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Genomic Adaptations to Extreme Salinity and pH in Ammonia-Oxidizing Bacteria

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by

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ABSTRACT

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Ammonia-oxidizing organisms are key contributors to the nitrogen cycle, yet little is known about their genomic adaptations to saline or alkaline conditions. This study combines physiological characterization and comparative genomics of ammonia-oxidizing bacteria (AOB) with a metagenomics to better understand potential adaptations to salt and high pH environments. Halotolerant AOB possess numerous adaptations that help regulate the osmotic stress caused by varying salt and pH conditions including osmolyte biosynthesis pathways and an inventory of transporters that helps regulate the electrochemical gradient imposed. Genomic analysis of *Nitrosomonas sp.* ANs5, an extremely alkalitolerant species within the *Nitrosomonas* lineage, identified multiple genomic features predicted to facilitate osmotic and pH homeostasis, including biosynthesis of compatible solutes and the presence of a V-type ATPase, unique within the *Nitrosomonas*. Ammonia-oxidizing enrichment cultures were established from an isolated hypersaline brine pool at the Carpinteria Salt Marsh, in which attempts to isolate the novel MAG, *Nitrosomonas sp.* CSM, were unsuccessful although the enrichment demonstrated faster growth at 800mM NaCl than 600 mM NaCl. Comparative genomic analysis revealed the combination of transporter and osmolyte biosynthesis adaptations that contribute to salt and pH tolerance across reference and newly sequenced AOB. Metagenomic read mapping indicated that halotolerant AOB co-inhabit environments where freshwater or halophilic AOB are distributed. These results

demonstrate some AOB are adapted to saline and alkaline environments and provides insight into the adaptations that permit their activity in extreme environments.

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Introduction

Nitrification is a chemoautotrophic process in which ammonia is oxidized to nitrate; up to four groups of microorganisms can be responsible. Ammonia-oxidizers, one group of nitrifiers, mediate the oxidative half of the nitrogen cycle and may be a source of nitrous oxide to the atmosphere, which serves an important role in the warming of our global climate[1]. Ammonia-oxidizing microorganisms produce nitrite or nitrate, which can be used by other organisms as a source of nutrients or terminal electron acceptor for anaerobic respiration. Phylogenetically, these microorganisms belong to either the ammonia-oxidizing archaea (AOA) in the phylum *Thermoproteota*[2] or the ammonia-oxidizing bacteria (AOB), which are split between *Gamma-* and *Betaproteobacteria*[3]. AOB and AOA have been detected globally in freshwater, brackish water , seawater, sediment, saline lake, soda lake, and in artificial environments[4–6].

Adaptations in AOA and AOB to extreme salinity and pH are not well understood and are important to study because ammonia-oxidizers have an important ecological role in diverse ecosystems by supporting key steps in the nitrogen cycle. The environmental factors that control nitrification in the environment include substrate availability, oxygen availability, salinity and pH[4,7,8]. Most of what is known about ammonia-oxidizers in saline systems has come from marine and wastewater systems[9]. Marine AOA, including *Nitrosopumilus* and *Candidatus* Nitrosopelagicus have been shown to require salt based on their marine origin and laboratory studies[10–12]. No salt tolerant terrestrial strains of AOA have been isolated despite being detected in saline conditions[13]. *Nitrosomonas* AOB species that have been enriched from desert soil have demonstrated tolerance to high saline conditions[14]. The most salt tolerant AOB have been identified within the genera

Nitrosococcus, which are *Gammaproteobacteria* typically found in marine systems[7]. AOA and AOB differ in their salt tolerance with conflicting reports of AOA being more dominant than AOB in activated sludge under salt stress[15], and reports observing the opposite in saline nitrification reactors[16]. The underlying mechanism of how AOA and AOB respond to salt stress has yet to be understood[13].

Environmental pH also determines the distribution and activity of the ammonia oxidizer clades by impacting the environmental availability of ammonia (pK_a= 9.25) through acid-base chemistry, which imposes nutritional and toxicity stress on these organisms[7,17]. Strains of the AOA lineage *Nitrosotalea* and AOB linages *Ca.* Nitrosoglobus and *Ca.* Nitrosoacidococcus have been isolated from acidic soils, noting that AOA harbor horizontally transferred genetic adaptations such as the proton-pumping V-type ATPase that confers acid tolerance by consistently extruding cytosolic protons[17,18]. However, few studies have conducted environmental surveys to assess the activity of AOA and AOB within alkaline, high pH systems[19,20]. Environmental surveys for ammonia-oxidizers in saline environments are typically associated with *Nitrosomonas halophila*, *N. europaea*, and *N. eutropha*[13,19–21].

Physiological Challenges of Extreme pH and Salinity

Alkaliphiles are organisms that thrive within high pH (> 8) environments. Microorganisms can be categorized into three categories relative to their growth characteristics ordered by increasing pH values: alkalitolerant, facultative alkaliphile, and obligate alkaliphiles (Table 1). At high pH, alkaliphiles face the challenge of maintaining an alkaline cytoplasm when the external environment is of an even higher pH, leading to a

transmembrane pH differential that reduces the energy yield of the proton motive force[22]. Haloalkaliphiles are organisms that thrive under high pH and salt environments. Microorganisms can be categorized into two categories relative to their growth characteristics ordered by increasing salt and flexible pH values: moderately haloalkaliphilic and extremely haloalkaliphilic (Table 1). Natronophiles are organisms that are similar to haloalkaliphiles; the main difference is their preferential growth in the presence of the Na^+ ion, which means they have a growth requirement of sodium ions through Na_2CO_3/$NaHCO_3$ (Table 1).

Extreme pH values pose a threat to the osmotic and energetic balance between the external and internal environment relative to the cell[22]. The pH also has an impact on the activity of the enzymes within the cell based on the folding and amino acid composition. Acidophiles maintain a cytoplasmic pH closer to neutral pH relative to the external environment and they cope with low pH conditions by actively pumping and exporting protons out of the cytoplasm[22,23]. Alkaliphiles maintain an alkaline cytoplasmic pH within two pH units of the external environment[23]. They cope with the scarcity of protons by downregulating proton export[22], exporting Na^+ or K^+ ions with antiporters that are importing H^+ ions[22,24], and through increased expression of the proton capturing F-type ATP synthase that may contain multiple c-subunits that modulate the H^+/ATP ratio[25,26].

Microorganisms have adopted two strategies to cope with high salinity: “salt-in” and “salt-out”[27]. The “salt-in” strategy, where microorganisms import K^+ ions to maintain an osmotic balance, is less energy intensive compared to the “salt-out” strategy. Halophilic Archaea of the family *Halobacteriaceae* and bacteria of the *Salinibacter* are among the few microorganisms that use the “salt-in” strategy[28]. Most microorganisms are capable of the

"salt-out" strategy that involves the extrusion of Na^+ ions and biosynthesis of organic solutes, which require a greater energetic investment from the organism[28]. Microorganisms can be categorized into four categories relative to their growth characteristics ordered by increasing salinity: halotolerant, facultative halophile, moderate halophile, and obligate extreme halophiles[29] (Table 1). Each of these categories grow optimally around neutral pH.

Genomic Adaptations to High pH and Salt

Genomic adaptations to pH homeostasis include sodium symporters, proton antiporters, and ATPases. Sodium-solute symporters couple the movement of sodium and compatible solutes into the cell serving as an osmolyte accumulation strategy while a concentration of sodium ions builds for active transporters to exclude sodium out of the cell. Na^+/H^+ antiporters, including those encoded by *mrp* genes, are responsible for the antiport of sodium and protons across the membrane that serve to regulate pH by importing protons into the cell while alleviating salt stress by exporting the sodium ions out of the cytoplasm[30–33]. Potassium-proton antiporters exchange potassium ions out of the cell for the import of protons, much like the antiporters mentioned prior, to help regulate pH within the cell and require sufficient potassium internally to fuel the import of protons[34,35]. The sodium-pumping V-type ATPase is capable of pumping sodium ions out of the cell at the cost of ATP[22].

Genomic adaptations to high salt conditions include adaptations in the transporter inventory, respiratory chain, and osmolyte biosynthesis. Na^+ transporters are more abundant and diverse in halophiles[36] all of which are intended to balance the osmotic stress experienced at high salt concentrations[37]. Na^+ ion transport can be coupled with H^+ ions to

regulate the pH in the cell[24]. Na^+/H^+ antiporters are important to the exchange of sodium and protons across the membrane[30]. For example, *mrp* and *nha* are Na^+/H^+ antiporters that move sodium ions out of the cell and protons into the cell[38]. Sodium-solute transporters can couple the transport of sugars, amino acids, or vitamins into the cell with the movement of sodium ions along the transmembrane salinity gradient to save energy moving these different compounds[39]. On a similar note, motile species utilize sodium powered flagella by permitting the entry of Na^+-ions along the salinity gradient, and thus leverage the electrochemical potential by converting it into mechanical function for the cell[40]. Magnesium (Mg^{2+}) ion import can assist with the extrusion of sodium ions when dealing with high salt conditions[22,41,42]. The calcium concentration within the cell can help with the signaling and activation of sodium extrusion mechanisms within the cell to help regulate salinity at high salt conditions. Import of potassium ions can help regulate the cytosolic Na^+/K^+ ratio that helps maintain homeostatic levels of sodium within the cell[34,43]. Channels facilitate the movement of ions in and out of the cell, for example, Cl^- channels facilitate the movement of chloride ions out of the cell, which at high concentrations can be toxic to the cell[44]. Ion transporters can offset the osmotic stress caused by high salt by maintaining the appropriate charge to maintain the proton motive force and homeostasis[27,45].

