



Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*

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Abstract

The description of comammox *Nitrospira* spp., performing complete ammonia-to-nitrate oxidation, and their co-occurrence with canonical β -proteobacterial ammonia oxidizing bacteria (β -AOB) in the environment, calls into question the metabolic potential of comammox *Nitrospira* and the evolutionary history of their ammonia oxidation pathway. We report four new comammox *Nitrospira* genomes, constituting two novel species, and the first comparative genomic analysis on comammox *Nitrospira*. Unlike canonical *Nitrospira*, comammox *Nitrospira* genomes lack genes for assimilatory nitrite reduction, suggesting that they have lost the potential to use external nitrite nitrogen sources. By contrast, compared to canonical *Nitrospira*, comammox *Nitrospira* harbor a higher diversity of urea transporters and copper homeostasis genes and lack cyanate hydratase genes. Additionally, the two comammox clades differ in their ammonium uptake systems. Contrary to β -AOB, comammox *Nitrospira* genomes have single copies of the two central ammonia oxidation pathway operons. Similar to ammonia oxidizing archaea and some oligotrophic AOB strains, they lack genes involved in nitric oxide reduction. Furthermore, comammox *Nitrospira* genomes encode genes that might allow efficient growth at low oxygen concentrations. Regarding the evolutionary history of comammox *Nitrospira*, our analyses indicate that several genes belonging to the ammonia oxidation pathway could have been laterally transferred from β -AOB to comammox *Nitrospira*. We postulate that the absence of comammox genes in other sublineage II *Nitrospira* genomes is the result of subsequent loss.

Additional information: All raw sequence data and genome sequences have been deposited at NCBI under the project PRJNA384587, with sequence accession for the raw sequence data: SRR5739198—SRR5739203; and for the draft genomes: GCA_002869925.1, GCA_002869845.1, GCA_002869885.1, GCA_002869855.1 and GCA_002869895.1 (GenBank assembly accessions). The file containing the protein clusters sequences is available on figshare (<http://dx.doi.org/10.6084/m9.figshare.4924748>).

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Introduction

Nitrification, the biological oxidation of ammonia to nitrate, is an essential process in terrestrial and aquatic environments as well as in water quality engineering applications. For the last century, nitrification was assumed a two-step process executed by two complementary functional groups, ammonia-oxidizing prokaryotes (AOP) and nitrite-oxidizing bacteria (NOB). Recently, several groups have shown that single microorganisms belonging to the genus *Nitrospira* can carry out the complete oxidation of ammonia to nitrate, a process abbreviated *comammox* [1, 2]. *Nitrospira* spp., long-recognized as nitrite-oxidizers, are widespread in both natural and engineered ecosystems associated with nitrogen cycling [3, 4]. The *Nitrospira* genus is extremely diverse and comprises at least six lineages, which frequently coexist [3, 5]. Comammox *Nitrospira* genomes described to date belong to *Nitrospira* lineage II, and comprise two clades (clade A and B) based on the phylogeny of their ammonia monooxygenases [1, 2]. Both clades were detected in samples retrieved from a groundwater well

and in a groundwater-treating rapid sand filter, together with conventional *Nitrospira* spp. [1, 6]. The diversity of *Nitrospira* spp. points towards ecological niche-partitioning; nitrite and dissolved oxygen concentrations are potential niche determinants for *Nitrospira* lineages I and II [7–9]. The recently described metabolic versatility of some *Nitrospira* spp., which includes formate, hydrogen, urea and cyanate metabolisms [10–12] may also contribute to their coexistence. Nevertheless, little is known about differences in the functional potential of the two comammox *Nitrospira* clades and niche-partitioning between comammox *Nitrospira* and AOP. Only the hypothesis that comammox *Nitrospira* could outcompete canonical AOB in surface-attached and substrate-limited environments has been proposed [13]. The evolutionary history of ammonia oxidation in *Nitrospira* is unknown. Based on their ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO) sequences, β -proteobacterial ammonia oxidizing bacteria (β -AOB) are most similar to comammox *Nitrospira* [1, 2, 6, 14]. Either AMO and HAO encoding genes are ancestral in *Nitrospira* spp. or were acquired by horizontal gene transfer from AOP, even though the high degree of phylogenetic divergence of these proteins between comammox *Nitrospira* and AOP makes a recent acquisition improbable [2].

To examine the functional potential which might allow niche-partitioning among comammox *Nitrospira* and between comammox *Nitrospira* and AOP, and to unravel the evolutionary history of ammonia oxidation in *Nitrospira*, we performed differential coverage genome binning on a *Nitrospira* composite population genome recovered from a metagenome in which comammox *Nitrospira*, canonical AOB and ammonia oxidizing archaea (AOA) co-occurred [3, 6]. Comparative genome analysis was conducted with the recovered genomes and high-quality published *Nitrospira* genomes. The evolutionary history of genes involved in ammonia oxidation in comammox *Nitrospira* was also explored with a special focus on understanding the potential role of horizontal gene transfer, gene duplication, and gene loss. For this purpose, we executed pairwise protein dissimilarity comparison between and within comammox *Nitrospira* clades as well as with other AOB, explored the genomic arrangement in the relevant pathways, and performed probabilistic and parsimony-based reconciliation analysis where trees for individual genes were compared to a clock-like species-tree constructed from ribosomal proteins. Our study revealed comammox-, comammox clade- and canonical *Nitrospira*-specific features related to nitrogen assimilation, electron donor versatility and substrate-limitation tolerance. Our analysis suggests that ammonia-oxidation related genes were transferred from β -proteobacterial ammonia oxidizers to comammox *Nitrospira*.

Materials and methods

Recovery of individual genomes

As described in Palomo et al. [6], DNA was extracted from triplicate 0.5 g samples taken from three adjacent (ca. 0.5 m apart; and two different depths from each) locations in a singular rapid sand filter (RSF) and subject to shotgun sequencing to describe the microbial community involved in groundwater purification. The work presented here focuses on the metagenomes from which a *Nitrospira* composite population genome (CG24) was recovered [6]. In order to separate the composite population genome into individual genomes, a strategy based on subsample assembly [15] followed by differential coverage binning was applied [16] (further details in Supplementary Information and Supplementary Figure S1). The quality of recovered genomes was evaluated using coverage plots from mmgenome [17], hidden Markov models (HMMs) for 107 essential single-copy genes, and CheckM [18] (further details in Supplementary Information). The selected draft genomes were refined using Anvi'o [19] to remove contigs with inconsistent coverage. Assembly quality was improved by alignment against related complete or draft genomes using the Multi-Draft based Scaffolder (MeDuSa) [20] and gaps were closed with GapFiller v.1.10 [21]. All raw sequence data and genome sequences have been deposited at NCBI under the project PRJNA384587, with sequence accession for the raw sequence data: SRR5739198–SRR5739203; and for the draft genomes: GCA_002869925.1, GCA_002869845.1, GCA_002869885.1, GCA_002869855.1 and GCA_002869895.1 (GenBank assembly accessions).

