50% complete (n = 645), aligned using MUSCLE (v3.8.1551, 40), and trimmed to remove gaps in > 50% of genomes using trimAl (v1.4.rev15, (Capella-Gutiérrez *et al.*, 2009)). The function *anvi-compute-functional-enrichment-in --annotation-source KOfam* was used on the *Nitrospirales* pangenome and between the AOA genera present in SR to identify KOfam-annotated genes that were significantly more common or "enriched" in different groups (Shaiber *et al.*, 2020). Only genomes > 90% completeness were used for testing gene enrichment between clade A comammox (n = 70) and clade B comammox (n = 32) and between different GTDB-defined *Nitrospirales* families, including NS-4 (n = 9), *Nitrospiraceae* (n = 149), and UBA8639 (n = 36) with UBA2166 being excluded due to low genome count (n = 1). ANI was calculated using FastANI (Jain *et al.*, 2018) via *anvi-compute-genome-similarity*. Gene sequences for 16S rRNA gene sequences were retrieved from *Nitrospirales* genomes using *anvi-get-sequences-for-hmm-hits --hmm-source Ribosomal_RNA_16S* and sequences > 1380 nt were aligned and then manually trimmed to the shortest sequence length in Geneious prime (v2024.0.7) before alignment with reference *Nitrospira* sequences as found in Daims *et al.* (2001, 2015) and Keuter *et al.* (2023). The function *anvi-get-sequences-for-gene-clusters* was used to obtain *amoA* and *narH/nxrB* sequences from pangenomes. Additionally, *Nitrospirales* genomes were queried via *blastp* (blast v2.15.0) using full-length *nxrA* and *nxrB* sequences from established NOB, including from *Nitrospira inopinata* and *Nitrobacter winogradskyi* using an e-value cutoff of 1e-20. Protein alignments were made using full length reference sequences and for *narG*/*nxrA* sequences > 500 aa, *narH*/*nxrB* sequences > 200 aa, and *amoA* sequences > 200 aa from the *Nitrospirales* genomes used in our pangenomic analysis. Guanidine degradation genes (Palatinszky *et al.*,

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2024) from Palsa-1315 sp013140715, *Nitrosomonas europaea* ATCC 19718, and *Nitrosospira multiformis* ATCC 25196 were queried against all *Nitrospirales* genomes via *blastp* using an e-value cutoff of 1e-20. POR (*porA*) and OGOR (*forA*) genes from *Nitrospira defluvii* (Lücker *et al.*, 2010) were queried against all *Nitrospirales* genomes via blastp using an e-value cutoff of 1e-150. Blast query lengths and accession numbers are available in Table A1. Alignments were carried out using Clustal Omega (Sievers *et al.*, 2011) unless otherwise stated. Phylogenetic trees were constructed using IQ-TREE (v2.0.3) (Minh *et al.*, 2020)) model finder -MFP to identify the best tree and 1000 bootstraps. Phylogenetic trees were visualized using FigTree (v1.4.4) (Rambaut, 2010) and iTOL (v6) (Letunic and Bork, 2024).

RESULTS

We recovered MAGs from Slate River floodplain sediments corresponding to several guilds of nitrifiers, including AOA, comammox, and putative NOB from within the order *Nitrospirales*. We did not recover any MAGs for AOB or established NOB. All nitrifier MAGs originated from sediments collected from depths shallower than 130 cm. Nitrifier MAGs had highest abundances in depths 50-110 cm and were largely absent from depths 130-150 cm (Fig. 1). Nitrifiers ranged from 0-9% relative abundance in MAG libraries with a median genome coverage of 5.3x (range = 0.7-45.5x, Fig. A1). In June of 2018, ammonia concentrations (measured via water extraction) were typically low (2 - 20 µM) with highest concentrations occurring at the shallowest depth, 50 cm, with a secondary peak in depths 130-150 cm (Fig. A2).