Components of the respiratory chain are also impacted by high pH and salinity. The proton motive force (PMF) is composed of two electrochemical gradients: the ΔpH represents the transmembrane pH gradient and the $\Delta\Psi$ represents the transmembrane electrical potential[22]. At high pH, the $\Delta\Psi$ is large due to the reversal of the ΔpH which contributes favorably to the ability to attract protons[22]. The proton motive force is generated by primary proton pumps in bacteria, which include respiratory chain pumps, light-driven

pumps, and proton-pumping ATPases[22]. Energy generated by the PMF is used to fuel active transport. For example, *nuo* subunits are proton-translocating NADH:ubiquinone oxidoreductases of complex I that pump protons out of the cytoplasm ahead of the F-type ATPase[46]. The F-type ATPase synthase is responsible for coupling the movement of protons into the cell with the generation of ATP[46], and with additional c-subunits, alkalitolerant organisms can generate a greater proportion of ATP per proton pumped across the membrane[25,26]. The Na^+-dependent ATPases function as pumps that couple the consumption of ATP with the movement of sodium out of the cell[47]. Sodium-transporting NADH:ubiquinone oxidoreductases, encoded by *nqr* genes, are essential to setting up the sodium motive force by pumping Na^+ ions out of the cytoplasm[48].

Finally, compatible solutes such as glycine betaine, glutamate, and proline can either be synthesized or transported to be utilized as an osmoprotectant to prevent reduce osmotic stress caused by high salt conditions[27] and then used as an anabolic carbon source. Glycine betaine transporters encoded by *opu* genes are multi-subunit genes that are responsible for the import of the osmolyte[49]. The synthesis of ectoine is encoded by the multi-subunit *ect* genes[50], while glutamate can be synthesized through reactions catalyzed by glutamate dehydrogenase (*gdh2*), glutamine synthetase (*glnA*), and glutamate synthase (*gltDB*)[13]. Synthesis of proline from other organic osmolytes is encoded by the multi-subunit *pro* genes[51]. Organic compounds such as ectoine, glutamate, and proline can be synthesized to serve the same role as glycine betaine, thus the presence of their synthesis or transport pathways can be critical to responding to environmental salinity[52,53].

Ammonia oxidizers at Extreme Salinity and Alkaline pH

Extreme conditions of salt and pH pose specific challenges for chemoautotrophic ammonia-oxidizing organisms, over and above the challenges experienced by other microorganisms. This may explain why they are typically restricted to salinities at or below seawater salinity of ~3% or ~34 g/L[28,54]. First, the energetic burden of maintaining homeostasis against high salt and pH conditions is particularly challenging because of the low free energy yield of ammonia oxidation (ΔG^0 ~ -307 kJ mol^{-1}), and ammonia oxidizers must use some of this energy to power carbon fixation. Second, alkaline conditions significantly shift the equilibrium between acid-base conjugates of ammonia-oxidizer substrates as the pK_a of NH_4^+/NH_3 is 9.25 at 25˚C[17]. High pH is thus beneficial from the standpoint of free energy yield, as NH_3 will be more abundant and is thought to be the substrate for the AMO enzyme. However, NH_3 is also toxic[55], thus NH_3 may reach toxic levels at high pH[56]. Finally, all known AOB use the Calvin-Benson-Bassham cycle for carbon fixation, which requires relatively high concentrations of pCO_2 as a substrate for RUBISCO[57]. At high pH, most inorganic carbon exists at as bicarbonate and carbonate ions[17]. Despite these challenges, there are some AOB that can tolerate intermediate salinities (between 10-20% or 1.2-2.8M NaCl)[28]. *Nitrosococcus halophilus* Nc4 is an AOB of the *Gammaproteobacteria* and the most halotolerant ammonia-oxidizer described with an upper salt threshold of 1.8M NaCl[58] (Table 2).

Both AOB[56] and nitrite-oxidizing bacteria (NOB) have been cultivated from alkaline systems[59]. Yet, much of what is known about pH homeostasis in nitrifiers has come from metabolic reconstructions of metagenome assembled genomes, which contain proton- or sodium-pumping ATPases and secondary active transporters[13,60,61]. Ammonia-oxidizers use

ammonia over ammonium as their preferred substrate and may have the ability to utilize urea, at acidic pH values, as an alternative source of nitrogen[62]. At high pH, the availability of CO_2 decreases and few AOB such as *N. eutropha* and *N. halophila* possess carboxysomes to concentrate CO_2 in order to have a carbon source to build biomass[56,63].

The first cultivated alkaline-adapted nitrifiers were of the genera *Nitrobacter*[59] and *Nitrosomonas*[56]. These include the ANs1-5 isolates from Mongolian soda lakes that vary in chemical composition and salinity[56]. The most alkalitolerant of these strains, ANs5, can grow up to a pH of 11.4, which is the highest of all chemolithoautotrophs[56]. The ANs isolates have previously been considered alkaliphilic eco-types of the type-strain *Nitrosomonas halophila* Nm1 that was originally isolated from seawater, and with whom they share greater than 70% DNA similarity[56,64]. Understanding the genes unique to the haloalkalitolerant AOB, *Nitrosomonas sp.* ANs5, may provide information regarding genes that confer tolerance to high pH that are not present in the halotolerant species or other reference AOB[17,64].

Objectives

This research aims to understand the genomic inventories of ammonia-oxidizing bacteria, with a focus on potential adaptations to extreme pH and salinity. This research also aims to genomically characterize a newly enriched AOB species and understand patterns in the environmental distribution across genera of AOB. Based on previous efforts that demonstrate *Nitrosomonas sp*. ANs5's ability to grow at high pH, we hypothesized ANs5 will have pH adaptations that are not shared with *Nitrosomonas halophila* Nm1 or other reference AOB. Given that *Nitrosomonas halophila* Nm1 and *Nitrosomonas sp.* ANs5

possess the same salinity optimum and tolerance range, ANs5 and Nm1 will share salt adaptation genes. Ammonia-oxidizing enrichments were established from an isolated coastal hypersaline brine pool and genome sequenced. Lastly, based on growth characteristics of AOB summarized in Table 1, we expect the distribution of halotolerant AOB species to overlap with freshwater and halophilic AOB, yet freshwater and halophilic AOB will not overlap in their environmental distributions. The objectives of this study are: 1) to physiologically characterize the response to gradients of salt and pH in an extremely alkalitolerant AOB species; 2) to enrich and genomically characterize halotolerant AOB from different environments to understand adaptations that confer salt and pH tolerance; 3) to assess the environmental distribution patterns of AOB relative to one another. Open questions we hope to answer are: What are the adaptive features that confer tolerance to high pH and high salt in ammonia-oxidizing bacteria? And what are distribution patterns of halotolerant and halophilic ammonia-oxidizing bacteria in the environment?

Materials and Methods

Enrichment of *Nitrosomonas sp.* CSM

Sampling was conducted in September 2023 at the isolated brine pool within the Carpinteria Salt Marsh Reserve. The pH of the brine pool is 7.5; the salinity has been reported as 11%[65]. Sediment samples with overlying water were collected with a push core, then brought to the lab for enrichment inoculation. 10 g of wet sediment were added to 100 mL of Watson Artificial Sea Water media based on a previous study[66], amended with 5 mM NH_4Cl with modifications in the NaCl and pH adjusted to 8.7 with NaOH. The remaining samples were kept at 4˚C. Enrichments were incubated at 18˚C under dark, aerobic conditions without shaking. Initial enrichments conducted at 700 mM and 1800 mM NaCl were monitored for three months before their termination due to the lack of detectable nitrite production activity. Fresh media was re-inoculated from the same samples at 600 mM and 800 mM NaCl in December 2023.

Nitrite production was monitored monthly with a colorimetric assay[67] to verify ammonia-oxidation activity, then enrichments were fed with additional 5 mM NH_4Cl. Cultures were monitored for changes in pH monthly and re-adjusted after feeding as they approached neutral pH values to prevent the enrichments from acidifying themselves in the media. As the isolation process progressed, the frequency of checks increased to every two weeks depending on nitrite production. Ten percent transfers were conducted monthly for up to four months before the initial dilution series. Contamination checks were conducted in screw top tubes up to the 10^{-9} dilution in nutrient rich media that contained 30 g/L of Tryptic Soy Broth, 0.5 g/L of $NaNO_3$, 1 g/L of NH_4Cl, and 5 g/L of KH_2PO_4. Samples of the growing enrichment (1 mL) were used to inoculate 10 mL of the soy broth media and were

incubated at 18˚C in dark conditions. Growth within the contamination checks were determined as positive for contaminants growing within the enrichment. After the third dilution series (16 months after the initial enrichment), suspected isolates were grown up in half liter batches prior to filtering for DNA extraction. Cryostocks of the enrichment culture were made by sampling 1 mL of growing culture with 1 mL of 15% glycerol before storing at -80˚C in cryovials.

