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Applied and Environmental Science

Metagenomic Evidence for the Presence of Comammox *Nitrospira*-Like Bacteria in a Drinking Water System

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ABSTRACT We report metagenomic evidence for the presence of a *Nitrospira*-like organism with the metabolic potential to perform the complete oxidation of ammonia to nitrate (i.e., it is a complete ammonia oxidizer [comammox]) in a drinking water system. This metagenome bin was discovered through shotgun DNA sequencing of samples from biologically active filters at the drinking water treatment plant in Ann Arbor, MI. Ribosomal proteins, 16S rRNA, and *nxrA* gene analyses confirmed that this genome is related to *Nitrospira*-like nitrite-oxidizing bacteria. The presence of the full suite of ammonia oxidation genes, including ammonia monooxygenase and hydroxylamine dehydrogenase, on a single ungapped scaffold within this metagenome bin suggests the presence of recently discovered comammox potential. Evaluations based on coverage and k-mer frequency distribution, use of two different genome-binning approaches, and nucleic acid and protein similarity analyses support the presence of this scaffold within the *Nitrospira* metagenome bin. The *amoA* gene found in this metagenome bin is divergent from those of canonical ammonia and methane oxidizers and clusters closely with the unusual *amoA* gene of comammox *Nitrospira*. This finding suggests that previously reported imbalances in abundances of nitrite- and ammonia-oxidizing bacteria/archaea may likely be explained by the capacity of *Nitrospira*-like organisms to completely oxidize ammonia. This finding might have significant implications for our understanding of microbially mediated nitrogen transformations in engineered and natural systems.

IMPORTANCE Nitrification plays an important role in regulating the concentrations of inorganic nitrogen species in a range of environments, from drinking water and wastewater treatment plants to the oceans. Until recently, aerobic nitrification was considered to be a two-step process involving ammonia-oxidizing bacteria or archaea and nitrite-oxidizing bacteria. This process requires close cooperation between these two functional guilds for complete conversion of ammonia to nitrate, without the accumulation of nitrite or other intermediates, such as nitrous oxide, a potent greenhouse gas. The discovery of a single organism with the potential to oxidize both ammonia and nitrite adds a new dimension to the current understanding of aerobic nitrification, while presenting opportunities to rethink nitrogen management in engineered systems.