AOA and non-AOA Thaumarchaea MAGs

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From SR floodplain sediments, we recovered 11 (7 after dereplication at 98% ANI) AOA MAGs from four genera from the *Nitrosopumilaceae*, including CSP1-1, *Nitrosotalea*, and two sister genera to *Nitrosotalea*, named TA-20 and UBA8516 in GTDB (RS220) (Table A2, Fig. 2). TA-20 was the most abundant AOA in SR sediments followed by CSP1-1, UBA8516, and *Nitrosotalea*, all of which were much less abundant (Fig. 1). AOA were most abundant from 70 to 110 cm at OBJ1 and 50 cm at OBJ2 (Fig. 1). Very few studies have generated MAGs for AOA from floodplain sediments; however, we can compare the SR AOA MAGs to the 2 *Thaumarchaeota* MAGs generated from the neighboring East River Basin (Matheus Carnevali *et al.*, 2021) and 27 *Thaumarchaeota* MAGs from the Wind River Basin (Reji *et al.*, 2022). East River floodplain sediments yielded only 2 *Nitrosopumilaceae* genomes from within the CSP1-1 and *Nitrosarchaeum* genera (Fig. 2). Wind River Basin MAGs originated from a great diversity of genera, including freshwater clades from within the *Nitrosopumilaceae* such as *Nitrosarchaeum*, *Nitrosotenuis*, and CSP1-1, as well as diverse genera from the *Nitrososphaeraceae* often clustering with AOA from sediments of the River Thames (Sheridan *et al.*, 2020) such as in the *Nitrososphaera*, 'TH1177', and 'TH5896' (Reji *et al.*, 2022). Although the 5 *Nitrosotalea*-like MAGs generated from SR were of medium quality (Table A2), many harbor genes for using alternative sources of ammonia, such as urease and a putative nitrilase (NIT1), and a smattering of flagellar genes indicating their possible motility (Fig. A3). MAGs from the CSP1-1 genus were found only at OBJ1 and are closely related to the original CSP1-1 MAG recovered from aquifer sediments in Rifle, CO (Hug *et al.*, 2016) and other MAGs generated from East River (Matheus Carnevali *et al.*, 2021) and Wind River Basin floodplain sediments (Reji *et al.*, 2022) (Fig. 2). SR

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CSP1-1 MAGs were of medium to high quality (Table A2) and harbored urease, chemotaxis, and flagellar genes (Fig. A3).

We also recovered two non-AOA *Thaumarchaeota* MAGs from the *Ca.* Gagatemarchaeaceae (formerly *Thaumarchaeota* Group I.1c) (Fig. 2), a family of aerobic heterotrophs (Sheridan *et al.*, 2023). These MAGs were present at low abundance from depths 50-90 cm but most abundant at 90 cm despite falling within or sister to the "topsoil" Ca. Gagatemarchaeum (Bog-1369 in GTDB) genus (Fig. 1). The undefined *Ca*. Gagatemarchaeaceae (UBA183 family in GTDB) MAG from SR is more complete than the Ca. Gagatemarchaeum (Bog-1369) MAG (Table A2) and harbors genes for aerobic respiration such as *coxD/ctaF, coxB/ctaC, coxAC* which encode cytochrome c oxidase. Both the *Ca.* Gagatemarchaeum (Bog-1369) and undefined *Ca*. Gagatemarchaeaceae (UBA183 family) MAGs generated from SR harbor genes for carbon monoxide oxidation (*coxMSL)* and a potassium transporter (*kdpABC*) aiding in pH homeostasis. Additionally, the undefined *Ca*. Gagatemarchaeaceae (UBA183) genus MAG also has a small conductance mechanosensitive channel, which may be used for osmoregulation in AOA (Mosier *et al.*, 2012; Ren *et al.*, 2019).

Clade B comammox MAGs

We recovered 6 (4 after dereplication) MAGs from the GTDB-defined genus "Palsa-1315" from within the *Nitrospiraceae*. Palsa-1315 MAGs are only of medium quality (Table A2); however, all but one harbor subunits for AMO and several encode HAO, *nirK*, and NAR/NXR genes (Fig. 3) supporting that these represent comammox organisms. Palsa-1315 is of greater relative

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abundance than AOA at most depths, has peak abundance occurring at 70 cm depth at OBJ1 and 90 cm depth at OBJ2, and is generally of greater relative abundance at OBJ1 than at OBJ2 (Figs. 1 & A1). These MAGs harbor genes for utilizing alternative sources of ammonia, including several that encode urea transporters and urease. One of these comammox MAGs encodes nitrile hydratase (*nthAB*) and another encodes cyanate transport and cyanase (*cynS*) genes (Fig. 3). Genes for guanidine degradation (guanidinase, guanidine carboxylase, carboxylguanidine deiminase, APC superfamily permease) are absent from these comammox MAGs, however, 3 MAGs contain an allophanate hydrolase (*atzF*). The allophanate hydrolase is collocated with 2 copies of biuret hydrolase (*biuH*) and an ABC transporter. A phylogeny of the two *biuH* genes indicates that one of these may be a triuret hydrolase (*trtA*) (Fig. A4). The presence of *atzF*, *biuH*, and *trtA* indicate these comammox may be capable of triuret (carbonyldiurea) or biuret (carbamoylurea) degradation as an additional source of ammonia.