Physiological Characterization and Genomic Sequencing of *Nitrosomonas sp.* ANs5

Growth of *Nitrosomonas sp.* ANs5 followed methods previously described in Sorokin et. al. 2001, with 10 mM NH_4Cl. Cultures were monitored for production of nitrite as a proxy for growth[67]. Cultures were maintained in 120 mL screw top bottles with 30 mL of media. In order to assess the growth at different salt and pH conditions, the media was chemically adjusted from the minimal medium with 10% HCl, 5-6M NaOH, and NaCl in each individual condition. Exponentially growing cultures were enclosed in a box while incubated at 30˚C, rotating at 100 rpm, and then transferred at 10-20% in order to acclimate the organism toward the ends of the salt and pH gradients from the demonstrated optimal conditions (Figure 1).

Eight individual cultures were combined for filtration for DNA extraction. DNA extraction used the DNEasy Blood & Tissue kit (Qiagen) with modifications as described in a previous study[68]. Illumina sequencing libraries were prepared using the tagmentation-based and PCR-based Illumina DNA Prep kit and custom IDT 10bp unique dual indices (UDI) with a target insert size of 280 bp. No additional DNA fragmentation or size selection steps were performed. DNA was sequenced at SeqCenter using Illumina NovaSeq X Plus

sequencing with 2 x 151bp paired end reads producing a total of 1,541,784 paired reads. Draft genomes of ANs5 were analyzed using the Kbase open-source platform for assembly and analysis[69]. Quality control and pre-processing were conducted with FASTQC[70] (v 0.12.1), TRIMMomatic[71] (v 0.36; sliding window = 5, min. quality = 20), and PRINSeq[72] (v 0.20.4), in that order. Quality filtered and trimmed reads were assembled using Unicycler[73] (v 0.4.8; min. contig length = 2000 bp, contig bridging threshold = 'bold'). The assembled genome (65x coverage) was annotated via the NCBI Prokaryotic Genome Annotation Pipeline[74] (PGAP). The assembly was then assigned taxonomic classification with GTDB-Tk[75] (v 2.3.2).

Metagenomic Sequencing and Assembly of *Nitrosomonas sp.* CSM

DNA was extracted as described above for ANs5 from 500 mL of enrichment culture after the third dilution series. Illumina sequencing libraries were prepared using the tagmentation-based and PCR-based Illumina DNA Prep kit and custom IDT 10bp unique dual indices (UDI) with a target insert size of 280 bp. No additional DNA fragmentation or size selection steps were performed. Libraries were sequenced at SeqCenter on an Illumina NovaSeq X Plus sequencer in a multiplexed shared-flow-cell run with 2 x 151bp paired-end reads producing a total of 3,581,000 paired reads for the lab enrichment associated with *Nitrosomonas. sp.* CSM. Quality check and assurance were conducted with FastQC[70] and Trimmomatic[71] before assembly using metaSPAdes[76] (v 4.2.0) and MEGAHIT[77] (v 1.2.9). Assemblies were binned with metaWRAP[78] (v 1.3.2), dereplicated with dRep[79] (v 3.4.2), and bin taxonomy was assigned with GTDB-Tk[75] (v 2.6.1). Assembly qualities were assessed with CheckM2[80] (v 1.1.0). The bin that was generated from the MEGAHIT

assembly classified as *Nitrosomonas* from the Carpinteria Salt Marsh lab enrichment metagenome was > 90% complete and < 2% contaminated, this was selected for comparative analyses after annotation with Prokka[81] (v 1.14.5) (Table 3).

Comparative Genomics

Nitrosomonas reference genomes were downloaded from the NCBI Genome database (Table 4). The bacterial phylogenomic tree was generated with GToTree[82] (v1.8.16), using the prepackage single-copy gene-set for proteobacteria (119 target genes). In brief, prodigal[83] (v2.6.3) was used to predict genes within input reference genomes provided as NCBI accessions and non-references provided as fasta files. Target genes were identified with HMMER3[84] (v3.4), individually aligned with MUSCLE[85] (v5.1.linux64), trimmed with trimAl[86] (v1.5.rev1), and concatenated prior to phylogenetic estimation with FastTree2[87] (v2.1.11). GTDB[88] taxonomy was added to connect full lineages to taxonomic IDs for input genomes provided as NCBI accessions. The final taxonomic ID was edited for the fasta provided genomes in iTOL[89] (v7.2). Cluster labeling of the AOB was assigned based on previous studies[3,90].

Two separate analyses were conducted with *Nitrosomonas sp.* ANs5 and *Nitrosomonas sp.* CSM to their closest cultured reference genome. Summary assembly statistics (Table 3) were generated with CheckM2[80] (v 1.1.0) and Quast[91] (v 5.0.2). Average nucleotide identify (ANI) between the assembled genomes and the closest reference genome was determined using skani[92] (v0.3.2). Each genome was imported into KBase and annotated with Prokka[81] (v 1.14.5). The predicted proteomes of each pair were aligned (with corresponding locus tags) with the 'Compare Two Proteomes' tool[93,94]. In short, the tool

performs an all-vs-all protein comparison based on BLAST output and computes the bi-directional-best-hits between a pair of species[94]. The application output is a dot plot matrix showing pairs of similar proteins determined from the algorithm similar to the BUS approach[93]. The aligned proteins (with locus tags) were exported, and data tables were manually curated in Excel, R, and amino acid sequences were extracted by locus tags with SeqKit[94]. Genes with bi-directional-best-hit percentages greater than 60% were considered shared genes[94,96]. Filters were applied in RStudio to identify genes absent in *N. halophila* or *N. aestuarii* but present in ANs5 or CSM to be assessed for further investigation. SeqKit was provided the Prokka annotated amino acid fasta files of each ANs5 and CSM with a text file containing unique locus tags corresponding to 321 genes in ANs5 and 738 genes in CSM. These files were used to generate amino acid fasta files of unique features in each organism. That subset of genes were assigned COG categories with reCOGnizer[97] (v 1.11.1)**.**

Phylogenetic Analysis of Select Protein-Coding Genes

Genes from reference organisms were located in GenBank based on the previous studies that focus on the phylogenetics of different V-type ATPase[98] and hydrogenases[99]. Amino acid sequence alignments were conducted with MUSCLE (v3.8.1551)[85] and trimmed with trimAl[86] (v1.5.rev1). After trimming, the aligned sequences were uploaded to the NCBI multiple sequence alignment viewer (v1.26.0) to enumerate the conserved positions across all sequences. For the hydrogenases, the amino acid sequence length of the alignments was 717 positions; 119 conserved positions were used in the analysis. For the V/A-type ATPases, the amino acid sequence length of the alignments was 620 positions; 375 conserved positions were used in the analysis. Phylogenetic trees were generated with IQ-

TREE[100]. The model for the hydrogenase tree was Q.pfam+I+G4 and the model for the V-type ATPase was LG+I+G4. The best fit model was determined with IQ-Tree's ModelFinder feature which improves the accuracy of the final tree based on the internal testing and ranking of different tree models[101].

Assessment of Genomic Inventory and Pangenomic Analysis

The pangenome analysis was conducted with the standard parameters of the anvi'o (v8) pangenome workflow[102]. *Nitrosomonas sp.* ANs5 and CSM were compared to twelve reference *Nitrosomonas* species from the NCBI GenBank Genome Database (Table 4). Genes were annotated by COG and KEGG databases and a pangenome was generated within anvi'o using 'anvi-pan-genome' with the DIAMOND aligner[102]. Gene clusters were annotated by binning sequences based on gene accessions annotated by COG and KEGG as related to salt or pH tolerance– this includes genes related to the electron transport chain, osmolyte biosynthesis pathways, metabolic pathways, or cytochrome oxidase genes[103,104]. The dataset from 'anvi-summarize' function was manually curated using Microsoft Excel prior to visualization within RStudio[105] with the ComplexHeatmap[106] package (v2.21.2).

Environmental Distribution Analysis

Twenty-three AOB genomes representing the genera *Nitrosomonas, Nitrosospira,* and *Nitrosococcus* were downloaded from the NCBI GenBank Genome database[107] and JGI IMG/M databases[108] (Table 5). The sourmash[109] (v4) tool was used to search for metagenomes in the SRA database that contain query genomes of AOB that were provided as nucleic acid fasta files. The resulting metagenomes from the sourmash search were

manually curated and downloaded (Table 6). CoverM[110] (v0.7.0) was used to determine the relative presence of AOB species within the downloaded metagenomes (Table 6). CoverM was run in the cluster utility mode to dereplicate and cluster the genomes with the following parameters: --checkm2-quality-report --ani 95 --precluster-ani 80 --precluster-method skani. CoverM was then run in genome mode to calculate coverage of genomes using the transcripts per million (TPM) calculation method that assumes no other metagenomic reads map to other genomes. Values are reported as reads per million (RPM) after being log transformed and visualized with R using the ComplexHeatmap[106] package (v2.21.2).