KEYWORDS: *Nitrospira*, comammox, drinking water systems

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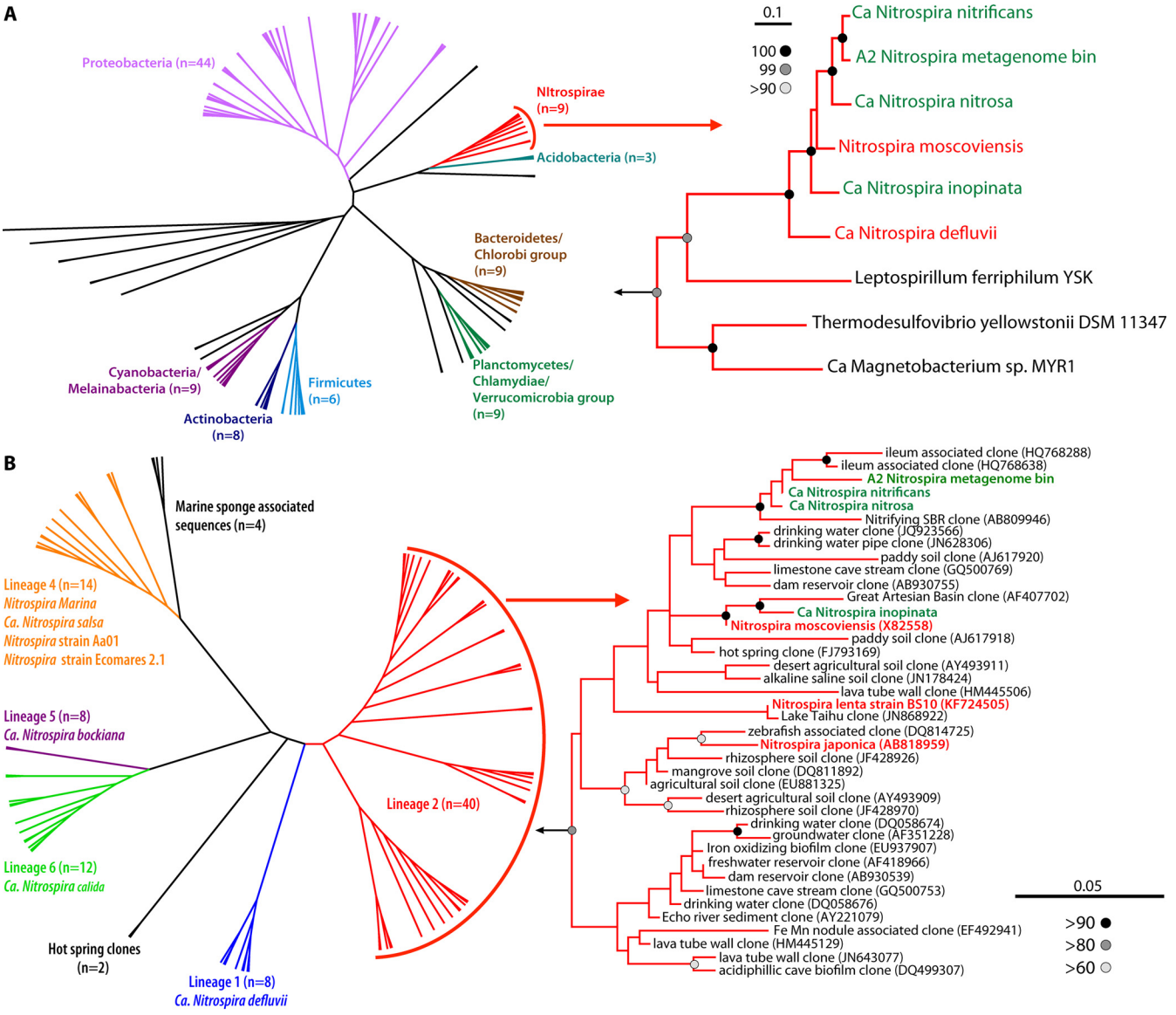
Comammox Nitrospira in the #H2Omicrobiome

Until recently, aerobic nitrification was considered to be a two-step process involving two functional guilds. The process of nitrification was considered split between ammonia-oxidizing bacteria (AOB) (1) and ammonia-oxidizing archaea (AOA) (2), which oxidize ammonia to nitrite, and strict nitrite-oxidizing bacteria (NOB) (3), which oxidize nitrite to nitrate. The phylogenetic distribution of AOB is limited to the *Betaproteobac-*

teria and *Gammaproteobacteria*; AOA fall within the *Thaumarchaea*, and NOB span the *Proteobacteria*, *Chloroflexi*, *Nitrospirae*, and *Nitrospinae*. However, the recent discovery of complete ammonia oxidizer (comammox) organisms, i.e., bacteria that completely oxidize ammonia to nitrate (4), within the genus *Nitrospira* has significantly changed our understanding of the aerobic nitrification process (5, 6). In the current study, we report metagenomic evidence of a *Nitrospira*-like organism that has the potential to perform both steps of the aerobic nitrification process and thus is likely to be a comammox bacterium. Specifically, it has genes to oxidize nitrite to nitrate (nitrite oxidoreductase) and possesses all genes required for ammonia oxidation, i.e., ammonia monooxygenase (*amoA*, *amoB*, *amoC*) and hydroxylamine dehydrogenase (also known as hydroxylamine oxidoreductase) (*hao*). This metagenome bin was discovered through shotgun DNA sequencing of samples from biologically active filters at a drinking water treatment plant (Ann Arbor, MI). IDBA-UD assembly (7), CONCOCT genome binning (8), and manual curation (see Text S1 in the supplemental material) resulted in 51 high-quality draft genomes with 2 to 755 scaffolds (median = 73), N_{50} ranging from 7.4 to 114 kbp (median = 86.9 kbp), and levels of completeness ranging from 77 to 100% (9). All genome bins were annotated by the Integrated Microbial Genomes Expert Review (IMG ER) system (10).