To better understand why Palsa-1315 comammox may be found in SR, we carried out a pangenomic comparison of the two GTDB genera that harbor comammox organisms. Analysis of high-quality Palsa-1315 genomes revealed most (20/32) harbor at least one subunit of ammonia monooxygenase (Fig. 4 & A5). The only other genus within the *Nitrospiraceae* harboring ammonia oxidation genes is *Nitrospira* D, with most (60/70) genomes also containing at least one ammonia monooxygenase gene (Fig. A5). Not all organisms within *Nitrospira* D are comammox, however, as this GTDB-defined genus includes established NOB like *Nitrospira lenta* and *Nitrospira moscoviensis* (Fig. 4). Comammox genomes from the two GTDB-defined genera, Palsa-1315 and *Nitrospira D*, correspond well to comammox clades B and A based on *amoA*

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phylogeny (Fig. A6). Previous comparative genomics of comammox and other *Nitrospira* NOB found that comammox organisms encoded more urease, urea transporters, and copper stress genes when compared to *Nitrospira* NOB*,* and that within the comammox clades *Nitrospira D* (clade A) contained an Rh-type ammonia transporter whereas Palsa-1315 (clade B) encoded Amt-type ammonia transporters (Palomo *et al.*, 2018). Additionally, the study observed that Palsa-1315 (clade B) encoded formate dehydrogenases and transport genes which allow for alternative energy metabolism using formate while *Nitrospira D* (clade A) genomes encoded sulfhydrogenases that play an unknown metabolic role (Palomo *et al.*, 2018), although these genes for alternative metabolisms are not necessarily exclusive to one clade or the other (Poghosyan *et al.*, 2019). We carried out a pangenomic comparison of comammox clades using many additional high-quality (> 90% complete) genomes now available for Palsa-1315 (clade B) and *Nitrospira D* (clade A). Urea utilization genes are found in a vast majority of genomes from both comammox clades and some Palsa-1315 (clade B) and *Nitrospira* D (clade A) also encode cyanase, nitrilase/nitrile hydratase, and biuret hydrolase (Fig. A5). We computed enrichment scores via *anvi'o* to test if gene clusters were found in significantly more *Nitrospira D* (clade A) or Palsa-1315 (clade B) genomes. As previously reported (Palomo *et al.*, 2018), we confirmed that formate dehydrogenase and formate transport are found in significantly ($p < 0.001$) more Palsa-1315 (clade B) genomes in contrast to *Nitrospira D* (clade A) genomes which are significantly ($p < 0.001$) more likely to harbor genes for hydrogenase maturation and expression and sulfhydrogenase, though these genes are not exclusive to one clade or the other and could be found in both (Fig. A5). Of particular relevance, Palsa-1315 (clade B) are significantly ($p < 0.001$) more likely to harbor aquaporin Z for adapting to rapid changes in osmolarity, which may

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occur in a dynamic floodplain while *Nitrospira*_D (clade A) are significantly ($p < 0.001$) more likely to harbor a small conductance mechanosensitive channel (*mscS*). In total, 70 genes are significantly enriched in *Nitrospira*_D (clade A) and 27 genes in Palsa-1315 (clade B).

Putative NOB from the Nitrospiraceae *and NS-4 family*

We recovered 11 MAGs from the *Nitrospirales* (excluding previously discussed comammox MAGs) that we investigated for their genetic potential for nitrite oxidation as MAGs from established NOB were conspicuously absent. *Nitrospirales* MAGs from SR originated from the "2-02-FULL-62-14" genus (*Nitrospiraceae*) and "NS-4" family. Currently, *Nitrospirales* contains 4 families in GTDB (RS220), including *Nitrospiraceae*, NS-4, "UBA8639", and "UBA2166". We generated a 16S rRNA gene phylogeny to compare sequences from these 4 families to the 7 established *Nitrospira* lineages as defined by 16S rRNA (Daims *et al.*, 2001, 2016; Keuter *et al.*, 2023) (Fig. A7). Based on 16S rRNA gene sequences, sequences from the GTDB-defined UBA8639 family fall into *Nitrospira* lineage IV, *Nitrospiraceae* fall into or are sister to *Nitrospira* lineages I, II, V, V|, and VII, and both NS-4 and UBA2166 are sister to the previously defined *Nitrospira* NOB lineages. Although MAGs generated from SR lack 16S rRNA genes, we observe that a sequence from a 2-02-FULL-62-14 (*Nitrospiraceae*) genome is sister to *Nitrospira* lineages I, II, and VII.

We recovered 3 MAGs (1 after dereplication) from 2-02-FULL-62-14. The 2-02-FULL-62-14 (*Nitrospiraceae*) from SR was only present in OBJ1 and were of low abundance (Fig. 1). 2-02-FULL-62-14 *narG*/*nxrA* and *narH*/*nxrB* genes fall within a clade that contains all of the