Results

Enrichment of halophilic AOB

Slow ammonia oxidation rates were observed in both the 600 mM and 800 mM NaCl enrichments, but the enrichments at 800 mM grew slightly faster (Table 7). Multiple dilution series were conducted to isolate the ammonia oxidizers from heterotrophic bacteria. Efforts to quantify the growth of the *Nitrosomonas sp.* CSM were unsuccessful due to near-zero growth after the 10^{-8} dilution was transferred into fresh media.

Salinity and pH Optima of ANs5

Nitrosomonas sp. ANs5 nitrite production ranged from 30 to 150 μmol h^{-1} and reached final cell densities of up to 10^7 mL^{-1}. ANs5 was able to grow at all salinities tested, from 0.2 M to 0.6 M NaCl, with a salinity optimum of 0.3 M (Figure 1A). ANs5 demonstrates the highest nitrite production at a total sodium concentration of 300 mM, and the rate begins to decrease above this value (Figure 1A). ANs5 was also able to grow at all tested pH from 7.5 to 11, with a pH optimum of 9.6 (Figure 1B). ANs5 demonstrated that pH 9.6 is conducive to the highest nitrite production rate, outside of this value, the nitrite production rate decreased (Figure 1B).

Gene Content and Phylogenomic Context of Newly Sequenced AOB

The draft assembly for *Nitrosomonas sp.* ANs5 contains 122 contigs with an N_{50} value of 49,119 (Table 3). The total genome size is 3.07 Mbp with a GC content of 52% and coding density of 85%. The genome contained 2,713 protein-coding genes and is 99.82% complete with 0.42% contamination. The draft assembly for *Nitrosomonas sp.* CSM

contained 265 contigs with an N_{50} value of 32,315 and L_{50} value of 28 (Table 3). Of the eight bins assembled from the lab enrichment culture metagenome, CSM's DNA represented 42.27% of the metagenome (Table 8), where 20.5% of the DNA reads remained unmapped. The remaining 37.24% of DNA reads were split between seven bins that were represented by four *Marinobacter sp.*, one *Maricaulis sp.*, a *Halomonas sp.*, and an unclassified bacterium (Table 8). The individual abundance, completeness, and contamination values are represented in Table 8. The total genome size for CSM is 3.33 Mbp with 3,025 protein-coding genes, with a GC content of 45% and coding density of 79%. The genome is 92.1% complete with 0.11% contamination. Strain ANs5 and *N. halophila* Nm1 share an average nucleotide identity (ANI) of 91.9% over an aligned fraction of 65.6%. Strain CSM and *N. aestuarii* Nm36 share an ANI of 83% over an aligned fraction of 27.4%.

Phylogenomic analysis of the two new AOB shows they fall within two distinct, well supported clades of AOB (Figure 2). ANs5 falls in the cluster 7 clade[3], which includes *N. halophila* Nm1*, N. europaea,* and *N. eutropha.* CSM forms a distinct lineage within the cluster 6b[3] *Nitrosomonas* that includes *N. marina* and is most closely related to *N. aestuarii* Nm36 (Figure 2).

Nitrosomonas sp. ANs5 and *N. halophila* Nm1 were compared to one another to find genes unique to ANs5 that may be uniquely linked to pH adaptations. Comparing the predicted proteomes, they share 2,328 genes of at least 60% amino acid similarity (Figure 5). ANs5 has 321 genes that are not shared with *Nm1* (Figure 3). Of these, 154 could be assigned to COG categories. Of these, four COG annotations associated with ATPases and antiporter functions were investigated further. Genes unique to ANs5 with locus tags

ABTW62_03510 and ABTW62_13425 are annotated as ATPases. Locus tag ABTW62_13135 from the metabolism category was annotated as *araJ*, an arabinose efflux permease that functions as a glucose-proton antiporter[111,112]. Lastly, locus tags ABTW62_13740 and ABTW62_13745 are annotated as antibiotic-proton antiporters, which suggest the coupling of macromolecules and proton transport.

Nitrosomonas sp. CSM and *N. aestuarii* were compared to find unique genes to CSM that may confer salt tolerance, given that CSM was enriched for at a higher salinity than *N. aestuarii* may tolerate and CSM originates from a site of higher salt content (Table 2). CSM and *N. aestuarii* Nm36 share 2,020 genes. CSM has 738 genes that are not shared with *N. aestuarii* Nm36 (Figure 4); COG categories could be assigned to 467 of these. Of these, four COG annotations associated with osmotic adaptations to the cell wall, sodium-proton antiport function, and metal cation pumping were identified. In CSM the locus tag IJPAFOND_00520 is annotated as OsmY, a gene encoding an osmotically inducible protein that facilitates cell volume recovery and assists with severe osmotic stress that can be induced by hypertonic or salt stress[113]. IJPAFOND_01089 is annotated as *nhaD,* encoding a Na^+/H^+ antiporter. Locus tag IJPAFOND_00762 encodes for *mrpG*, a subunit of the *mrp* complex antiporter responsible for the exchange of Na^+ and H^+ across the membrane[38], which *N. aestuarii* lacks all subunits to form a functional *mrp* complex (Figure 5). Locus tag IJPAFOND_00818 is annotated as a glycine-serine hydroxymethyltransferase whose function is to catalyze the interconversion between serine and glycine, both serving as osmolytes under high salt stress[114,115]. Locus tag IJPAFOND_01245 is annotated as an arabinose efflux protein *araJ*, also found in ANs5[116].

Comparative Genomic Analysis

Nitrogen metabolism gene inventory

All the analyzed *Nitrosomonas* genomes share the core metabolic genes associated with ammonia-oxidation but differ in their complement of urease subunits and genes involved in the formation of nitrogen-oxides (Figure 5A). All AOB share a complete set of ammonia monooxygenase (*amo*) genes for the first step of ammonia oxidation (Figure 5A). All AOB share the gene that encodes hydroxylamine oxidoreductase, catalyzing the second step of ammonia oxidation leading to the production of nitric oxide and/or nitrite (Figure 5A). All *Nitrosomonas* have at least one component of the nitrifier-denitrification pathway, including the electron carrying cytochromes c552 and the nitrous oxide generating cytochrome P460 (Figure 5A). *N. communis* is the only AOB that lacks *nirK* that encodes for nitrite reductase and catalyzes the production of nitric oxide from nitrite (Figure 5A). All the genomes contained genes for at least one component of the nitric oxide reduction pathway (*nor*), with the exception of *N. marina*, which contained none (Figure 5A). Both newly sequenced AOB contain *nirK,* and lack *norB*, *norC* and any urease subunits (*ure*) (Figure 5A).

Hydrogenases

Of the 14 AOB genomes examined, all but three possess genes that encode for hydrogen metabolism (Figure 5B) but differ in their complement of hydrogenase genes. More than half of the analyzed AOB contain *hypX/hoxX*, *hypB*, or *hyaD*/*hybD*. HypX is a hydrogenase maturation factor involved in hydrogenase biosynthesis[117,118] (Figure 5B). *hypB* encodes a protein that directs the incorporation of the metal center within hydrogenases[118]

(Figure 5B). *hyaD/hybD* gene that is a group 1 membrane bound hydrogenase responsible for the uptake and oxidation of hydrogen with aerobic respiration[99,118,119] (Figure 5B). The majority of these AOB genomes contain the group 3 hydrogenase, *hoxH* gene, that encodes a bidirectional $NADP^+/NAD^+$ reducing NiFe hydrogenase capable of energy conservation[99,118] (Figure 5B). More than half of the AOB contain the group 3 hydrogenase, *hoxY* gene, that encodes a small subunit of the bidirectional NAD^+-reducing hydrogenase containing an iron-sulfur cluster that catalyzes the reversible reaction with *hoxH*[99,118] (Figure 5B). The group 4 hydrogenase encoded by *hyfB* gene is responsible for transporting protons as a formate hydrogen-lyase, and this is shared with only five AOB in the dataset (Figure 5B). Lastly, less than half of the analyzed AOB possess the group 4d hydrogenase encoded by the *ehbD* gene, a membrane bound hydrogenase that couples H_2-oxidation to ferredoxin reduction to generate reducing equivalents for carbon fixation[119] (Figure 5B).