One of the genomes was phylogenetically assigned to the *Nitrospira* genus (JGI GOLD identification number Ga0074138) by Amphora2 (11). This 4.1-Mbp genome consists of 61 scaffolds (N_{50} = 150.74 kbp) with a GC content of 55%, 4,196 coding sequences, a complete 5S rRNA gene, and partial 16S and 23S rRNA genes. The genome bin was 88% complete, with 2.8% likely contamination based on 182 markers (9). The Amphora2 phylogenetic assignment was confirmed by Ribosomal Database Project (RDP) classification (12) of the 574-bp partial 16S rRNA gene in this genome (100% confidence). Maximum-likelihood phylogenetic analyses (13) of 16 syntenic ribosomal proteins (14) (Fig. 1A; see also Fig. S1 in the supplemental material) and the 16S rRNA gene (Fig. 1B) and Bayesian phylogenetic analyses (15) of the *nxrA* gene (Fig. 1C; Text S1) indicated that this metagenome bin is related to *Nitrospira* lineage II bacteria. The metagenome bin contains two copies of *nxrA*, both of which have high levels of similarity to *Nitrospira* lineage II bacterial genes (Fig. 1C). We were unable to recover the *nxrB* gene in the metagenome bin and suspect that this was due to assembly issues, as both copies of *nxrA* were found at the ends of their respective scaffolds. Indeed, mapping of the reads extracted for reassembly indicated that 391 paired-end reads mapped to the *nxrB* genes of organisms in *Nitrospira* lineage II, confirming that this gene was present but not assembled. As with *Nitrospira moscoviensis*, the new metagenome bin also contained a full suite of genes for urea transport and its degradation to ammonia (16).

The newly described metagenome bin contained genes involved in ammonia oxidation (*amoA*, *amoB*, *amoC*, and *hao*) on a single 92.7-kbp scaffold (scaffold 158; scaffold range, 101 to 161) in close succession on a region beginning at 69 kbp (Fig. 1D). This observation is consistent with reports of recently described comammox bacteria that have radically altered our understanding of the nitrogen cycle (5, 6) and provides further indication of the metabolic versatility of *Nitrospira* bacteria (16–18). In addition to finding the *amo* and *hao* genes, we found several genes on scaffold 158 and a second scaffold (scaffold 123) within this metagenome bin with a gene arrangement similar to that of the recently published comammox bacteria. First, the *hao* gene cluster was preceded by several genes associated with heme export and cytochrome *c* biogenesis (*ccmA* to *-H*), a feature not seen in any published AOB genomes and thus suggested as a diagnostic feature for comammox *Nitrospira* (5) (Fig. 1D). Second, we found a gene that likely codes for membrane-bound protein OrfM based on its similarity to the corresponding gene in "*Candidatus* Nitrospira inopinata" (identity = 69%, E value = 5e−132, bit score = 368) (5). However, IMG annotation and subsequent independent annotation efforts suggested that it was a hypothetical protein. Third, we found homologues of *amoD* (*n* = 2) and *amoE* arranged in close succession on scaffold 123, genes that were originally annotated as encoding hypothetical proteins. Their