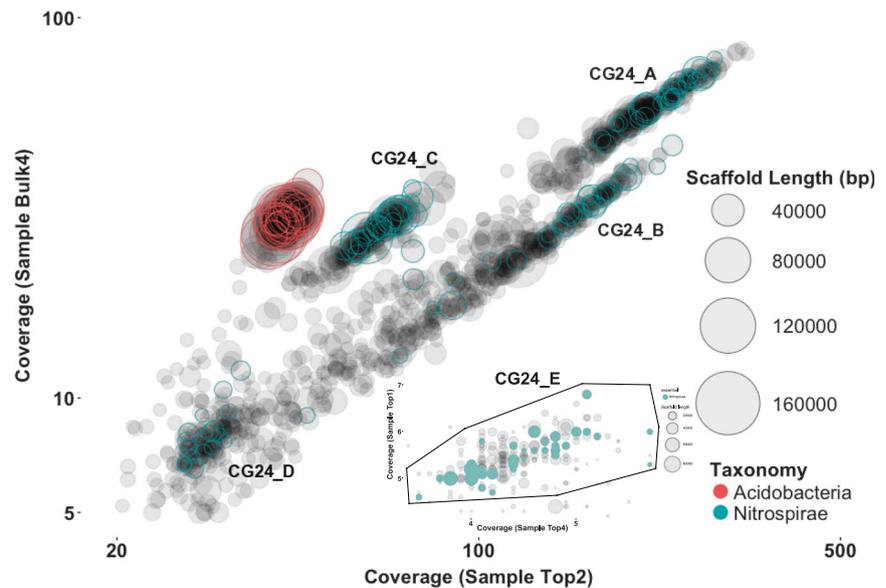
Comparative genome analysis

High quality published *Nitrospira* genomes (more than 90% complete and with less than 5% redundancy) were also included in the comparative genomic analysis. Gene calling on the recovered genome bins and published genomes was performed using Prodigal v.2.62 [22]. Annotation was conducted in RAST [23] and protein functional assignments of genes of interest were confirmed using KEGG and blastp. Pangenomic analysis was executed using the meta-pangenomic workflow of Anvi'o [19] with default parameters with the exception `--maxbit = 0.3` (further details in Supplementary Information). All comparative genome information was visualized using the program `anvi-interactive` in Anvi'o [19].

Sequence dissimilarity and gene synteny analysis

Pairwise amino acid dissimilarities (Supplementary Information) among the genomes for 18 housekeeping genes, 27

Fig. 1 Differential coverage plot of two metagenomes obtained from Islelvbro waterworks at different sampling depth. Scaffolds are displayed as circles, scaled by length and colored according to phylum-level taxonomic affiliation. Only scaffolds >4 kbp are shown. A second differential coverage plot showing the CG24_E bin extraction (enclosed by the polygon) is presented



ammonia oxidation-related proteins and 14 syntenic ribosomal proteins (Supplementary Table S1) were calculated using Clustal Omega v.1.2.1 [24]. Gene arrangement of ammonia oxidation related genes was visualized using the R package genoPlotR [25].

Construction of clock-based species-tree and guest-tree sets

The software BEAST, version 2.4.4, was used to construct a dated species-tree using a relaxed log-normal clock model [26, 27]. The input data was a set of 14 ribosomal proteins assumed to be inherited mostly vertically (Supplementary Information and Supplementary Table S1). The timetree.org resource [28] together with the substitution rate of 16S rRNA [29] were used as a main starting point for finding calibration information for internal nodes (Supplementary Information).

For each investigated gene the protein sequences were aligned using the G-INS-i method in MAFFT [30]. The software MrBayes version 3.2.6 [31] was then used to reconstruct phylogenies (further details in Supplementary Information).

Reconciliation analysis

The analysis of event probabilities was done using the software JPrIME-DLTRS version 0.3.6 [32] (Supplementary Information). The clock-based tree mentioned above was used as the reference species-tree for all analyses (Supplementary Information). For each gene, the sample file containing 1500 midpoint-rooted trees constructed using MrBayes was used as a guest-tree set from which JPrIME-

DLTRS could sample guest-tree topologies. MCMC was allowed to run for 4 million iterations with a thinning of 200, and running 3 parallel chains for each gene (Supplementary Information). To investigate which branches the transfer events most likely involved, we used the software ecceTERA version 1.2.4 [33] (Supplementary Information). The software SylvX version 1.3 [34] was used for visualizing reconciliations computed by ecceTERA.

Phylogenetic analysis

Phylogenetic trees for AmoA and HaoA were constructed using MrBayes. The substitution model for both data sets was LG with gamma-distributed rates (determined using jmodeltest), using 5 million iterations for 2 sets of 3 chains, with a thinning of 5000 and a burn-in of 25%. Convergence was checked using Tracer and by computing the potential scale reduction factor (ensuring that R was close to 1.0 for all parameters) and the average standard deviation of split frequencies (which was well below 0.01 in both cases).