Ion transport

The analyzed AOB share carbon-ion symporters, cation import, and NADPH generating strategies. Transporter genes within the first bracket are shared with the majority of the AOB within the dataset. The shared genes encode Na^+/H^+-dicarboxylate symport (*gltP*), K^+ import (*trkAG*), Mg^{2+} uptake (*mgtE*), and proton-transporting $NAD^+/NADP^+$ transhydrogenase (*pntAB*) (Figure 5C). From the first group, only two or three genes are missing that encode K^+/H^+ antiport (*pha* genes), K^+ import (*kef*), citrate-succinate antiport (*citT*), or Na^+/Ca^{2+} antiport (*yrbG*) (Figure 5C). Genes within the second bracket are shared in approximately half of the AOB within the dataset, representing Na^+/H^+ antiport (*nha* genes), magnesium import (*corA*), bicarbonate-sodium symport (*bicA*), proline-sodium

symport (*putP*), alanine-sodium symport (*alsT*), and the efflux of divalent metals and monovalent ions (*czcO*) (Figure 5C).

The analyzed AOB differ in their transporter inventory involved in the regulation of protons, osmolytes, and ions. Not all freshwater *Nitrosomonas* possess sequences that encode for Na^+/H^+ or K^+/H^+ antiporters (Figure 5C). *Nitrosomonas communis* has *mrp* genes and lacks *pha* genes while the opposite is true for *N. ureae*. *N. oligotropha* lacks *mrp*, *pha*, and *kef* genes (Figure 5C). Transporters within the third bracket are sporadically present in AOB, encoding Na^+/H^+ antiport (*mrpBDEFG*, *nhaP*), sodium-phosphate symport (*nptA*), sodium-solute symporters (BASS, *yidk*), Ca^{2+}/H^+ antiport (*gdt1*), Ca^{2+}-activated chloride channel (*yfbK*), a chloride channel (*clcA*), and ATP-consuming glycine betaine import (*OpuC*) (Figure 5C).

Osmolyte Biosynthesis and Metabolism

All analyzed AOB share genes for the synthesis and interconversion of organic osmolytes and amino acids proline, glutamate, and glutamine, while less than half contain genes for ectoine synthesis (Figure 5D). All AOB share *proABC* genes, that encode the synthesis of proline from L-glutamate (Figure 5D). All analyzed AOB share the glutamate dehydrogenase gene (*gdh2*) responsible for the synthesis of glutamate from 2-oxo-glutamate and glutamine synthetase (*glnA*) that can convert between L-glutamine and L-glutamate (Figure 5D). The analyzed AOB differ in their ability to synthesize other osmolytes from internal reserves such as trehalose, ectoine, and the irreversible 2-oxo-glutamate to L-glutamate. Of the six AOB that share the *treS* gene, encoding for the synthesis of trehalose from maltose, three are of cluster 7 and three are members of the 6b cluster (Figure 5D).

Only three members possess *ectABC* genes encoding for ectoine synthesis from L-aspartate-4-semialdehyde. Hydroxyectoine is an organic osmolyte produced by extremophilic bacteria to survive high stress environments and the *ectD* gene, shared by three AOB in the dataset, encodes the synthesis of hydroxyectoine from L-ectoine[120] (Figure 5D). Only five of the analyzed AOB possess proteins that are responsible for the unidirectional conversion of 2-oxo-glutamate to L-glutamate and are encoded by the *gltDB* genes (Figure 5D).

Carbon Concentration Mechanisms

Carboxysomes concentrate CO_2 within the cell. Only four AOB possess genes that encode the carboxysome (Figure 5D): *Nitrosomonas sp.* ANs5, *N. halophila, N. eutropha,* and *N. nitrosa*. Four AOB in the dataset lack carbonic anhydrase, that is responsible for the conversion of bicarbonate to carbon dioxide (Figure 5D).

Respiratory Chain

The analyzed AOB share most genes in the electron transport chain but differ in their gene content responsible for sodium pumping. All AOB share the F-type ATP synthase and proton-pumping *nuo* genes that form Complex I (Figure 5E). A significant amount of AOB genomes possess the ability to pump sodium out of the cell with sodium-transporting NADH:ubiquinone oxidoreductases (*nqr* genes), none of which are freshwater species. Two closely related halotolerant AOB, *Nitrosomonas sp.* ANs5 and *N. halophila* Nm1, are the only two species that have the V-type ATPase capable of pumping sodium or protons out of the cell (Figure 5E).

The analyzed AOB differ in their complement of terminal oxidases. All *Nitrosomonas* genomes analyzed contain the subunits of the cytochrome bc_1 complex (*petABC*) encoding the ubiquinol-cytochrome c reductase Fe-S, -b, and -c1 subunits, respectively[121] and cytochrome bo_3 terminal oxidase (*cyoABC*) (Figure 5F). *Nitrosomonas eutropha* is the only species with the *cyoD* gene encoding the 4th subunit of the cytochrome bo_3 terminal oxidase that is essential to the binding of copper ions to the catalytic site of the enzyme complex (Figure 5F)[122]. Only two AOB, *Nitrosomonas sp.* ANs5 and *N. halophila* Nm1, possess sequences for *appBC* encoding the cytochrome bd-II terminal oxidase responsible for reducing O_2 to H_2O without pumping protons out of the cell[46,123,124] (Figure 5F). *N. eutropha* is the only AOB that possesses the *ccoO* gene encoding the cbb_3-type cytochrome oxidase capable of pumping multiple protons out of the inner membrane per electron and can reduce nitric oxide to nitrous oxide (Figure 5F)[125]. The terminal oxidases present in AOB suggest a diverse composition in the respiratory system of the *Nitrosomonas* genus.

Phylogenetic Analysis of V-type ATPases

AOB of the *Betaproteobacteria* and *Gammaproteobacteria* possess sodium-pumping V-type ATPases based on their phylogenetic relationship relative to protein sequences that have been experimentally verified[98], including assertions of their ion specificity[7,9,126] (Figure 6). The *Nitrosococcus* ATPases form a distinct clade from the archaeal proton-pumping ATPases that have been experimentally characterized in *Sulfolobus acidocaldarius* and *Methanosarcina barkeri* (Figure 6). The *Nitrosomonas* ATPases, present only in ANs5 and *Nitrosomonas halophila* Nm1, form a distinct clade most closely related to the bacterial

sodium-pumping ATPases that have been experimentally characterized in *Calormator fervidus* (Figure 6). These features function by exporting Na^+-ions at the expense of ATP[22]. All V-type ATPase gene accession used to make the phylogenetic tree are available in Table 9.

Phylogenetic Analysis of Hydrogenases

Ammonia-oxidizing bacteria of the *Betaproteobacteria* and *Gammaproteobacteria* clades possess different [NiFe]-hydrogenases involved in hydrogen metabolism. The *Gammaproteobacteria* AOB possess the [NiFe]-hydrogenase that clusters with group 3b, an NADP reducing hydrogenase that is sensitive to O_2 (Figure 7). *Betaproteobacteria* AOB possess [NiFe]-hydrogenase (*hoxH*) that clusters with group 3d, a bidirectional NADP/NAD reducing hydrogenase that can consume H_2 or generate protons[99,118] (Figure 7).

Pangenomic Analysis of the Genus Nitrosomonas

The *Nitrosomonas* pangenome contained a total of 41,786 protein coding genes organized into 12,059 gene clusters. 998 gene clusters are shared between the 14 AOB species. There were 11,061 gene clusters that were not shared amongst all species.

Environmental Distribution Patterns of *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus*

The metagenomic distribution of AOB species depends on their reported growth characteristics and isolation source. Halotolerant AOB species overlap in metagenomes with freshwater or halophilic AOB (Figure 8). Environments in which halotolerant and freshwater AOB species overlap in distribution are freshwater and soil environments (Figure

8). Halotolerant and halophilic AOB species *Nitrosomonas sp.* CSM and *Nitrosococcus halophilus* Nc4 overlap in their distribution within the dried salt lake in the dataset (Figure 8). *Nitrosomonas europaea* has the highest presence within wastewater metagenomes and is the one species to commonly share their distribution with other halotolerant species within bioreactors, wastewater, activated sludge, and alkaline environments (Figure 8). *Nitrosomonas sp.* Nm120, Nm134, and Nm141, commonly overlap within estuaries, sediment, freshwater and marine metagenomes. Freshwater and halophilic AOB do not overlap in metagenomes (Figure 8).

Discussion

Enrichment of Halophilic AOB

Nitrosomonas sp. CSM has proven to be difficult to isolate at the 0.8M NaCl condition; it is important to note that this salinity is greater than the max tolerance of the closest relative *N. aestuarii*. One potential next step that could lead to its isolation is a second round of selective treatment (i.e. amend with hydroxylamine) to select against the contaminants within the lab enrichment as shown in a previous study[58].

Salinity and pH Optima of ANs5

We observed the highest rate of nitrite production by Ans5 between 0 and 0.4M NaCl, which is similar to that of ANs1[56]. ANs5 demonstrates the fastest growth rates at a pH of 9.6; this finding agrees with the alkaline shift in ANs5*'s* physiology as previously demonstrated by activity percent[54]. *Nitrosomonas* sp. ANs5 has demonstrated its alkalitolerant capabilities that can serve as the basis of gene expression studies to investigate the pH regulation network we have yet to understand.