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established *Nitrospira* NOB lineages and is sister to an *Nitrospina* and anammox clades (Figs. 5 & A8). MAGs harbor genes for the reverse tricarboxylic acid cycle (rTCA) cycle, including ATP citrate-lyase (*aclAB*) and two oxygen-tolerant, 5-subunit POR/OGOR (Yamamoto *et al.*, 2006; Lücker *et al.*, 2010), as well as oxidative phosphorylation genes for complexes I, II, III, and V indicating these organisms may be capable of carbon fixation and aerobic metabolism (Table A3). In addition to ammonia (*amt*) and nitrite transporters (NRT), this lineage also encodes genes for cyanase (*cynS*), a nitrate/nitrite/cyanate ABC transporter (*nrtABCD*), urease (*ure*), and urea transport (*urt*) (Fig. 4). We suggest the 2-02-FULL-62-14 organisms may be NOB and their genomic content supports the potential use of alternative N sources to produce ammonia for biomass or potentially cross-feeding with AOA (Koch *et al.*, 2015; Palatinszky *et al.*, 2015) (Fig. 4). MAGs also harbor pilus assembly and twitch motility genes, as well as formate dehydrogenase genes, that are commonly found in *Nitrospira* NOB (Figs. 4, Table A3) and may allow for metabolic flexibility. These MAGs lack genes for other dissimilatory N and sulfur cycling processes.

Most of the non-comammox *Nitrospirales* MAGs recovered from the SR floodplain fall within two genera of the NS-4 family. The NS-4 family currently consists of 4 genera ("SCTG01", "NS-11", "NS-12", and "NS-4") represented by 8 genomes in GTDB (RS220). The 2 genomes in GTDB (RS220) in the SCTG01 genus originated from nutrient-limited groundwater of the Department of Energy's Oak Ridge Field Research Center (ORFRC) in Bear Creek Valley watershed of Oak Ridge, Tennessee, US (Tian *et al.*, 2020) and Alberta, Canada groundwater. All of the remaining NS-4 family genomes in GTDB (RS220) originate from samples from meadow soils (ranging from

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10 to 40 cm depth) in the Angelo Coast Range Reserve, CA, including the MAG that is the namesake for the lineage. The NS-4 family MAGs from these meadow soils predominantly fall into the NS-12 genus (Crits-Christoph *et al.*, 2018; Diamond *et al.*, 2019). We recovered 8 MAGs from the NS-4 family, including 3 (1 after dereplication) from the GTDB-defined genus SCTG01 and 5 (3 after dereplication) from a previously undescribed lineage of the NS-4 family, including one 95.9% completeness and < 1.8% contamination. This novel lineage is sister to the GTDB-defined genera NS-4 and NS-11, which consist of MAGs from meadow soils, in a concatenated ribosomal protein tree (Fig. 4). The MAGs from this newly described lineage have strong genome similarity with one another (96-99% ANI), 75-79% ANI with other NS-4 lineages, and ~75% ANI with established *Nitrospira* NOB (Fig. A9).

Several MAGs from both the SCTG01-genus and novel NS-4 lineage harbor NAR/NXR genes (Fig. 3). Both *narG*/*nxrA* and *narH*/*nxrB* sequences fall within an NS-4 family clade and are closely related to established *Nitrospira* NOB sequences from the *Nitrospiraceae* and UBA8639 (*Nitrospira* lineage IV) families and sister to anammox and *Nitrospina nxrA* (Figs. 5 & A8). MAGs generated from the SR floodplain in both the SCTG01 genus and novel NS-4 lineage harbor genes for oxidative phosphorylation (complexes I-V), the rTCA cycle, including ATP-citrate lyase and generally two copies of the 5-subunit POR/OGOR, formate dehydrogenase, and Ni/Fe-hydrogenase III genes (*hyc*) (Fig. 4, Table A3). MAGs also harbor genes commonly found in *Nitrospira* NOB, such as *nirK* and chlorite dismutase (*cld*) (Fig. 4). NOB from different genera, including *Nitrospira*, *Nitrospina*, *Nitrobacter*, and *Nitrococcus*, encode *cld* (Maixner *et al.*, 2008; Kostan *et al.*, 2010; Mlynek *et al.*, 2011; Barnum and Coates, 2023) that may be used in chlorite

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protection during chlorate reduction (Lees and Simpson, 1957; Hynes and Knowles, 1983) perhaps carried out by NXR as can happen with NAR (Hochstein and Tomlinson, 1988). Based on the genomic content of these MAGs, we suggest they may be NOB. Both lineages encode genes for the potential use of cyanate as a N source and assimilatory nitrite reduction to ammonia and all but one lack urease (Fig. 3). The novel NS-4 lineage MAGs harbor many flagellar and pilus assembly genes along with chemotaxis genes, but they lack twitch motility genes (Table A3). They also encode several methods for osmoregulation, including several potassium and compatible solute uptake genes, aquaporin NIP, and mechanosensitive channels (Table A3).