Statistical analysis

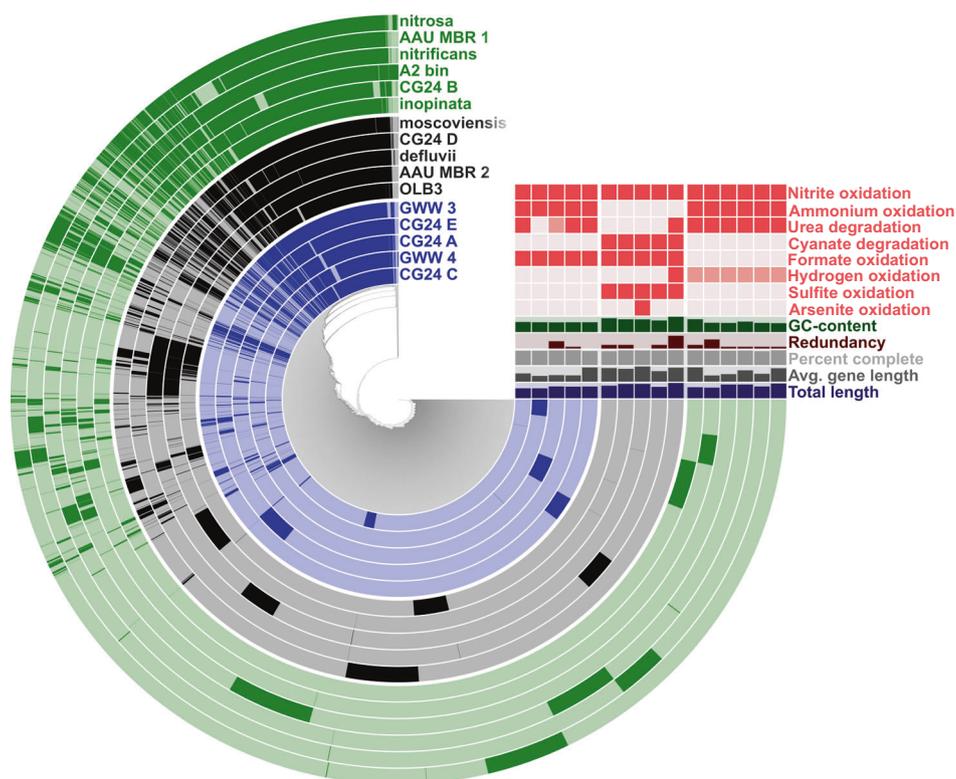
All statistical tests were performed using R v3.3.1 [35]. Correlations between genomes phylogenetic distance (based on 14 concatenate ribosomal proteins) and sequence divergence for housekeeping or the ammonia-oxidation related proteins were determined using a linear regression model. Significances between two slopes were calculated using ANOVA test. Significances between two means of sequence dissimilarities for either housekeeping proteins or

Table 1 Characteristics of examined genomes

Bin ID or reference	CG24_A	CG24_B	CG24_C	CG24_D	CG24_E	A2_bin	AAU_MBR1	AAU_MBR2	Ca N. inopinata	Ca N. nitrificans	Ca N. nitrosa	OLB3	GW3	GW4	<i>N. mosco-viensis</i>	Ca N defluvii
Size (bp)	3,555,106	3,236,455	3,000,613	3,392,746	3,477,523	4,111,999	3,525,021	4,296,753	3,295,117	4,117,083	4,422,398	3,754,263	3,501,770	2,981,542	4,589,485	4,317,083
GC Content	55.7	55.3	56.1	57.8	55.8	55.1	55.1	59.1	59.2	56.6	54.8	60.4	55.8	55.9	62.0	59.0
N50 (bp)	137,244	240,795	172,703	66,498	113,824	150,736	30,005	73,098	3,295,117	174,194	727,365	121,772	184,662	32,587	4,589,485	4,317,083
Number of scaffolds	58	36	28	135	63	61	225	172	1	36	15	79	38	167	1	1
Number of coding sequences	3697	3437	3044	3388	3628	4316	3607	4129	3308	4162	4380	3734	3433	3077	4572	4132
Number of tRNAs	42	35	44	39	36	42	39	49	49	46	49	40	43	37	50	49
Completeness (107 HMM)	95	95	97	97	100	93	90	99	100	100	100	94	98	94	100	100
Redundancy (107 HMM)	2.9	2.0	0.0	1.0	0.9	1.0	2.1	1.0	2.8	0.9	0.9	2.0	1.0	1.0	2.8	0.9
Completeness (CheckM)	88	92	92	92	94	88	86	97	97	97	97	91	92	84	96	98
Redundancy (CheckM)	5.1	6.9	2.7	2.7	3.2	2.8	2.7	5.5	4.8	2.7	2.3	4.3	3.2	1.8	6.6	2.3
Comammox affiliation	clade B	clade A	clade B	No	clade B	clade A	clade A	No	clade A	clade A	clade A	No	clade B	clade B	No	No
<i>Nitrospira</i> Lineage	II	II	II	II	II	II	I	I	II	II	II	I	II	II	II	I
Reference study	This study	This study	This study	This study	This study	[14]	[1]	[1]	[1]	[2]	[2]	[82]	[1]	[1]	[11]	[51]

*Minimum sequence length in which 50% of the assembled bases occur

Fig. 2 Communality and uniqueness in the *Nitrospira* pangenome as derived from the clustering of 16 genomes based on 12,337 protein clusters (PCs). Each radial layer represents a genome, and each bar in a layer represents the occurrence of a PC (dark presence, light absence). Clade A and Clade B comammox *Nitrospira* genomes are denoted in green and blue, respectively



ammonia-oxidation related proteins were evaluated using Welch Two Sample t-test.

Results and Discussion

Genome extraction from the metagenome

Metagenomes from adjacent locations in a RSF were used for individual genome extraction [6]. Five *Nitrospira* genomes (CG24_A, CG24_B, CG24_C, CG24_D, CG24_E) were recovered from the metagenomes using differential coverage binning [16] (Fig. 1). The characteristics of these and other genomes used in this study are shown in Table 1. The size of the comammox genomes assembled here range from 3.0–3.6 Mb, with a completeness of 95–100%. Based on phylogenetic analysis of 14 syntenic ribosomal proteins, all genomes belong to *Nitrospira* sublineage II (Supplementary Figure S2). Genes required for complete ammonia oxidation (*amo* and *hao* operons) were detected in four of the genomes. Based on *amoA* phylogeny CG24_A, CG24_C and CG24_E belong to comammox clade B and CG24_B to comammox clade A (Supplementary Figure S3). CG24_A was most abundant at an average relative abundance of 18.6% (of all metagenome sequence reads) followed by CG24_B (8.6%), CG24_C (4.6%), CG24_D (2.4%, the only canonical NOB *Nitrospira* recovered) and CG24_E (1.1%). (Supplementary Figure S4).

Comparative genomics

The newly recovered genomes were compared with each other and publicly-available, high-quality *Nitrospira* genomes, including in total 11 comammox and five canonical *Nitrospira* (Table 1). Based on average amino acid identity (AAI) analysis, the 16 studied *Nitrospira* genomes constitute 11 different species, seven of them comammox *Nitrospira* (at species level cut-off of 85% AAI [36]. CG24_A, CG24_D and CG24_E are divergent enough from each other and previously published genomes to be separate species (Supplementary Figure S5).