A
Proteobacteria (n=44)
NItrospirae (n=9)
Acidobacteria (n=3)
Bacteroidetes/ Chlorobi group (n=9)
Planctomycetes/ Chlamydiae/ Verrucomicrobia group (n=9)
Firmicutes (n=6)
Actinobacteria (n=8)
Cyanobacteria/ Melainabacteria (n=9)
0.1
100
99
>90
Ca Nitrospira nitrificans
A2 Nitrospira metagenome bin
Ca Nitrospira nitrosa
Nitrospira moscoviensis
Ca Nitrospira inopinata
Ca Nitrospira defluvii
Leptospirillum ferriphilum YSK
Thermodesulfovibrio yellowstonii DSM 11347
Ca Magnetobacterium sp. MYR1
B
Marine sponge associated sequences (n=4)
Lineage 4 (n=14)
Nitrospira Marina
Ca. Nitrospira salsa
Nitrospira strain Aa01
Nitrospira strain Ecomares 2.1
Lineage 5 (n=8)
Ca. Nitrospira bockiana
Lineage 6 (n=12)
Ca. Nitrospira calida
Hot spring clones (n=2)
Lineage 1 (n=8)
Ca. Nitrospira defluvii
Lineage 2 (n=40)
ileum associated clone (HQ768288)
ileum associated clone (HQ768638)
A2 Nitrospira metagenome bin
Ca Nitrospira nitrificans
Ca Nitrospira nitrosa
Nitrifying SBR clone (AB809946)
drinking water clone (JQ923566)
drinking water pipe clone (JN628306)
paddy soil clone (AJ617920)
limestone cave stream clone (GQ500769)
dam reservoir clone (AB930755)
Great Artesian Basin clone (AF407702)
Ca Nitrospira inopinata
Nitrospira moscoviensis (X82558)
paddy soil clone (AJ617918)
hot spring clone (FJ793169)
desert agricultural soil clone (AY493911)
alkaline saline soil clone (JN178424)
lava tube wall clone (HM445506)
Nitrospira lenta strain BS10 (KF724505)
Lake Taihu clone (JN868922)
zebrafish associated clone (DQ814725)
Nitrospira japonica (AB818959)
rhizosphere soil clone (JF428926)
mangrove soil clone (DQ811892)
agricultural soil clone (EU881325)
desert agricultural soil clone (AY493909)
rhizosphere soil clone (JF428970)
drinking water clone (DQ058674)
groundwater clone (AF351228)
Iron oxidizing biofilm clone (EU937907)
freshwater reservoir clone (AF418966)
dam reservoir clone (AB930539)
limestone cave stream clone (GQ500753)
drinking water clone (DQ058676)
Echo river sediment clone (AY221079)
Fe Mn nodule associated clone (EF492941)
lava tube wall clone (HM445129)
lava tube wall clone (JN643077)
acidiphillic cave biofilm clone (DQ499307)
0.05
>90
>80
>60
C
Nitrospira moscoviensis (NITMOv2_4534)
Nitrospira moscoviensis (NITMOv2_0255)
Nitrospira moscoviensis (NITMOv2_4028)
Nitrospira moscoviensis (NITMOv2_4033)
Nitrospira moscoviensis (NITMOv2_4538)
Ca Nitrospira inopinata (NITINOP_0879)
A2_Nitrospira_metagenome_gene_2619758441
Ca Nitrospira nitrosa (COMA1_11047)
Ca Nitrospira nitrificans (COMA2_50263)
Ca Nitrospira nitrificans (COMA2_220001)
Ca Nitrospira nitrificans (COMA2_220022)
A2_Nitrospira_metagenome_gene_2619758370
Ca Nitrospira nitrosa (COMA1_10021)
Nitrospira Lineage II
Ca Nitrospira defluvii (NIDE_3237)
Ca Nitrospira defluvii (NIDE_3255)
Nitrospira Lineage I
Ca Scalindua brodae GN
Ca Brocadia sinica JPN1
Ca Kuenenia stuttgartiensis
Planctomycetes
0.1
D
Scaffold 158
CcmA-H
Heme export & cytochrome biogenesis proteins
Hydroxylamine dehydrogenase
hao haoB
Cytochrome c-type (NapC)
69162
80600
Cytochrome c554
Cytochrome C Oxidase, cbb3 type, subunit III
Likely OrfM
Ammonia monooxygenase
partial amoC amoA amoB
92746
Putative NO-forming Nitrite reductase
PA domain containing protein
MOSC domain containing protein
Scaffold 123
partial amoC
Likely amoD2 amoD1 amoE1
1
CopD CopC
Bacterioferritin
7224
Copper binding/ resistance proteins