Nitrite oxidation may be carried out by uncultured lineages from the Nitrospirales

We found *narG/nxrA* and *narH*/*nxrB* genes in genomes from 3 of the 4 families in the *Nitrospirales*, including *Nitrospiraceae* (*Nitrospira* lineages I, II, V, VI, VII), UBA8639 (*Nitrospira* lineage IV), and NS-4 (Fig. 4). A vast majority of *narG/nxrA* and *narH*/*nxrB* genes from the *Nitrospirales* fall into one clade that is sister to *Nitrospina* and anammox genes (Figs. 4 & A5). A majority of GTDB species representatives from the *Nitrospirales* encode genes annotated as *narH/nxrB* (197/256) (Fig. 4). Annotation through *anvi'o* rarely annotates genes as *narG*/*nxrA* within *Nitrospirales* despite the presence of many known NOB (*nxrA*-containing organisms). A *blastp* query using full-length *Nitrospira* and *Nitrobacter nxrA* sequences (Table A1) against the *Nitrospirales* genomes in our pangenome yielded at least one hit (e-value < 1e-20, > 500 aa length) from half of genomes (130/256) that largely fall within a *Nitrospirales* clade (Fig. 4).

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To better understand the uncultured NS-4 family compared to the *Nitrospira* NOB-containing *Nitrospiraceae* and UBA8639, we tested for genes that are significantly more common (enriched) in the different GTDB families. Urease and urea transporter genes are significantly ($p < 0.001$) more common in genomes from the *Nitrospiraceae*; however, these genes are annotated in genomes throughout the *Nitrospirales* (Figs. 4 & A10). Unsurprisingly, the *Nitrospiraceae* family is also significantly ($p < 0.001$) enriched in genes encoding AMO and HAO as it is the only family containing comammox organisms (Figs. 4 & A10). A gene commonly found in *Nitrospira* NOB, chlorite dismutase, is significantly enriched ($p < 0.001$) in *Nitrospiraceae* and NS-4 families though it is also found in a few UBA8639 genomes (Fig. 4 & A10). In addition to being prevalent in *Nitrospira* D (clade A) genomes, hydrogenase and accessory genes are found in a few other genomes of the *Nitrospiraceae* as well as the UBA8639 family (Fig. A10). *Nitrospira* can have metabolic flexibility (Koch *et al.*, 2015; Poghosyan *et al.*, 2019; Bayer *et al.*, 2021; Leung *et al.*, 2022) and genes annotated as formate dehydrogenase are found in a few genomes from all 4 *Nitrospirales* families (Figs. 4). NS-4 and UBA8639 are enriched ($p < 0.001$) in osmoprotectant transport system and *tkr*/*ktr* potassium uptake system genes. Aquaporin NIP is enriched ($p < 0.001$) in the NS-4 family whereas glycine betaine and spermidine/putrescine transporters are enriched ($p < 0.001$) in UBA8639 and a large mechanosensitive channel and KUP potassium uptake gene are enriched ($p < 0.001$) in the *Nitrospiraceae* family, highlighting their different potential strategies for osmoregulation.

DISCUSSION

Oligotrophic AOA and non-AOA Thaumarchaea MAGs recovered from floodplain sediments

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AOA are highly diverse in the riparian floodplain sediments of the Upper Colorado River Basin and Wind River Basin in the western US (Cardarelli *et al.*, 2020; Reji *et al.*, 2022), but AOA have not previously been examined within the Slate River, CO floodplain. Few distinct lineages of AOA were recovered from SR and East River floodplain sediments in comparison to the great diversity of AOA lineages recovered from the Wind River Basin near Riverton, WY (Reji *et al.*, 2022), which could be due in part to the higher salinity and drier conditions compared to SR and East River systems. SR AOA fell predominantly into *Nitrosotalea*-like genera in sharp contrast to MAGs recovered from East River or Wind River floodplain sediments which yielded no representatives from these clades (Fig. 2). East River samples were collected from 10-25 cm, much shallower than the samples collected at SR which could also explain some of the variability between the AOA genera recovered between these two nearby sites.

Nitrosotalea and *Nitrosotalea*-like AOA are found in oligotrophic freshwater environments, including lakes (Herrmann *et al.*, 2009; Auguet *et al.*, 2011; Auguet and Casamayor, 2013; Bollmann *et al.*, 2014), groundwater (van der Wielen *et al.*, 2009; Reed *et al.*, 2010), floodplain sediments (Cardarelli *et al.*, 2020), and topsoil (Vuong *et al.*, 2022), as well as acidic soils (Gubry-Rangin *et al.*, 2010, 2011). *Nitrosotalea* AOA have also been shown to survive extreme conditions and respond rapidly to ammonia inputs in semi-arid top soils (Vuong *et al.*, 2022). The recovery of less diverse AOA than found in other floodplains of the Western US (Cardarelli *et al.*, 2020; Reji *et al.*, 2022) and predominantly from *Nitrosotalea*-like genus, TA-20, likely indicates the floodplain sediments of SR are oligotrophic in terms of ammonia and that AOA may need to respond rapidly to pulses of ammonia and changes in water table height.