Phylogenomic Context and Gene Content of Newly Sequenced AOB

Nitrosomonas sp. ANs5 is distinct from all other reference AOB; the next closest relative is *N. halophila* Nm1, with which it shares ~92% ANI. ANs5 falls within the cluster 7 *Nitrosomonas,* which also includes halotolerant members *N. halophila* Nm1*, N. europaea,* and *N. eutropha,* this finding is in agreement with previous relationships shown with 16S trees for the ANs1-5 species[3,19].

The placement of *Nitrosomonas sp.* CSM is a novel finding relative to the other previously sequenced AOB, and it is distinct from all other reference AOB. The next closest relative of CSM is *N. aestuarii* and they share 75% ANI. These species belong to the same cluster 6b species with an obligate salt requirement and agrees with previous literature[127].

Unique genes present in *Nitrosomonas sp.* ANs5, when compared to *N. halophila*, are either poorly characterized as ATPases with no described function or suggest the exchange of protons across the membrane with macromolecules such as sugars or antibiotics. The *araJ* gene present in *Nitrosomonas* sp. ANs5 is a glucose-proton antiporter previously described in *Staphylococcus epidermidis*[112] and references within fail to establish a connection between homologous glucose transporters present in *E. coli* to pH regulation[111]. The antibiotic-proton antiporter has been previously described as a proton-dependent multidrug efflux systems[128], where the arabinose-proton transport system is not associated with the transmembrane proton gradient or the ATP-dependent multidrug efflux systems[116]. Genes unique to *Nitrosomonas sp.* ANs5 require further analyses to understand their role in pH regulation.

Nitrosomonas sp. CSM has unique genes, not shared with *N. aestuarii*, that may be involved in the regulation of osmotic stress and genes that help maintain salt and pH homeostasis through antiport functions or osmolyte interconversion. The genome of *Nitrosomonas* sp. CSM contains the *osmY* gene that has been linked to a growth response to extreme osmotic stress[113], which may be induced by the extremes of environmental salinity and/or alkalinity. The *nhaD* gene, present in CSM, is key for salt and pH homeostasis in microorganisms that reside in salty or alkaline environments[129,130] (Figure 5C). *Nitrosomonas sp.* CSM possesses multiple subunits of the *mrp* complex, that *N. aestuarii*

lacks, that encode a Na^+/H^+ antiporter responsible for the exclusion of Na^+ and H^+ import that has been previously reported to regulate environmental salt and pH[31,131] (Figure 5C). The glycine-serine hydroxymethyltransferase facilitates the interconversion between serine and glycine which are two osmolytes that help with the osmotic stress induced by high salt[114,115]. CSM has the *araJ* gene, as previously mentioned, encoding a glucose-proton antiporter that has the potential to regulate pH at the exchange of sugar molecules for the import of protons[111]. Genes unique to *Nitrosomonas sp.* CSM require further analyses to understand their role in salt and pH homeostasis.

Significance of Missing Urease Genes in Nitrogen Inventory

The finding of ANs5 lacking urease genes is in agreement with previous reports of cluster 7 organisms lacking this feature[56,127] (Figure 5A). The finding of CSM lacking urease genes disagrees with previous reports regarding cluster 6b organisms, which have been shown to harbor these genes[127] (Figure 5A).

Significance of Hydrogenase Inventory

The presence of hydrogenase genes in the genomes of AOB highlight the metabolic versatility in the *Nitrosomonas* genus. Hydrogenases and H_2-oxidation have been detected in aerobic and anaerobic microbes from multiple species within the bacterial, archaeal, and eukaryotic phyla[119]. Genes *hypX*/*hoxX* and *hypB* are relevant for hydrogenase maturation factors involved in hydrogenase biosynthesis and metal center incorporation which are significant in the synthesis and proper functioning of the enzymes[117,118]. The *hyfB* gene has been previously implicated in the production of H_2 under aerobic conditions[132] and

maintaining pH homeostasis[133]. Hydrogenases encoded by the *hyaD*/*hybD* gene are responsible for the uptake and oxidation of hydrogen and have been detected in aerobic hydrogenotrophs[99,118,119]. The *hoxYH* genes encode the small and large subunit of a bidirectional NADP/NAD reducing [NiFe]-hydrogenase capable of energy conservation in bacteria and archaea, both subunits are required for proper functionality[99,118] (Figure 7). The *ehb* hydrogenases share sequence similarity with subunits of complex I of the respiratory chain[134] and are driven by the proton motive force[135]. The *ehb* type hydrogenases found in archaea[118] are membrane-bound features that have a role in H_2-oxidation and anabolic processes, and when interrupted, are detrimental to CO_2 assimilation[136]. We propose that *ehb* type hydrogenases are used as a source of reducing power to drive carbon fixation[99]. Some AOB members in the analysis can thrive in a range of salinities and oxygen concentrations where the presence of hydrogenases may allow an alternative source of energy generation when ammonia is low and reducing power for anabolic functions.

Significance of Ion Transport Inventory

The transport inventory across AOB species suggests that the difference between halotolerant and freshwater species may come down to the combination of transporters in the genome. The transport features that are partly missing in all of the freshwater AOB are responsible for the antiport of cations with protons, efflux of divalent metals and monovalent ions, and symport of osmolytes with sodium ions into the cell, all of which may play a role in salt and pH regulation in AOB (Figure 5C). *mrp* genes have been previously shown to help regulate both salt and pH stress within the microbial cell that has been previously overlooked[13] and only just recently been identified within the AOB of the

Nitrosomonas genus[137]. The *pha* genes are similar to *mrp* genes in terms of helping with osmotic stress due to salt or pH conditions[138]. The chloride channels help regulate the concentration of chloride ions within the cell and can provide tolerance to salt and pH stress in microorganisms[44]. The calcium-sodium antiporter may be key under high Ca^{2+} conditions and has been previously identified in salt tolerant AOB and AOA[13]. The *gdt1* gene has been previously identified in yeast[139] and human cells as having a role in influencing pH homeostasis[140–143]. Genes encoding the symport of osmolytes with sodium ions have been previously identified in AOA and AOB[13] and discussed as a method to bring in amino acids along the salinity gradient to save energy and offset osmotic stress in salt tolerant organisms[37,144,145]. Potassium regulation with *pha*, *kef*, and *trk* genes protect the organism from high internal concentrations of the ion and assist with pH homeostasis[35,146]. Magnesium uptake is a passive method of importing the ion and has been previously identified in haloarchaea[147] and in salt tolerant ammonia-oxidizing microorganisms[13] as osmoadaptations that stabilize enzyme functionality[148]. Antiporters are key for the exchange of cations out of the cell for the import of protons which is significant for the regulation of both environmental salt and pH[149].

Significance of Osmolyte Synthesis and Carbon Processing Capabilities

All analyzed AOB share genes that encode for proline synthesis, a compatible solute that is associated with adaptations to salt and pH stress[28,150]. However, AOB that also contain genes that encode the synthesis of ectoine and hydroxyectoine, may be better equipped to deal with increased osmotic stress[27,28,151]. Trehalose synthesis, which we identified in six AOB, has been previously discussed as beneficial to managing osmotic

stress due to salt or pH[152,153] and has previously been identified in ammonia-oxidizing organisms from saline-sodic soils[13]. The capabilities to synthesize osmolytes shared across AOB seem to be limited to proline synthesis and glutamate-glutamine interconversion, while AOB with the ability to synthesize trehalose or ectoine have the potential to reside in higher salt ranges.

Carbon concentrating features such as carboxysomes provide a carbon source for fixation and has been previously identified in other nitrifiers that inhabit saline-alkaline environments[60,61]. At high pH this feature is essential when CO_2 is in such low concentrations[17]. The carbonic anhydrase in AOB helps with the interconversion of bicarbonate to carbon dioxide and provides a source of carbon that may be used by AOB for anabolic processes[154].

Significance of Respiratory Chain

The shared F-type ATP synthase couples proton movement with ATP formation and complex I subunits serve as Na^+/H^+ antiporters setting up the electron transport chain in addition to regulating sodium and proton concentrations across the membrane[46]. *nqr* genes are key to the respiratory chain by pumping sodium ions into the cell through the interaction with NADH-ubiquinone oxidoreductase[46,155]. The shared cytochrome bc_1 complex and cytochrome bo_3 terminal oxidase are key for setting the proton motive force by contributing to the generation of the proton gradient through the pumping of protons out of the cell[46]. The cytochrome bo_3 terminal oxidase has been previously identified in AOB although a previous study has only detected this feature in two cluster 7 AOB members[90]. The presence of the bd-II terminal oxidases is a new finding for members of the *Nitrosomonas* genus and may

provide an advantage in microaerobic conditions[46,124]. The presence of the cbb_3-type cytochrome c oxidase in *Nitrosomonas eutropha* is in agreement with a previous study that has identified this feature in the species[90].