Comparison of the 16 *Nitrospira* genomes was based on SEED subsystems (further details in Supplementary Information and Supplementary Figure S6) and pangenomic analysis (Fig. 2). The 59,744 coding sequences (CDS) of the 16 *Nitrospira* genomes clustered into 12,337 protein clusters (PCs), with a core *Nitrospira* genome consisting of 1382 PCs. The core genome includes genes for the nitrite oxidation pathway, the reductive tricarboxylic acid cycle for CO₂ fixation (rTCA), the gluconeogenesis, the pentose phosphate, and the oxidative TCA cycle. Chlorite dismutase and copper-containing nitrite reductase (*nirK*) are also present in the core genome (Fig. 3). 35 comammox-specific PCs were identified; 16 and 3 of these PCs had highest sequence similarity to homologs in β -AOB and methane oxidizers, respectively (Supplementary Table S2). In addition, we detected 57 and 52 comammox clade A and clade

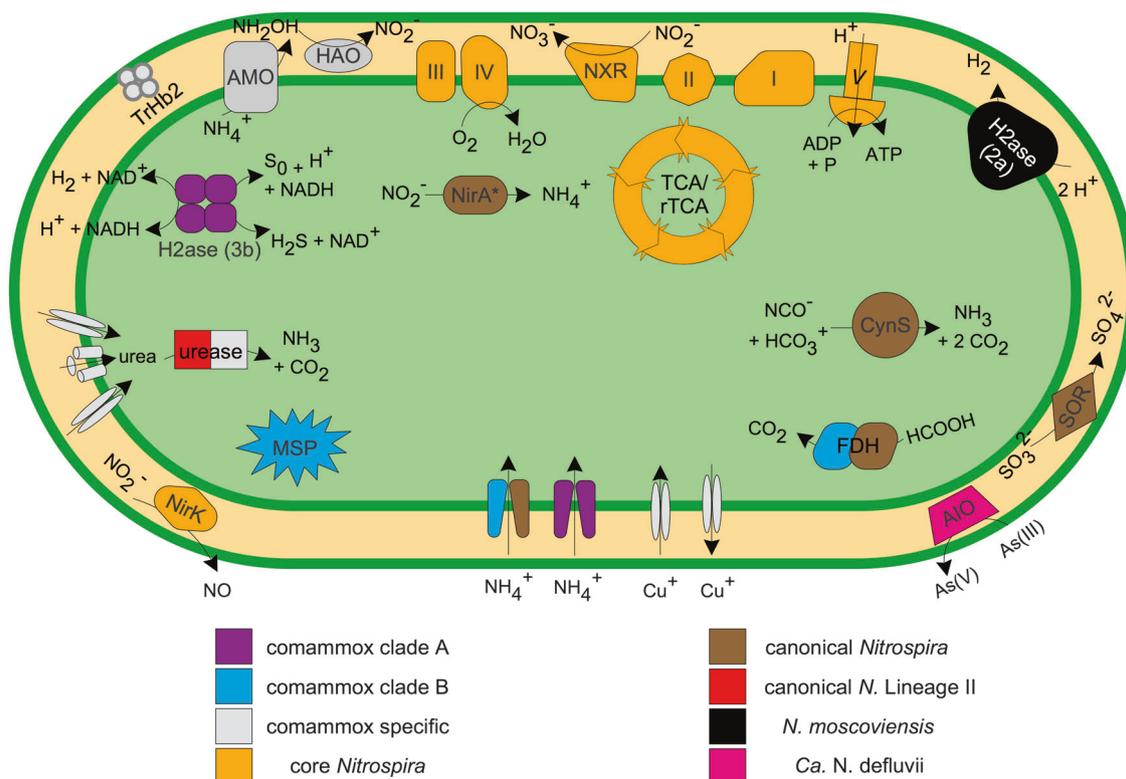


Fig. 3 Cartoon of core and specific key metabolic features in the *Nitrospira* pangenome, as predicted from genome annotation. AIO arsenite oxidase, CynS cyanate hydratase, FDH formate dehydrogenase, H2ase hydrogenase, MSP methionine salvage pathway,

SOR sulfite dehydrogenase, TrHb2 2/2 hemoglobin group 2. Enzyme complexes of the electron transport chain are labeled by Roman numerals. * *N. moscoviensis* possesses an octaheme nitrite reductase (ONR) putatively involved in nitrite reduction to ammonia

B-specific PCs, respectively (Supplementary Table S2 and Fig. 3). The specific metabolic characteristics inferred from the annotations of genes with known homologs, and identified with the pangenomic analysis are described below.

Nitrogen metabolism

All the recovered genomes encoded the nitrite oxidoreductase (NXR), consistent with other *Nitrospira* spp. The copy number of NXR in the investigated genomes varied from one to two in most of the comammox genomes (except *Ca. N. nitrificans*, which has four copies), to five in *N. moscoviensis* (Supplementary Table S2). The ammonia oxidation pathway (AMO structural genes *amoCAB* and the putative AMO subunits *amoEDD2*, as well as genes for *HaoAB* and the associated cytochromes *CycAB*) is present in four of the newly recovered genomes (CG24_A, CG24_B, CG24_C and CG24_E).

Regarding nitrogen uptake, clade B and canonical *Nitrospira* genomes encode MEP-type ammonia transporters, which are also found in AOA [37]. Clade A genomes encode an ammonia transporter with high homology (>70% amino acid similarity) to Rh-type transporters found in most of β -AOB (Supplementary Table S3). Experimental analysis

in *Nitrosomonas europaea* showed that its Rh-type transporter proteins have ammonium affinity in millimolar range and high uptake capacity [38], and similar results were observed for Rh-type ammonia transporter in other organisms [39]. In contrast, investigated MEP-type ammonium transporters have higher affinity (micromolar range) and lower uptake capacity [40, 41]. Whether these observations are also valid for *Nitrospira* spp. awaits experimental confirmation. Besides the uptake of exogenous ammonia by *Nitrospira*, ammonia can be produced through urea degradation as most of the comammox genomes harbor urease genes. This enzyme, which is functional in *N. moscoviensis* [11], is not detected in genomes of *Nitrospira* sublineage I. The comammox genomes contain a diversity of urea transporters. In addition to the high affinity urea ABC transporters (*urtABCDE*) present in some *Nitrospira* spp. such as *N. lenta* [11], comammox genomes also harbor two additional urea transporters: an outer-membrane porin (*fmdC*) involved in uptake of short-chain amides and urea at extremely low concentrations [42], and a urea carboxylase-related transporter (*uctT*). These extra urea transporters present in comammox *Nitrospira* might confer a competitive advantage in urea uptake with respect to other *Nitrospira* in environments with low and/or fluctuating urea

concentrations. On the other hand, cyanate hydratase genes (*cynS*) are only detected in canonical *Nitrospira*. This may confer a benefit over comammox *Nitrospira* in environments with very low ammonium concentrations and the presence of cyanate coupled with reciprocal feeding with ammonia oxidizers [12]. All genomes except *Ca. N. inopinata* encode a $\text{NO}_2^-/\text{NO}_3^-$ transporter gene (*narK*). Furthermore, canonical *Nitrospira* genomes contain additional transporters for the uptake of nitrite: lineage I *Nitrospira* and CG24_D genomes harbor a nitrite transporter (*nirC*) and a formate/nitrite family transporter, while *N. moscoviensis* and CG24_D contain a nitrite-nitrate ABC transporter (*nrtABC*). The comammox genomes lack an assimilatory nitrite reductase (*nirA*) or octaheme nitrite reductase (ONR) (which has been proposed to be associated with nitrite reduction to ammonia in *N. moscoviensis* [11]), which would prevent comammox *Nitrospira* growth in the presence of nitrite as sole N source, as observed in *Ca. N. inopinata* [1].