levels of identity to the corresponding genes in "*Ca*. Nitrospira inopinata" were 66% (E value = 1e−92) and 66.5% (E value = 4e−97) for *amoD* and 65% (E value = 2e−86) for *amoE*. These three genes were preceded by two genes annotated as encoding bacterioferritin and succeeded by the copper binding/resistance genes *copC* and *copD*, an arrangement consistent with that of the recently described comammox *Nitrospira* organism (5, 6). The *copD* gene was followed by a partial *amoC* gene at the 5′ end of scaffold 123, which is potentially contiguous to the partial *amoC* gene found at the 3′ end of scaffold 158. Despite these highly conserved features between our *Nitrospira* metagenome bin and the three other genome bins described recently, it is important to note that our observation is purely metagenomic in nature. Further experimental analyses need to be performed to (i) estimate the abundance of comammox *Nitrospira* organisms in the sampled drinking water filters and (ii) confirm the expression of these genes and link them to ammonia/nitrite oxidation.

To assess whether the presence of ammonia oxidation genes in the *Nitrospira*-like metagenome bin was an artifact, we considered the potential for (i) misannotation, (ii) misassembly, and (iii) incorrect genome binning. First, IMG ER annotation indicated strong support for *amoA*, *amoB*, and *hao* against sequences in the KEGG, InterPro, TIGR, and Pfam databases. *amoC* was confirmed only against the Pfam database (E value = 1.5e−25), potentially because it was a partial gene at the end of the scaffold. Second, we considered the likelihood of misassembly of scaffold 158 using coverage-per-base information (Fig. S2A and S2B) and also checked the phylogenetic signal along the length of the scaffold (Text S1). Our analyses confirmed that the scaffold not only was properly assembled (there is strong support for this conclusion from properly mapped reads) but also had regions of similarity to *Nitrospira*-like bacteria across nearly the entire scaffold length, including ribosomal protein L31P found on this scaffold, whose best hit was *Nitrospira* sp. ENR4 (NCBI accession number CUQ65102.1, total score = 132, query coverage = 100%, E value = 3e−38, percent identity = 88%), an enrichment of "*Ca*. Nitrospira inopinata." Finally, we checked whether the scaffold was correctly binned into the *Nitrospira*-like metagenome bin. To do this, we compared the coverage per sample of scaffold 158 (Fig. S2C) and the k-mer frequency distribution (Fig. S2D) to those of other scaffolds in this metagenome bin. Both analyses suggested that this scaffold was not an outlier with respect to the other scaffolds in the bin. We also performed an Emergent Self-Organizing Map (ESOM) analysis (19) to test whether an independent binning approach would place the scaffold within this metagenome bin (Fig. 2A). ESOM analysis identified three outlier scaffolds unrelated to ammonia oxidation (Fig. 2B; Text S1), while the scaffold with ammonia oxidation genes binned with the *Nitrospira*-like metagenome bin (Fig. 2C). Based on these three lines of evidence (annotation, assembly, and binning), we conclude that the scaffold with ammonia oxidation genes belongs to the *Nitrospira*-like metagenome bin and thus suggests the presence of a comammox bacterium in the drinking water system of Ann Arbor, MI. The *amoA* gene of the newly reported *Nitrospira*-like metagenome bin clustered closely with the novel *amoA* gene of comammox *Nitrospira* bacteria. Phylogenetic analyses (15,