Significance of V-type ATPase

Based on the presence and clustering with neighboring sequences of the V-type ATPases present in *Nitrosomonas sp.* ANs5 and *N. halophila* Nm1 (Figure 4D), we suggest that the V-type ATPase in these organisms is the Na+ pumping variant and is used to drive sodium extrusion at the cost of ATP (Figure 8). This was previously suggested as the functional significance of the V-type ATPase in *Nitrosococcus oceani*[7,9,126]. The V-type ATPase seems to be a key adaptation in AOB that are capable of tolerating a wider range of pH and salinities[22,54]. This is the first report of the V-type ATPases in the genus *Nitrosomonas* (Figure 4D). The phylogenetic relationship between the *Gamma*- and *Betaproteobacteria* V-type ATPase sequences suggest they are distantly related based on the clustering with archaeal and bacterial-like sequences, respectively (Figure 8).

Significance of Environmental Distribution of AOB

Nitrosomonas species share their distribution with other AOB genera and are dominant representatives within wastewater and man-made environments. The distribution of AOB species has been previously reviewed and discussed as depending on environmental conditions such as substrate concentration, environmental pH, oxygen, temperature, and salinity[4,6,156].

Nitrosomonas sp. CSM and *Nitrosococcus halophilus* are the only organisms that inhabit hypersaline environments in this analysis (Figure 9). Overlapping distribution between CSM and *Nitrosococcus halophilus* in a dried salt lake is not surprising due to the fact that both organisms originate from saline systems (Table 4). They also share some characteristics in their sites of origin and reported growth characteristics (Table 4, Table 5)[58,65].

The analyzed dataset shows a shared distribution across genera in soil and freshwater environments which agrees with a previous reviews on the reported distribution of *Nitrosomonas* and *Nitrosospira* AOB[6,157] (Figure 9). We do not see overlap in marine environments between genera in this dataset and that could be due to the chosen set of metagenomes; this finding doesn't agree with previous descriptions of the distribution of *Nitrosomonas* and *Nitrosospira* AOB[6,157] (Figure 9). Marine and freshwater environments are the most likely to have mixed genera of AOB, where the *Nitrosomonas* species have the greatest likelihood to co-inhabit environments with the *Nitrosospira* and/or *Nitrosococcus* organisms[6].

Nitrosomonas species in the dataset are the most dominant of wastewater environments within the dataset and agrees with known distributions of these organisms[6,157] (Figure 9). Wastewater environments have been known to host various *Nitrosomonas* and *Nitrosospira* species, though *Nitrosomonas* sequences within these environments are typically closely related to *N. europaea* and *N. eutropha*[6]. Estuarine, freshwater, and sediments are more likely to contain sequences that are Nm 143-like, cluster 6A *Nitrosomonas* species, and *Nitrosospira* species[6]. The distribution of *Nitrosospira* and *Nitrosomonas* species within soil and freshwater systems in the dataset agrees with previous descriptions of their distribution[6,157] (Figure 9). Terrestrial systems and soils are more commonly inhabited by

Nitrosospira species while cluster 7 *Nitrosomonas* species are less common in these systems[6]. *Nitrosospira* and *Nitrosococcus* species within this dataset do not share their distribution with one another (Figure 9).

Conclusions

This study compares the genomic inventory of *Nitrosomonas* AOB and the environmental distribution across genera of AOB. Several genomic traits were conserved across the *Nitrosomonas* genus including: 1) nitrogen and hydrogen utilization, 2) ATP synthesis through the respiratory chain and cytochrome oxidases, 3) transport linked to salt and pH regulation, and 4) osmolyte synthesis pathways. The aforementioned genes are associated with salt-tolerance mechanisms of active ammonia-oxidizers. Genes that are involved in the export of sodium ions for the import of protons, sodium pumping at the cost of ATP, and the biosynthesis of organic compatible solutes are all examples of genomic features that enable some AOB species to survive in a wider salt range than other organisms. AOB possess a wide range of strategies to regulate the ion concentration within the cell to help with osmotic stress induced by increasing environmental salt and pH conditions. Some AOB have adapted their genomes to metabolize trace gasses such as hydrogen, that may be internally produced. Therefore, the genus wide and strain specific adaptations detailed in this study highlight the strategies AOB have developed to respond to environmental changes such as salinity and pH, which ultimately have an impact in their ecological distribution and activity.

Tables and Figures

Growth Characteristics	pH range (Optimum pH)	Salt range (Optimum salinity)	Representative bacterial species
Freshwater	5-8 (6.5-7.5)	< 3% (w/v) NaCl, < 0.3M	*Escherichia coli*
Low-salt-tolerant natronophile	8-10.5 (9.5-10.0)	0.3-1.5M (0.6M) of total Na+	*Thioalkalimicrobium aerophilum* AL3
Moderate natronophile	8-10.5 (9.0-10.0)	0.5-2M (1M) of total Na+	*Ectothiorhodospira haloalkaliphila*
Extreme natronophile	8-10.7 (9.5-10.2)	1.5-4.4M (2-3.5M) of total Na+	*Thioalkalivibrio versutus*
Moderate haloalkaliphile	8-10.5 (9.0-10.0)	3-20% (5-12%) (w/v) NaCl	*Ectothiorhodospira haloalkaliphila*
Extreme haloalkaliphile	8-10.7 (9.5-10.2)	10-30% (12-25%) (w/v) NaCl	*Thioalkalivibrio versutus*
Alkalitolerant	6.5-9 (6.5-7.5)	0.6-16% (w/v) NaCl	Alkalitolerant *Bacillus* species (e.g., *Bacillus horikoshii*)
Facultative alkaliphile	7.5-11 (7.5-10)	0.05-0.1M of total Na+	*Bacillus pseudofirmus OF4*
Obligate alkaliphile	9.0-11.5 (9.5-10.5)	0.05-2M (0.05-0.2M) of total Na+	*Bacillus alcalphilus* ATCC 27647
Halotolerant	5.0-8.5 (6.8-7.5)	0-15% (1-5%) (w/v) NaCl	*Salinivibrio costicola*
Facultative halophile	6.0-11 (6.8-7.5)	0-35% (6-12%) (w/v) NaCl	*Planococcus halophilus*
Moderate halophile	5.0-8.5 (6.8-7.5)	3-15% (7-12%) (w/v) NaCl	*Halomonas halophila*
Obligate extreme halophile	5.2-8.5 (7-7.5)	12-30% (15-25%) (w/v) NaCl	*Salinibacter ruber*

Table 1: Growth characteristics and respective salt and pH categories adapted from Banciu and Sorokin, et al. 2013. *Springer Netherlands.* The natronophile category salt range is depicted with just the sodium ion (Na^+).

Species	Salt Requirement	Salt optimum	Max tolerance	Origin	Salt %	Group	Source(s)
Nitrosomonas oligotropha Nm45	-	< 100 mM	150 mM	Freshwater, Soil	< 2%	Freshwater	Soliman and Eldyasti (2018)
Nitrosomonas ureae Nm10	-	< 100 mM	200 mM	Freshwater, Soil	< 2%	Freshwater	Soliman and Eldyasti (2018)
Nitrosomonas communis Nm2	-	< 100 mM	250 mM	Soil	< 2%	Freshwater	Soliman and Eldyasti (2018)
Nitrosospira multiformis ATCC 25196	-	< 100 mM	200 mM	Soil	< 2%	Freshwater	Soliman and Eldyasti (2018)
Nitrosospira briensis C-128	-	<250 mM	250 mM	Soil	< 2%	Freshwater	Soliman and Eldyasti (2018)
Nitrosospira lacus APG3	ND	ND	ND	Lake Sediment	< 2%	Freshwater	Urakawa et al. (2015)
Nitrosomonas nitrosa Nm90	-	< 100 mM	300 - 500 mM	Eutrohpic Environments	< 3 - 5%	Halotolerant	Soliman and Eldyasti (2018)
Nitrosomonas eutropha C91	-	< 100 mM	400 mM	Sewage	<3%	Halotolerant	Soliman and Eldyasti (2018)
Nitrosomonas europaea ATCC 19718	-	0 mM	400 - 500 mM	Sewage, Water, Soil	<4%	Halotolerant	Soliman and Eldyasti (2018)
Nitrosomonas supralitoralis APG5	-	< 100 mM	~600 mM	Beach Sand	<5%	Halotolerant	Urakawa et al. (2022)
Nitrosomonas cryotolerans ATCC 49181	+	300 mM	~600 mM	Marine	< 5%	Halotolerant	Jones et al. (1988)
Nitrosomonas mobilis 1	+	50 mM	~700 mM	Brackish Water	0.2-6%	Halotolerant	Wang et al. (2016)
Nitrosomonas aestuarii Nm36	+	300 mM	600 mM	Brackish Water	0.2-5%	Halotolerant	Soliman and Eldyasti (2018)
Nitrosomonas marina Nm22	+	350 mM	~800 mM	Marine	0.2-.0.9M (1-6%)	Halotolerant	Soliman and Eldyasti (2018)
Nitrosomonas sp. ANs5	+	300 mM	>600 mM	Saline Soda Lake	0.2-.0.9M (1-6%)	Extremely alkalitolerant, low-salt-tolerant natronophile, Halotolerant	This study Sorokin et al. (2001)
Nitrosomonas halophila Nm1	+	300 mM	900 mM	Marine	0.2-.0.9M (1-6%)	Halotolerant	Koops et al. (1991) Purkhold et al. (2000)
Nitrosomonas sp. CSM	ND	ND	~800 mM	Hypersaline Brine Pool	11%	Halotolerant	This study
Nitrosococcus oceani ATCC 19707	+	~500 mM	1100 -1200 mM / 1.1 - 1.2 M	Marine	4-10%	Moderate Halophile	Campbell et al.(2011)
Nitrosococcus watsonii C-113	+	~600 mM	ND	Marine	6-15% depends on max	Moderate Halophile	Campbell et al.(2011)
Nitrosococcus wardiae D1FHS	+	700 mM	1600 mM / 1.6 M	Marine Sediment	6-15%	Moderate Halophile	Wang et al. (2016)
Nitrosococcus halophilus Nc4	+	~700 mM	1800 mM / 1.8 M	Saline Lagoon	6-15%	Moderate Halophile	Campbell et al. (2011) Koops et al. (1990)

Table 2: Salt requirements, optima, and origin of reference ammonia-oxidizing bacteria of the *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* genera organized by increasing tolerance, optima, and requirements. Information was sourced from the literature within the source column. ND = Not Determined / Not Described.