Alternative electron donors

Some *Nitrospira* can utilize substrates beyond nitrite as electron donors including formate and hydrogen [7, 10, 11, 43]. The genes coding for formate dehydrogenase (*fdh*) are present in canonical *Nitrospira* and comammox clade B but were not detected in comammox clade A. This could provide an opportunity for niche-differentiation particularly in oxic–anoxic transition zones where formate can be found as a product of fermentation. With respect to hydrogen oxidation, the group 2a [NiFe] hydrogenase and accessory proteins involved in aerobic hydrogen oxidation in *N. moscoviensis* [10] are absent from the other *Nitrospira* genomes. However, all comammox clade A genomes encode a complete group 3b [NiFe]-hydrogenase. Although this bidirectional hydrogenase (sulfhydrogenase) is extensively distributed across the bacterial domain [44], little is known about its actual role (further details in Supplementary Information). Comammox genomes do not contain genes for a periplasmic sulfite:cytochrome c oxidoreductase (sulfite dehydrogenase, *sorAB*), which is characteristic of canonical *Nitrospira*. These genomes also possess the genetic inventory for sulfur assimilation, suggesting that this sulfite dehydrogenase might be involved in sulfite oxidation, as suggested for another NOB (*Nitrospira gracilis*) [45]. Lastly, a complete arsenite oxidase was exclusively found in *Ca. N. defluvii*. Although all examined genomes harbor arsenic resistance genes, only *Ca. N. defluvii* has the genetic potential for energy conservation during arsenite oxidation (Supplementary Information).

Energy conservation and transduction

All *Nitrospira* genomes harbor two homologous sets of genes encoding the complex I of the electron transport chain (NADH:ubiquinone oxidoreductase, NUO). One of these NUO lacks the subunits involved in NADH binding (NuoE and NuoF). This feature has been observed in other prokaryotes [46]; in *Nitrospira* spp. this incomplete complex I may be used for reverse electron transport from quinol to low-potential ferredoxin, as proposed in *Nitrospira gracilis* [45], thereby providing reduced ferredoxin required for CO_2 fixation in the rTCA cycle. Additionally, CG24_A, *Ca. N. nitrosa*, AAU_MBR1, A2_bin and *N. moscoviensis* possess a third complete homologous set of genes coding for complex I, which is divergent from the other two, and is closely related to the γ -proteobacterial clade E type. This type of complex I has also been found in other distantly related organisms, including *Nitrosospira multififormis* and some *Bacteroidetes* spp., suggesting acquisition by horizontal gene transfer [47]. Although the exact function of this additional complex I is not known, differential expression of distinct complex I isozymes has been observed in other microorganisms, pointing towards physiological versatility [48, 49]. Also, differences with respect to complex II, the succinate:quinone oxidoreductase (SQR), were noted across genomes. SQR connects the TCA cycle to the quinone pool. This complex is classified in five types based on the number of membrane-bound domains and haem content [50]. *Nitrospira* genomes do not all contain the same SQR type. Clade B, CG24_D, and genomes belonging to *Nitrospira* lineage I contain a SQR type E, whereas clade A genomes and *Nitrospira moscoviensis* possess a SQR type B. Differences were also detected for the cytochrome *bc* complex (complex III) which is involved in the transfer of electrons from quinol to a *c*-type cytochrome. The canonical *Nitrospira* genomes contain at least two copies of complex III (three in *N. moscoviensis* and OLB3), while the comammox genomes harbor just one copy of this complex. With respect to cytochrome oxidases (complex IV), the studied genomes harbor a CDS similar to the the uncharacterized but functionally transcribed ‘*cyt bd*-like oxidase’ (NIDE0901) of *Ca. N. defluvii* which was proposed to be a terminal oxidase [51]. In addition to this putative terminal oxidase, canonical *Nitrospira* (except CG24_D), comammox clade A genomes (excluding *Ca. N. nitrosa*) and the clade B GWW3 bin contain the *cydAB* genes encoding a heterodimeric *cyt. bd* quinol oxidase. The reaction catalyzed by this respiratory oxidase is not coupled to proton pumping [52], and may be important when electrons derive from low potential electron donors.

Carbon metabolism

Comammox *Nitrospira*, like other sequenced *Nitrospira* genomes [51], encode genes for glycolysis, gluconeogenesis, the pentose phosphate pathway and the TCA cycle. Moreover, clade B comammox genomes and AAU_MBR2 carry an acetate permease gene (*actP*). *Nitrospira* genomes have the potential to degrade catechol and protocatechuate (Supplementary Table S2). Although these degradation pathways are only partially complete in *Nitrospira* genomes, comammox *Nitrospira* possess an intradiol ring-cleavage dioxygenase, which could transform catechol derivatives to TCA cycle intermediates [55]. Additionally, comammox clade B and *N. moscoviensis* harbor a 4-oxalocrotonate tautomerase, essential in the conversion pathway of various aromatic compounds such as catechol to intermediates for the TCA cycle [54]. The *phaZ* gene which codes for polyhydroxybutyrate (PHB) depolymerase involved in PHB degradation was exclusively found in *Nitrospira* comammox genomes. Genes related to PHB synthesis are not found in the studied genomes, hence whether the PBH polymerase is a relic or has any functional role remains unknown. Overall, the presence of enzymes and pathways involved in different carbon sources degradation suggests that comammox *Nitrospira* have the potential to grow mixotrophically as reported for other *Nitrospira* spp. [43, 55, 56].