FIG 1 (A, left) Radial cladogram based on RAxML-based maximum-likelihood phylogeny (500 bootstraps, gamma distribution model, and LG+F substitution model) constructed using 16 syntenic ribosomal proteins, with prominent phylum-level affiliation of branches indicated. The reference sequence from the phylum *Aquificae* was used as the outgroup for this analysis. (Right) Expanded view showing the placement of the *Nitrospira* metagenome bin within the phylum *Nitrospirae*, with >90% bootstrap support indicated. The comammox *Nitrospira* species are in green, while strict NOB are in red. A detailed annotated tree is provided in the supplemental material, while the concatenated alignment used to perform phylogenetic analyses is available on figshare (http://dx.doi.org/10.6084/m9.figshare.1619897). (B, left) Radial cladogram based on RAxML-based maximum-likelihood phylogeny (1,000 bootstraps, gamma distribution model, GTR substitution model) constructed using 16S rRNA genes from 87 reference sequences within the genus *Nitrospira* and the partial 16S rRNA gene within the *Nitrospira* metagenome bin. The different lineages are in different colors. (Right) Expanded view of *Nitrospira* lineage 2, showing the placement of the 16S rRNA genome from the *Nitrospira* metagenome bin alongside recently published comammox *Nitrospira* organisms. Comammox *Nitrospira* bacteria are in green, while strict NOB are in red. (C) Bayesian inference phylogeny (20,000 generations, standard deviation = 0.02) *nxrA* genes from *Nitrospirae* and *Planctomycetes*, with the root placed on outgroup *Nitrococcus mobilis* (class *Gammaproteobacteria*). Nodes with >99% bootstrap support are indicated with black circles. The *nxrA* genes from the *Nitrospira* metagenome bin cluster within lineage 2. (D) Arrangement of genes in the region from kbp 69.1 to 92.7 of scaffold 158 with ammonia oxidation genes and those on scaffold 123 with an arrangement similar to that of comammox *Nitrospira* bacteria. Hypothetical proteins are colored in gray, while genes annotated as coding for hypothetical proteins but showing homology to *orfM*, *amoD*, and *amoE* are also marked. The solid line indicates continuity between two fragments of scaffold 158, while the dotted line indicates likely connectivity between scaffold 123 and scaffold 158.