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CSP1-1 is the only genus represented in MAGs from SR, East River, and Wind River sediments, highlighting that this AOA genus can be present in distinct floodplain environments across the Western US. Notably, CSP1-1 has been identified as a fourth freshwater clade (in addition to *Nitrosarchaeum*, a subset of *Nitrosopumilus*, and *Nitrosotenuis*) with highest abundances in riverine environments (Ren and Wang, 2022). CSP1-1 MAGs from SR harbor many genes related to motility (Fig. 3), indicating this lineage may be well suited for a more oligotrophic or fluctuating environment as well.

Non-AOA *Thaumarchaeaota* from SR originated from or sister to the *Ca*. Gagatemarchaeum genus. *Ca.* Gagatemarchaeum (Bog-1369) is defined by larger genome sizes, acid tolerance, and more genes for utilizing exogenous organic substrates than the subsoil *Ca.* Subgagatemarchaeaum (UBA183 in GTDB) genus (Sheridan *et al.*, 2023), supporting that this genus is better adapted to fluctuating environments such as floodplains. This could explain why this “topsoil” clade is found at deeper depths within SR soils. Although several novel non-AOA *Thaumarchaeota* MAGs were also recovered from Wind River floodplain sediments (Reji *et al.*, 2022), the *Ca.* Gagatemarchaeaceae family was not represented among them (Fig. 2).

Clade B comammox are abundant nitrifiers and may utilize alternative sources of ammonia

All known comammox bacteria are found within the *Nitrospiraceae* and fall into two distinct clades, generally referred to as clade A and clade B (Daims *et al.*, 2015). Comammox bacteria are thought to be adapted to oligotrophic environments given the kinetics of their metabolism

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(Costa *et al.*, 2006; Pinto *et al.*, 2016; Kits *et al.*, 2017; Sakoula *et al.*, 2021). Further work is required to understand the different niches of *Nitrospira D* (clade A) and Palsa-1315 (clade B) comammox in terrestrial environments, especially given that some clade A lineages can be found in copiotrophic environments (C. Li *et al.*, 2021; Li *et al.*, 2023; Gao *et al.*, 2023). Palsa-1315 (clade B) comammox are found in oligotrophic terrestrial environments and more likely to co-occur with AOA in low-ammonia soils (Gao *et al.*, 2023; Li *et al.*, 2023) and were also the predominant nitrifiers in oligotrophic Antarctic soils and lake sediments (Han *et al.*, 2024). Palsa-1315 (clade B) comammox were also dominant in paddy fields with variable wet-dry cycles, irrigation, and fertilization conditions, indicating they may be able to thrive better in highly fluctuating environments (Gao *et al.*, 2022). Many comammox organisms may be able to use alternative N sources such as urea, cyanate, nitriles, or biuret based on their genomic content (Figs. 4 & A5), which could support their success in oligotrophic or fluctuating environments such as floodplains. The Palsa-1315 (clade B) comammox MAGs generated from SR harbor the genetic potential to utilize urea as an alternative ammonia source with one MAG also harboring genes for utilizing cyanate (Fig. 4). Urea can be used to select for comammox over other ammonia oxidizers (J. Li *et al.*, 2021; Zhao *et al.*, 2021) as well as allow for the coexistence of comammox and *Nitrospira* NOB better than ammonia-fed systems (Vilardi *et al.*, 2024).

Several Palsa-1315 MAGs from SR also harbored genes for degrading biuret (*biuH*, *atzF*), a polyurea molecule generated via pyrolysis of urea during urea fertilizer manufacture or by degradation of cyanuric acid (Aukema *et al.*, 2020)- a product of s-triazine pesticide degradation. Biuret is more slowly remineralized than urea in soils but can serve as a N source

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for microorganisms (Robinson *et al.*, 2018) and support nitrification at a slow rate in soils (Xue *et al.*, 2022). In most microbial genomes, biuret hydrolases are found independent of a cyanuric acid hydrolase gene (*atzD*), indicating that biuret degradation arose independently of s-triazine ring metabolism (Robinson *et al.*, 2018). All 3 comammox MAGs lack *atzD* but encode 2 co-located genes annotated as *biuH,* one of which may be a triuret hydrolase (*trtA*) (Fig. A4) which could allow for triuret degradation through the biuret degradation pathway (Tassoulas *et al.*, 2021). Like biuret, triuret is also an impurity in urea fertilizer, but it can also occur naturally, likely from purine or uric acid metabolism under oxidative stress (Venable, 1918; Gersch *et al.*, 2009; Kim *et al.*, 2009). Cultivation of terrestrial comammox strains and determination of nitrification kinetics would improve our understanding of the possible different niches occupied by terrestrial *Nitrospira D* (clade A) versus Palsa-1315 (clade B) organisms (Li *et al.*, 2023). For example, enrichment of *trtA* and *biuH*-encoding comammox could allow for measurement of nitrification rates on N sources such as biuret and triuret. Additionally, the ability of Palsa-1315 (clade B) comammox to act as NOB in the SR floodplain requires further investigation. While *Nitrospira inopinata* (clade A) has a lower nitrite affinity than *Nitrospira* NOB (Kits *et al.*, 2017), *Ca.* Nitrospira kreftii (clade A) can have a very high affinity for nitrite in line with *Nitrospira* NOB (Sakoula *et al.*, 2021). It is plausible that comammox in SR may act as NOB when conditions favor it as Palsa-1315 MAGs harbor nitrite transport genes and one MAG also encodes an assimilatory nitrite reductase (*nirA*) (Fig. 3).