Stress response, resistance and defense

Genes encoding catalase (*cat*) or superoxide dismutase (*sod*), providing protection against reactive oxygen species (ROS), are absent from the closely related comammox genomes *Ca. N. nitrosa* and AAU_MBR1 as well as from *Ca. N. defluvii*. On the other hand, CG24_A and *N. moscoviensis* harbor diverse genes related to ROS protection including one catalase and one peroxidase (two in both cases for *N. moscoviensis*), and two dissimilar superoxide dismutases. The remaining genomes encode for either catalases or superoxide dismutases and all genomes harbor the putative ROS defense mechanisms predicted for *Ca. N. defluvii* (consisting of cyt. *c* peroxidases, thioredoxin-dependent peroxiredoxins, manganese, bacterioferritin and carotenoids) [51]. Contrary to canonical *Nitrospira*, the comammox genomes contain a 2/2 hemoglobin type II (TrHb2), which has been associated with oxidative stress resistance and oxygen scavenging [57, 58]. Thus, the distinct capacity to deal with oxidative stress within *Nitrospira* spp. may enable these organisms to coexist by occupying different microniches in environments with oxygen gradients such as biofilms and flocs. Most of the genomes encode two proteins homologous to RsbUV, related to environmental stress response, and comammox clade A

genomes possess a fusion protein distinct from other *Nitrospira* spp. that is homologous to RsbUVW. *N. moscoviensis* also encodes all the genes for the stressosome (*rsbRSTX*), a complex that controls several signaling pathways in response to diverse environmental stresses [59] (further details in Supplementary Information). Hence, based on these genomes characteristics, comammox clade A and especially canonical *N. moscoviensis* might be better adapted to changing environmental conditions than other *Nitrospira*. On another note, genes associated with low-resource environments were encountered in some of the *Nitrospira* genomes. Comammox *Nitrospira* may have higher requirements for copper than canonical *Nitrospira* as ammonia monooxygenase contain Cu as cofactor. Different from canonical *Nitrospira*, the comammox genomes contain Cu homeostasis genes (*copCD* and *copAB*) with high sequence similarities to homologs in β -AOB. These proteins confer higher Cu²⁺ tolerance or increased Cu²⁺ uptake [60, 61] and may allow comammox *Nitrospira* to survive in conditions of low Cu²⁺ availability. Canonical *Nitrospira* genomes harbor the genes for the cytochrome *c* biogenesis system II. In contrast, comammox genomes contain the genes for the cytochrome *c* biogenesis system I. While system II requires less energy for cytochrome synthesis, system I is advantageous in iron-limited environments as this system has hemes with higher iron affinity [62]. As nitrifiers have a high iron requirement for their [FeS] cluster- and heme-containing enzymes, this could provide an important competitive advantage for comammox *Nitrospira* in iron-limited environments [63]. Comammox *Nitrospira* clade B genomes contain the methionine salvage pathway which was not detected in clade A or canonical *Nitrospira* genomes (Fig. 3 and Supplementary Table S2). This pathway is involved in the recycling of sulfur-containing metabolites to methionine and is upregulated under sulfur-limiting conditions [64, 65]. Thus, comammox clade B could better persist during periods of sulfur depletion. Genes related to arsenic and mercury resistance were found in all studied genomes. Especially striking were the cases of comammox CG24_B and AAU_MBR1 which contain phylogenetically distinct chromate, arsenic and mercury resistance proteins in a putative integrative conjugative element (Supplementary Information).

Comparison between comammox *Nitrospira* and ammonia oxidizing microorganisms

Comammox *Nitrospira* generally harbor a single copy of AMO and HAO genes (*Ca. N. nitrosa* contains two *amoA* genes and all comammox genomes harbor extra *amoC* besides the one present in the *amo* operon). A similar scenario is observed for γ -proteobacterial AOB (γ -AOB) and AOA. In contrast, β -AOB genomes are characterized by the

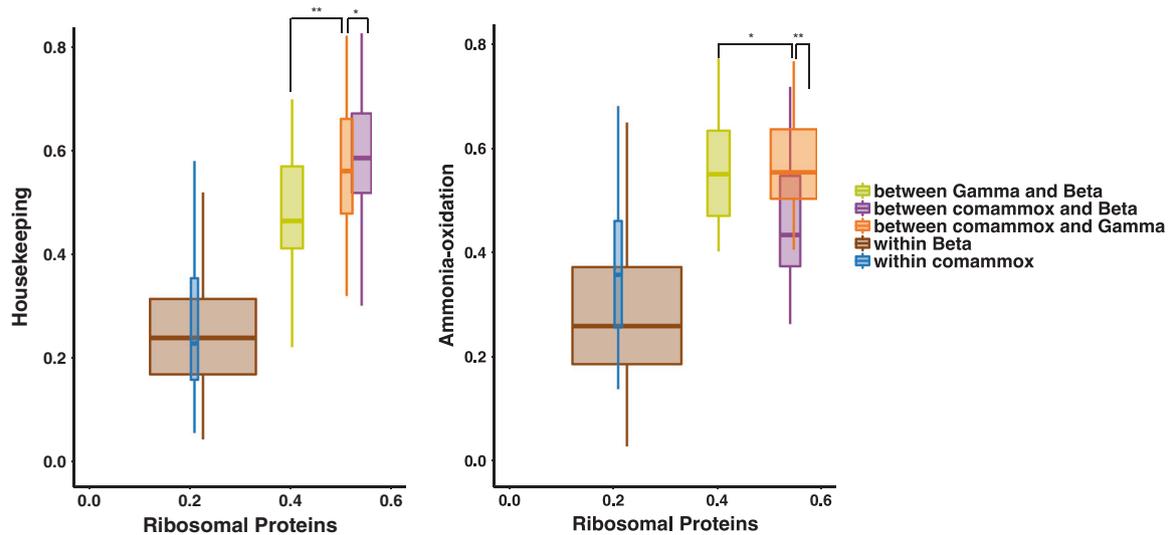


Fig. 4 Relationship between average phylogenetic distance of genomes and protein sequence divergence for housekeeping proteins (left) and for ammonia-oxidation related proteins (right) for comammox *Nitrospira*, β -AOB and γ -AOB genomes. Boxplot are colored according to the groups to which the compared genomes belong. The y-axis shows the pairwise protein dissimilarity (fraction of differing

amino-acid sites) for a set of 18 housekeeping proteins or 27 ammonia-oxidation related proteins, while the x-axis shows the corresponding pairwise dissimilarity for a set of 14 ribosomal proteins. Asterisks(*) and (**) indicates non-significant ($P > 0.01$) and significant ($P < 0.01$) difference between means of sequence dissimilarities of two studied groups, respectively