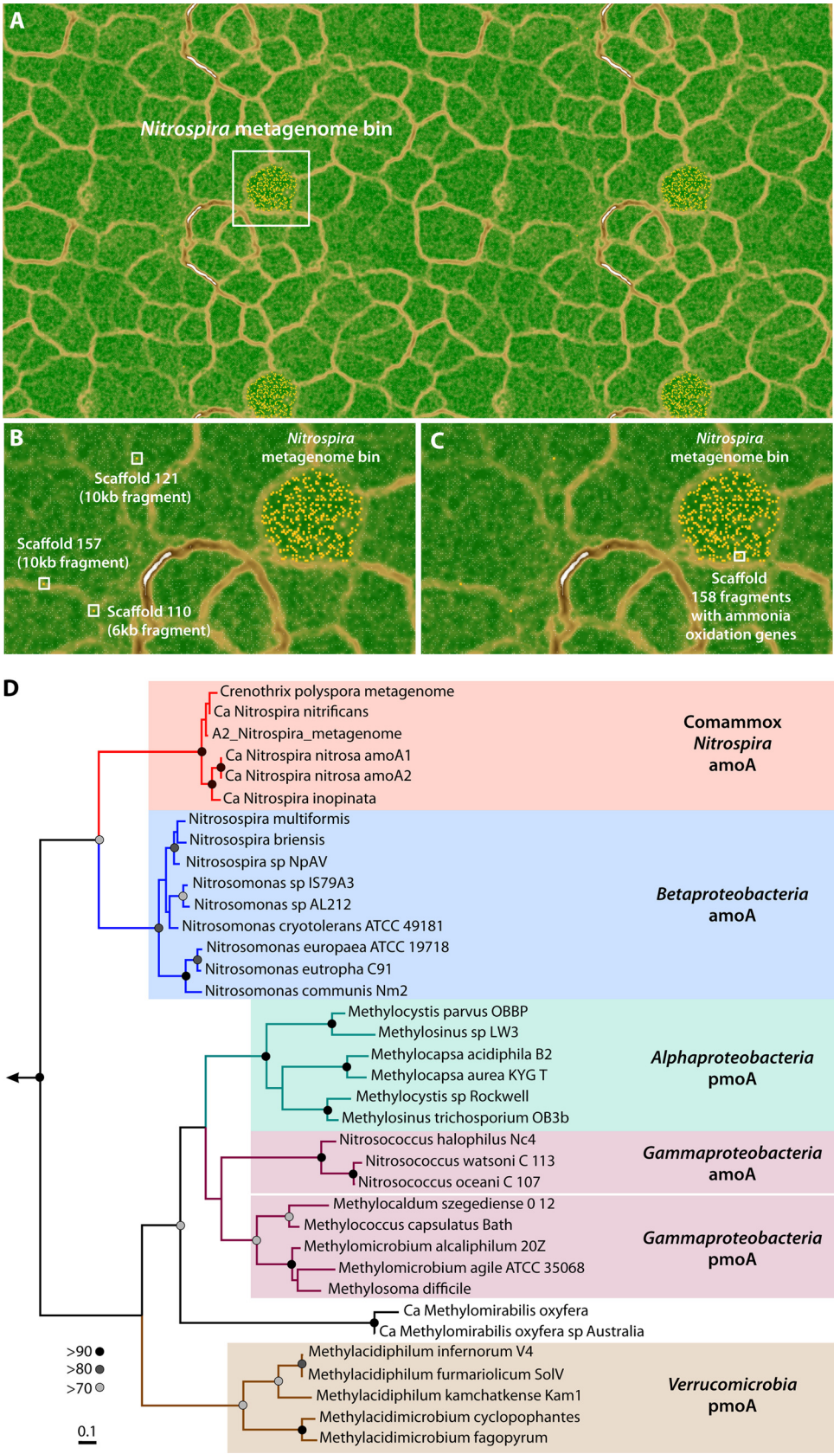


FIG 2 (A) Tiled view of an ESOM map constructed using all 51 metagenome bins assembled from the samples collected in this study, with the white square encompassing the *Nitrospira*-like metagenome bin. Some metagenome bins expand over the edge of a single ESOM grid. Hence, a tiled view consisting of four copies

(Continued)

16) indicated that it branches from betaproteobacterial *amoA* but clusters closely with *pmoA* found in the metagenome of *Crenothrix polyspora* (Fig. 2C), a gammaproteobacterial methane oxidizer detected in a drinking water treatment plant in Germany (20), and "*Ca.* Nitrospira nitrificans" (6). It is interesting to note that one of the eight copies of 16S rRNA found in the *Crenothrix polyspora* metagenome on IMG belongs to a *Nitrospira*-like organism, suggesting that the gene annotated as *pmoA* might in fact be comammox *amoA*.

As part of this study, we also assembled a draft metagenome bin of *Nitrosomonas*-like AOB (GOLD identification number Ga0074132). However, due to the highly fragmented nature of this metagenome bin (598 scaffolds, N_{50} = 7.4 kbp), we were unable to recover all genes associated with nitrification. Nonetheless, its close affiliation with bacteria within the genus *Nitrosomonas* suggests that other canonical AOB were also present in these filters. To check for the presence of bacterial and archaeal *amoA* genes in the drinking water filter samples, we annotated the master assembly against a custom database of bacterial and archaeal *amoA* genes (Text S1). Only four significant *amoA* hits were detected in the master assembly, with two of these mapping to *Betaproteobacteria*-like *amoA*. The *amoA* gene within the *Nitrospira* metagenome bin was also detected in the master assembly, and we found an additional comammox-like *amoA* gene that was not present in any of the genomes assembled from this data set. Differences in coverage of the scaffolds on which these *amoA* genes were present across samples suggest that the four *amoA* genes (two comammox, two *Betaproteobacteria*) belong to four different organisms. The phylogenetic affiliations of all of these *amoA* genes and coverages per sample can be seen in Fig. S4.