The presence and abundance of Palsa-1315 (clade B) comammox and TA-20 AOA, the lack of AOB, and the widespread genetic potential to utilize alternative sources of ammonia such as

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urea, cyanate, nitriles, biuret, and triuret in nitrifier MAGs support that the SR floodplain is a low-ammonia flux system. Our previous study of MAGs involved with C1-cycling at SR highlighted *Candidatus* Methylomirabilis genomes that lacked the ability for nitrite-dependent anaerobic methane oxidation (n-DAMO) and limited evidence of nitrate-dependent methanotrophy by *Candidatus* Methanoperedens (Rasmussen *et al.*, 2024). This current study supports that nitrification in subsurface floodplain sediments may be subject to oligotrophic conditions and select for nitrifiers that can respond to pulses of ammonia or utilize alternative N sources. Recent studies have highlighted the dominance of nitrite/nitrate dependent methanotrophs in Amazon River floodplain sediments (Gontijo *et al.*, 2021) and proposed a link between methanotrophy and nitrification in Amazon River floodplain sediments (Monteiro *et al.*, 2022). However, SR conditions in deeper sediments (> 30 cm) may not be as conducive to this coupling.

Established NOB missing from SR

We did not recover MAGs from established NOB in this study. We did recover MAGs from uncultured *Nitrospirales* lineages that we suggest are putative NOB based on their genomic content, including the presence of NAR/NXR genes closely related to sequences from known *Nitrospira* NOB and rTCA cycle genes. The most abundant putative NOB in SR is from a novel lineage from the NS-4 family. MAGs from these NS-4 organisms harbor many motility genes (Table A3). In the dynamic floodplain environment where ammonia oxidation may be occurring rapidly after pulses of ammonia transit through the system, it may be beneficial for SR NOB to be motile and respond to the resulting nitrite pulses. The putative NOB MAGs also encode

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several different genes related to osmoregulation such as putrescine transporters, osmoprotectant transport systems, mechanosensitive channels, and various aquaporins that could be beneficial for responding to changes in water table height in the floodplain (Table A3). In contrast to the comammox and putative NOB from the 2-02-FULL-62-14 (*Nitrospiraceae*), urease and urea transport genes were not common in the novel NS-4 genus though 1 MAG did encode *cynS* for potentially using cyanate as an alternative source of ammonia (Table A3). This novel lineage also encodes multiple nitrite transporters (*NRT2*, *nirC*) and assimilatory nitrite reductase (*nirA*) which may be necessary to compete in an ammonia-limited environment.

Nitrospirales *order may harbor nitrite oxidizers outside of established* Nitrospira *NOB*

In addition to established NOB within *Nitrospira* (now defined as several genera within the GTDB-defined *Nitrospiraceae* and UBA8639 family), our pangenomic analysis supports that most taxa within the order *Nitrospirales* have the genetic potential for nitrite oxidation (Fig. 4). Phylogenetic placement of *narG*/*nxrA* and *narH*/*nxrB* sequences supports that microorganisms in uncultured genera from the *Nitrospiraceae* and *UBA8639* as well as the NS-4 could contain *nxrA* and *nxrB* as they form a single clade sister to *Nitrospina* and *anammox* (Figs. 5 & A8). Further culture-based and biochemical research is necessary to confirm that genes annotated as NAR/NXR from uncultured lineages of the *Nitrospirales* are nitrite oxidoreductases and that the organisms within the NS-4 family can carry out nitrite oxidation. However, the genomic content and phylogenic placement of relevant genes support that organisms outside of the established *Nitrospira* NOB lineages, such as the NS-4, may be NOB. *Nitrospira* are known for being incredibly diverse and ubiquitous in the environment and our work highlights that other related

lineages may be NOB adapted to different niches such as oligotrophic or highly-variable environments.