presence of two or three copies of *amo* and *hao* gene clusters. Differences are also detected for genes related to NO_x metabolism. Copiotrophic AOB genomes generally contain cytochrome *c* nitric oxide reductase (cNOR), heme-copper nitric oxide reductase (sNOR), nitrosocyanin, and cytochrome P460. In the case of oligotrophic AOB genomes, they lack sNOR and in some cases nitrosocyanin or cNOR (Supplementary Table S3). On the other hand, comammox *Nitrospira* genomes do not have the four mentioned proteins related to NO_x metabolism as also observed for AOA genomes (Supplementary Table S3). This lack of NO_x metabolism related proteins may be associated with low ammonium concentration environments where nitrosative stress would be minor. Nevertheless, the investigated comammox *Nitrospira* genomes contain two proteins putatively linked to resistance to nitrosative stress: a NO-responsive regulator (*nnrS*) and a 2/2 hemoglobin type I (TrHb1), that are not found in AOA. The different AOP groups also clearly differ in their carbon fixation pathways. AOB and AOA utilize the oxygen-tolerant Calvin–Benson–Bassham and hydroxypropionate–hydroxybutyrate cycles, respectively, while comammox *Nitrospira* possess the microaerophilic related rTCA pathway [66]. Furthermore, canonical AOP genomes encode the low-affinity *aa3*-type heme-copper oxidase (*Nitrosomonas eutropha* also contains a high-affinity cytochrome *c* oxidase *cbb3*). In contrast, comammox *Nitrospira* harbor cytochrome *bd*-like oxidases. Cytochrome *bd* oxidases have high affinity for O_2 [67] and are expressed under O_2 -limited conditions in other

microorganisms [68, 69], but it remains unknown whether this is also the case for the cyt *bd*-like oxidases of *Nitrospira* spp. Additionally, the 2/2 hemoglobin type II (TrHb2), associated with oxidative stress resistance and oxygen scavenging, which was detected in comammox *Nitrospira*, is not universal for AOB (only present in *Nitrosospira* spp.) and has not been detected in AOA. It is to be experimentally confirmed whether these low oxygen-tolerance features are also physiologically expressed in comammox *Nitrospira* and may provide an advantage for growth in microaerophilic environments. Another characteristic of comammox *Nitrospira* is their genetic potential to compete under phosphorous and copper limiting conditions. Comammox *Nitrospira* genomes contain an alkaline phosphatase (*phoD*), which has been detected to be highly expressed under phosphorus limitation and starvation in other microorganisms [70, 71]. This enzyme was not detected in AOA genomes and is not universal in AOB (putative homologs are present in *Nitrosomonas* sp. Is79A3, *Nitrosococcus halophilus* and *Nitrosospira* spp.). In relation to Cu homeostasis, while *copCD* genes are present in both AOB and comammox genomes, the genes *copAB* detected in comammox *Nitrospira* are not common for AOB as just few species harbor homologs of these genes (*Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosospira multiformis* and *Nitrosospira* sp. Nv17). Although AOA seem to have a high Cu^{2+} demand due to their high presence of copper-containing proteins, their Cu^{2+} acquisition mechanisms are unknown [72]. These two aspects could be of great importance as both phosphorous and copper

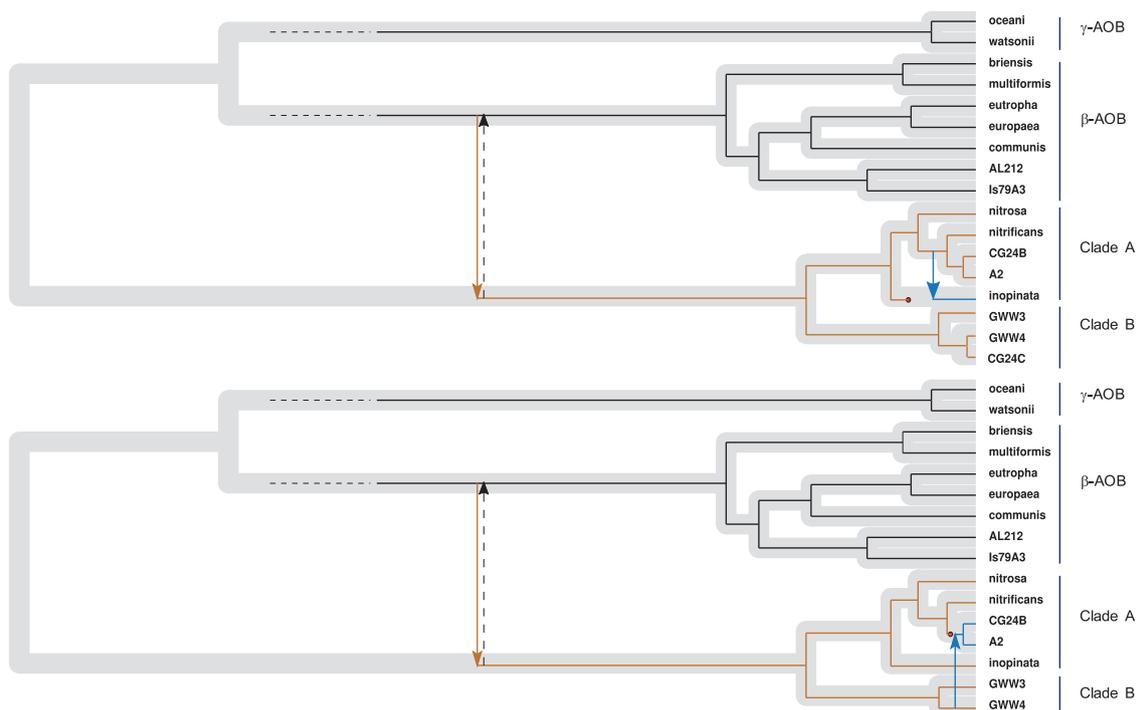


Fig. 6 Reconciliation of functional gene trees (based on amino acid sequences. Top. *AmoA*; bottom. *HaoA*) with species tree for comammox *Nitrospira*, β -AOB, and γ -AOB genomes. The species tree, based on 14 ribosomal proteins, is shown in gray with the gene-

trees super-imposed on top in narrower black lines. Arrows and red dots denote transfer and loss events, respectively. The displayed tree is the most parsimonious tree. Dashed arrows indicate an alternative reconciliation scenario

This pairwise dissimilarity analysis indicates that the evolutionary history of the ammonia oxidation-related genes is complex, and might indicate the occurrence of horizontal transfer(s) between comammox *Nitrospira* and β -AOB.

We, subsequently, analysed the genomic arrangement of ammonia oxidation-related genes for all comammox genomes and for representative β -AOB genomes (Fig. 5 and Supplementary Figure S9). For all comammox genomes except for *Ca. N. inopinata*, the AMO and HAO gene clusters as well as the cytochrome *c*-biosynthesis genes are situated in the same genomic region. This is not the case for AOB genomes. Additionally, comammox genomes have two copies of *amoD*, while AOB only have one copy. Among the comammox genomes, clade B genomes uniquely contain a duplication of one of the cytochrome *c*-biosynthesis proteins (*CcmI*). Clade A genomes have two duplicated hypothetical proteins-coding genes next to the *hao* operon, while comammox clade B and AOB genomes have only one copy (more details of genomic arrangement analysis in Supplementary Information). Shared features in the genomic regions encoding the ammonia oxidization pathways between the two comammox clades that are not shared with other ammonia oxidizers, suggests that the comammox clades have a common ancestor for this specific genetic capacity.