The presence of the complete ammonia oxidation capacity in *Nitrospira* organisms has significant implications for the nitrogen cycle, particularly if this organism is widespread. Using *amoA* sequence matches in the NCBI database and their associated environmental ontologies, we find support for previous detection of this *Nitrospira*-like organism in engineered systems (n = 8), soil ecosystems (n = 19), and groundwater (n = 10). This suggests that comammox *Nitrospira* organisms may be of importance for both engineered and natural systems. Interestingly, most of the matches in the NCBI database were attributed to methane-oxidizing bacteria, as also highlighted by Daims et al. (5). The recently published discovery of a comammox *Nitrospira* organism (5, 6) in combination with our finding strongly suggests that microbial contributions to the nitrogen cycle in engineered and natural environments will need to be reevaluated. The presence of a comammox is also congruent with previous observations of abundances of *Nitrospira*-like bacteria that were significantly higher than those of AOB/AOA based on 16S rRNA gene assays (21–23), indicating that comammox activity likely contributed substantially to nitrate formation in these environments. While direct evidence of the conversion of ammonia to nitrate by a *Nitrospira* organism was provided (5, 6), it will be critical to build on this initial work to understand the extent to which comammox organisms contribute to ammonia and nitrite oxidation in the wide range of environments where nitrogen cycling is important. This might be particularly critical for

Figure Legend Continued

of the ESOM grid is shown to allow for visualization of metagenome bins at the edge as contiguous clusters. This results in all metagenome bins included in the ESOM analyses appearing four times in the tiled view. (B) Enlarged view of panel A indicating three scaffold fragments that were outliers based on ESOM analyses. (C) Enlarged view of panel A showing fragments of scaffold 158 containing ammonia oxidation genes that were binned with the *Nitrospira* metagenome. The ESOM binning procedure and contents of the three outlier scaffolds/scaffold fragments are presented in Text S1 in the supplemental material. (D) RAxML-based maximum-likelihood tree constructed using amino acid sequences of the *amoA* gene in the *Nitrospira* metagenome bin and *pmoA*/*amoA* sequences from a range of ammonia-oxidizing bacteria/archaea and methane-oxidizing bacteria, including the *Nitrospira* comammox. The tree was built from a trimmed muscle alignment using the Dayhoff model for protein evolution, gamma distribution model, and 500 bootstraps using the archaeal *amoA* gene of *Nitrosopumilus maritimus* as the outgroup. Branches are colored according to phylogenetic affiliation, and node support of >70% is indicated. This placement of the *amoA* gene from the *Nitrospira*-like genome and overall tree topology were also confirmed by neighbor-joining analysis (500 bootstraps) and the unweighted pair group method with arithmetic mean (UPGMA) (500 bootstraps) in Geneious and Bayesian phylogeny inference (20,000 generations) (Fig. S3).

wastewater treatment systems that rely on partial nitrification followed by anammox processes (24) or short-cut nitrification-denitrification (25) for reducing energy costs of nitrogen removal. Similarly, nitrification in biofilms, a predicted ecological niche for comammox bacteria (4), is a considerable concern in drinking water distribution systems (26), and strategies devised to inhibit AOB/AOA may or may not yield optimal results if comammox activity primarily drives ammonia oxidation. On the other hand, the benefits of comammox bacteria can be exploited for ammonia removal from drinking water sources through promoting their activity in biofiltration systems, such as the system from which the currently described *Nitrospira*-like metagenome bin was obtained.

Raw reads and all draft genomes are available through NCBI BioProject PRJNA301005. The draft genomes and annotation information can be accessed through IMG ER using JGI GOLD identification numbers Ga0074129-141 and Ga0077522-560. JGI GOLD, IMG-ID, and NCBI accession numbers for the *Nitrospira* metagenome bin discussed in this paper are Ga0074138, 2619618852, and LNDU00000000, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://dx.doi.org/10.1128/mSphere.00054-15.

Text S1, DOCX file, 0.03 MB.
Figure S1, PDF file, 0.01 MB.
Figure S2, JPG file, 2.5 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.

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A.J.P. performed the sampling. A.J.P., D.N.M., U.Z.I., and Q.M.B.D.L.S. analyzed the data. A.J.P., G.J.D., and L.R. wrote the paper.

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