Conclusions

SR floodplains host nitrifiers such as *Nitrosotalea*-like AOA and Palsa-1315 (clade B) comammox that are adapted to oligotrophic environments, implying that the studied SR floodplain is a low ammonia flux system. Although established NOB were not recovered in SR MAGs, we recovered putative NOB from several genera from within the *Nitrospirales*, including from the 2-02-FULL-62-14 genus (*Nitrospiraceae*) and from the uncultured NS-4 family. Our genomic analysis indicates these organisms could be NOB, however, biochemical and physiological experiments are necessary to confirm these uncultured lineages are capable of nitrite-oxidation. We hypothesize that these putative NOB may be adapted to oligotrophic environments and respond quickly to pulses of nitrite based on the ecophysiological properties of the ammonia-oxidizers in the system. Our analysis supports that the potential for nitrite oxidation may be widespread in the *Nitrospirales* order, though further cultivation and sequencing of non-*Nitrospira* lineages is clearly needed. Perhaps pulses or perturbations, such as precipitation or flooding events, select for nitrifiers that can rapidly respond to ammonia inputs that allow for transiently high nitrification rates.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

AVAILABILITY OF DATA AND MATERIALS

The metagenomes analyzed this study are available under NCBI BioSample accession no. SAMN42042672, SAMN42042677, SAMN42042680, SAMN42042681, SAMN42042702, SAMN42042711, SAMN42042712, SAMN42042720, SAMN42042721, SAMN42042722,

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SAMN42042724, and SAMN42042725. MAGs generated in this study are available under NCBI BioProject accession no. PRJNA1142551.

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TABLE AND FIGURE LEGENDS

Figure 1 Abundance of nitrifier and non-AOA *Thaumarchaea* in SR MAG library by depth and location. Nitrifier guilds are grouped by GTDB-assigned family. Abundance shown in reads per kilobase of genome per gigabase of metagenome (RPKG).

Figure 2 Concatenated phylogeny of 17 ribosomal protein genes from *Thaumarchaeota* (*Nitrososphaeria*) generated using IQ-TREE model LG+F+R10 and 1000 bootstraps. MAGs generated from SR are in bold color, the Wind River Basin (Reji et al., 2022) in dark brown, and East River (Matheus Carnevali et al. 2021) in light brown. Labels indicate GTDB taxonomy.

Figure 3 Presence of relevant genes in nitrifier MAGs from SR. Genes include: ammonia monooxygenase (AMO: *amoABC*), nitrate reductase/nitrite oxidoreductase (NAR/NXR: *narGH*/*nxrAB*), nitrite reductase (*nirK*), ammonium transporter (*amt*), biuret hydrolase (*biuH*), triuret hydrolase (*trtA*), allophanate hydrolase (*atzF*), nitrate/nitrite/cyanate ABC-type transporter (*nrtABCD/cynABCD*), cyanase (*cynS*), nitrile hydratase (*nthAB*), nitrilase (NIT1/*ybeM*), nitrite transporter (*NRT2/narK/nrtP/nasA*, *nirC*), assimilatory nitrite reductase (*nirA*), urea transport system (*urtABCDE*), urease (*ureABC*), urease accessory proteins (*ureEFGHJ*). Diamond shapes indicate species representatives.

Figure 4 Phylogeny of 20 concatenated ribosomal genes from *Nitrospirales* generated with IQ-TREE model JTT+R9. External strip color indicates GTDB-defined family and *Nitrospira* lineage for select strains. SR MAGs are in bold. The presence of genes of interest are indicated in the righthand panel including genes encoding at least one subunit of the following: AMO; ammonia monooxygenase (*amoABC*), HAO; hydroxylamine dehydrogenase (*hao*), NAR/NXR; nitrate/nitrite oxidoreductase (*narGH*/*nxrAB*), nitrite reductase (*nirK*), urease (*ureABC*), cyanase (*cynS*), nitrile hydratase (*nthAB*), biuret hydrolase (*biuH*), allophanate hydrolase (*atzF*), chlorite dismutase (*cld*), formate dehydrogenase, Ni/Fe-hydrogenase I (*hya*), Ni/Fe-hydrogenase I (*hyc*), hydrogenase accessory genes (*hypABCDEF*), sulfhydrogenase (*hydABDG*), sulfite dehydrogenase (*sorAB*), arsenite oxidase (*aoxAB*), rTCA; reverse tricarboxylic acid cycle, Pyruvate ferredoxin oxidoreductase/2-oxoglutarate:ferredoxin oxidoreductases (POR/OGOR), ATP citrate lyase (*aclAB*, *ACLY*), cytochrome bd (*cydAB*). Black node labels show nodes with >= 90% bootstrap support. Tree is midpoint rooted.

Figure 5 IQ-TREE using model LG+R8 of an amino acid alignment of 460 *narG*/*nxrA*. Sequences from SR are bold, *nxrA* sequences in light pink, *narG*/*nxrA* sequences originating from *Nitrospirales* queried in this study in teal. Black node labels show nodes with >= 90% bootstrap support. Tree is midpoint rooted.

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