A reconciliation analysis was performed to stringently examine the possible occurrences of gene duplication(s), gene loss(es), and horizontal gene transfer(s). First, we constructed a clock-model based species-tree from the 14 ribosomal proteins (Supplementary Table S1), including the 16 examined *Nitrospira* genomes, *Leptospirillum ferrooxidans* (of the Nitrospirae phylum), eight and two publically available β -AOB and γ -AOB genomes, respectively, and two additional non-nitrifier β - and γ - proteobacterial genomes closely related to β -AOB and γ -AOB, respectively. (Supplementary Figure S10 and Supplementary Table S4). Gene trees were, subsequently, constructed for the ammonia oxidation-related genes under investigation (Supplementary Table S4). The probabilistic (Bayesian) analysis provided strong support for horizontal transfer of the majority of the investigated genes (Supplementary Table S6): for 12 genes, the posterior probability for at least one transfer event was between 95 and 100%, for other 10, the posterior probability was between 80 and 95%. For all investigated genes, we found very strong support (95–100%) for either a transfer or a duplication event.

The evolutionary history of *amoA* and *haoA* was further investigated. For *amoA*, the reconciliation model suggests a gene transfer event from β -AOB to an ancestor of comammox *Nitrospira* (Fig. 6). In addition, a transfer of *amoA* from the common ancestor of *Ca. N. nitrosa* and

AAU_MBR_1 to *Ca. N. inopinata* was inferred. This transfer would explain the deviation in the tetranucleotide pattern of the *amoCAB*-containing region compared to the genome-wide signature identified in *Ca. N. inopinata* by Daims et al. [1]. Furthermore, unlike the other comammox genomes, *Ca. N. inopinata* does not have the *amo* operon located in the same genomic region as the *hao* operon and the genes for cytochrome c-biogenesis system II (Supplementary Figure S9).

The high dissimilarity between the *amoA* sequences of the otherwise closely related clade A and clade B comammox genomes (Supplementary Figure S10), may be explained by an adaptation of the comammox organisms to different niches. Based on the pairwise dissimilarity sequence analysis, clade B comammox genomes have a faster evolution rate of the ammonia oxidation-related proteins than clade A genomes ($P < 0.0001$; only for *AmoA* $P < 0.0005$), while the two clades do not have a different evolution rate for the housekeeping protein ($P > 0.5$).

Regarding *haoA*, the reconciliation model predicted two transfer events (Fig. 6). As for *amoA*, a transfer from β -AOB to an ancestor of comammox *Nitrospira* is suggested. Another transfer was inferred from clade B to the ancestor of the clade A genomes A2 and CG24_B. Consistent with this hypothesis, A2 and CG24_B contain genes coding for a copper-containing nitrite reductase next to the HAO cluster as also observed for clade B genomes (Supplementary Figure S10).

Besides the scenarios predicted from the reconciliation model, alternative scenarios cannot be ruled out due to the uncertainties associated with the limited number of sequenced *Nitrospira* and AOB genomes. An alternative scenario would be HGT of *amoA* and *haoA* from comammox *Nitrospira* to β -AOB (Fig. 6). In another possible scenario, both comammox *Nitrospira* and β -AOB would have acquired the ammonia-oxidation genes from an unknown third donor. This final scenario was tested in our reconciliation analysis by including the option of investigating transfers to and from unsampled species. The potential involvement of an unknown donor in the evolutionary history of *amoA* is not discarded, although these scenarios are less parsimonious than the one showed in Fig. 6. On the other hand, no evidence of HGT from an unsampled species was detected for *haoA*.

As comammox clade A and clade B do not form a monophyletic group within *Nitrospira* sublineage II (Supplementary Figure S2), loss events for both *amoA* and *haoA* are expected in some of the *Nitrospira* spp. that phylogenetically placed between these clades such as *N. moscoviensis* and CG24_D. One possible explanation for loss of ammonia oxidation capacity maybe the postulated trade-off between rate and yield of ATP production [13]. Under this hypothesis, shortening the nitrification pathway would lead to an increased specific growth rate which could be

advantageous in some scenarios. Thus, the lack of the putatively acquired AMO and HAO-related genes in extant canonical *Nitrospira* spp. could be the result of selection for optimal pathway length.

The clock-based species-tree estimated the separation between clade A and clade B comammox genomes to have occurred approximately 300 ± 90 MYA, while the split between *Nitrospira* lineage I and II was dated at 375 ± 50 MYA (Supplementary Figure S10). Our model point towards an earlier transfer event for both *amoA* and *haoA* although it was not possible to date the HGT event with a high level of certainty. Hence, it remains unknown whether additional loss events would be required to explain the lack of comammox genes in *Nitrospira* genomes belonging to lineages other than lineage II.

Conclusions

In summary, our findings reveal diverse genetic capabilities of the two comammox clades, canonical *Nitrospira* and canonical ammonia oxidizing prokaryotes. The absence of NO_x metabolism pathways in the studied comammox genomes, as observed in AOA and some oligotrophic AOB strains in comparison with copiotrophic AOB, and the diverse genetic capacity for tolerance of low micronutrient concentrations, together with the dominant detection of comammox *Nitrospira* in substrate-limited environments might indicate an oligotrophic lifestyle of comammox *Nitrospira*. In fact, an oligotrophic lifestyle has been experimentally demonstrated in *N. inopinata* [76], the only comammox isolated to date.

Additionally, we identified a high probability of transfer events between β -proteobacterial ammonia oxidizers and comammox *Nitrospira* for genes belonging to the ammonia oxidation pathway. Together, these results expand our knowledge of the ecology and evolution of the recently discovered comammox *Nitrospira*. Further discovery and characterization of new comammox *Nitrospira*, as well as canonical NOB and AOP genomes will help confirm these observations.

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Authors contributions The study was conceived by AP, BFS and TSP. AP performed the genome binning and comparative genomic analyses. AGP conducted the evolutionary analysis supported by AP. AP led interpretation of the results supported by SJF, AD and AGP. AP drafted the manuscript helped by SJF, AGP, and BFS; all authors contributed to its revision, and approved the final submission.

Compliance with ethical standards

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

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