Statistics	*Nitrosomonas sp. ANs5*	*Nitrosomonas halophila*	*Nitrosomonas aestuarii*	*Nitrosomonas sp. CSM*
NCBI Bioproject Number	PRJNA1125436	PRJEB16612	PRJNA444064	PRJNA1460379
Contigs > 2 kbp	122	134	44	156
Total Contigs	122	157	58	265
Total Coding Sequences	2,713	2,985	3,201	3,025
Coding Density	0.85	0.86	0.85	0.79
Genome Size (Mbp)	3.06	3.2	3.4	3.2
N50	49.1 kb	39.5 kb	193.1 kb	32.9 kb
L50	21	23	7	28
GC (%)	52.34	53	44.34	44.67
Completeness (%)	99.82	97.4	100	92.13
Contamination (%)	0.42	4.6	0.08	0.11
Source	This Study	Refseq: GCF_900107165.1	Refseq: GCF_003046585.1	This Study

Table 3: Assembly summary statistics of *Nitrosomonas sp.* ANs5, CSM, *N. halophila* Nm1, and *N. aestuarii* generated with CheckM2 (v1.1.0) and Quast (v5.0.2) outputs.

Species	Accession
Nitrosomonas halophila Nm1	GCF_900107165.1
Nitrosomonas cryotolerans ATCC 49181	GCF_900143275.1
Nitrosomonas oligotropha	GCF_009833085.1
Nitrosomonas supralitoralis	GCF_003011925.2
Nitrosomonas ureae	GCF_001455205.1
Nitrosomonas marina	GCF_900110145.1
Nitrosomonas aestuarii	GCF_003046585.1
Nitrosomonas nitrosa	GCF_003051105.1
Nitrosomonas communis	GCF_001007935.1
Nitrosomonas eutropha C91	GCF_000014765.1
Nitrosomonas europaea ATCC 19718	GCF_000009145.1
Nitrosomonas mobilis	GCF_900103035.1

Table 4: List of reference AOB of the *Betaproteobacteria* with corresponding accession used within this study.

Species	Accession
Nitrosospira lacus	GCF_000355765.4
Nitrosospira briensis C-128	GCF_000619905.2
Nitrosospira multiformis ATCC 25196	GCF_000196355.1
Nitrosomonas nitrosa Nm90	GCF_003051105.1
Nitrosococcus sp. Sol14	MG-RAST: DAAN_MAGs
Nitrosomonas sp. CSM	NA
Nitrosomonas aestuarii Nm36	GCF_003046585.1
Nitrosomonas cryotolerans ATCC 49181	GCF_900143275.1
Nitrosomonas europaea ATCC 19718	GCF_000009145.1
Nitrosomonas eutropha C91	GCF_000014765.1
Nitrosomonas halophila Nm1	GCF_900107165.1
Nitrosomonas sp. ANs5	GCF_050584665.1
Nitrosomonas sp. Nm143	PRJNA444086
Nitrosomonas marina Nm22	GCF_900110145.1
Nitrosomonas mobilis 1	GCF_900103035.1
Nitrosomonas supralitoralis APG5	GCF_003011925.2
Nitrosomonas sp. Nm141	PRJNA444065
Nitrosomonas sp. Nm120	PRJNA370067
Nitrosomonas sp. Nm134	PRJNA444066
Nitrosomonas sp. Nm84	PRJNA444087
Nitrosococcus halophilus Nc4	GCF_000024725.1
Nitrosococcus oceani ATCC 19707	GCF_000012805.1
Nitrosococcus wardiae	GCF_004421105.1

Table 5: List of AOB species, used in the distribution analysis, with their corresponding accession (if available).

Metagenome	Habitat Type
ERR1294727	Alkaline
ERR2752150	Marine
ERR4182670	Soil
ERR7250480	Soil
ERR7250482	Soil
ERR9670067	Brackish
ERR9709080	Dried Salt Lake
ERR9709082	Soil
SRR10598224	Groundwater
SRR12337189	Marine Sediment
SRR12532595	Drinking Water
SRR12705451	Mine
SRR12951186	Mine
SRR13122810	Marine
SRR13696273	Alkaline
SRR1570765	Sand
SRR15927520	Estuary
SRR15927523	Estuary
SRR16248219	Alkaline
SRR16287267	Bioreactor
SRR16693997	Bioreactor
SRR1763383	Wastewater
SRR1763384	Wastewater
SRR18218232	Plant associated Desert Soil
SRR18218237	Desert Soil
SRR18218243	Soil
SRR20705928	Agricultural Soil
SRR21020893	Agricultural Soil
SRR21120500	Unvegetated Wetland Soil
SRR21266981	Sediment
SRR21517855	Bioreactor
SRR21542051	Hypersaline
SRR21705950	Sediment
SRR21824725	Sand
SRR21824726	Sand
SRR21824728	Coastal Brine
SRR21892050	Freshwater
SRR22387923	Groundwater
SRR22808125	Marine Sediment
SRR22808126	Sediment
SRR22870106	Sediment
SRR22956010	Activated Sludge
SRR23086401	Sediment
SRR24605838	Antarctic Soil
SRR24956259	Agricultural Soil
SRR25229492	Landfill
SRR25377788	Alkaline
SRR25399862	Dairy Facility
SRR25607932	Soil
SRR25607939	Agricultural Soil
SRR25638219	Soil
SRR25654127	Marine
SRR26176488	Wastewater
SRR26421363	Mangrove
SRR26421365	Mangrove
SRR26965286	Freshwater
SRR27028764	Marine
SRR27256817	Salt Pan
SRR27428931	Freshwater
SRR28493908	Freshwater
SRR28842982	Alkaline
SRR30281129	Salt Lake
SRR30281353	Salt Lake
SRR30566754	Soil
SRR30566756	Wastewater
SRR30568137	Sediment
SRR30641127	Wastewater
SRR30731182	Soil
SRR30765335	Soil
SRR30982337	Wastewater
SRR30982338	Wastewater
SRR4027579	Soil
SRR5678814	Soil
SRR5678936	Soil
SRR5679043	Agricultural Soil
SRR5690349	Soil
SRR5690606	Agricultural Soil
SRR6058007	Marine
SRR6425777	Soil
SRR6689525	Bioreactor
SRR8707056	Sediment
SRR8863432	Groundwater
SRR8925779	Bioreactor
SRR8931189	Groundwater
SRR9691048	Soil
SRR9721663	Soil
SRR27869039	Hypersaline
SRR26473340	Hypersaline
SRR24224779	Sediment
SRR14492182	Soil

Table 6: List of metagenomes used in the distribution analysis organized by accession and habitat type.

1st Dilution	Week 1	Week 2	Week 3	Week 4	Week 5				
Dilution Factor	-1	-2	-3	-4	-5	-6	-7	-8	-9
CSM at 600 mM	+	+	+	+	+	-	-	-	-
CSM at 800 mM	+	+	+	+	+	-	-	-	-
2nd Dilution	Week 1	Week 2	Week 3	Week 4	Week 5				
Dilution Factor	-1	-2	-3	-4	-5	-6	-7	-8	-9
CSM at 600 mM	+	+	+	+	-	-	-	-	-
CSM at 800 mM	+	+	+	+	+	+	+	-	-
3rd Dilution	Week 1	Week 2	Week 3	Week 4					
Dilution Factor	-1	-2	-3	-4	-5	-6	-7	-8	-9
CSM at 600 mM	+	+	+	+	-	-	-	-	-
CSM at 800 mM	+	+	+	+	+	+	-	-	-
4th Dilution (Post-sequencing)	Week 1	Week 2	Week 3	Week 4	**Week 5**				
Dilution Factor	-1	-2	-3	-4	-5	-6	-7	-8	-9
CSM at 600 mM	**+**	-	-	-	-	-	-	-	-
CSM at 800 mM	+	+	+	+	+	+	-	-	-

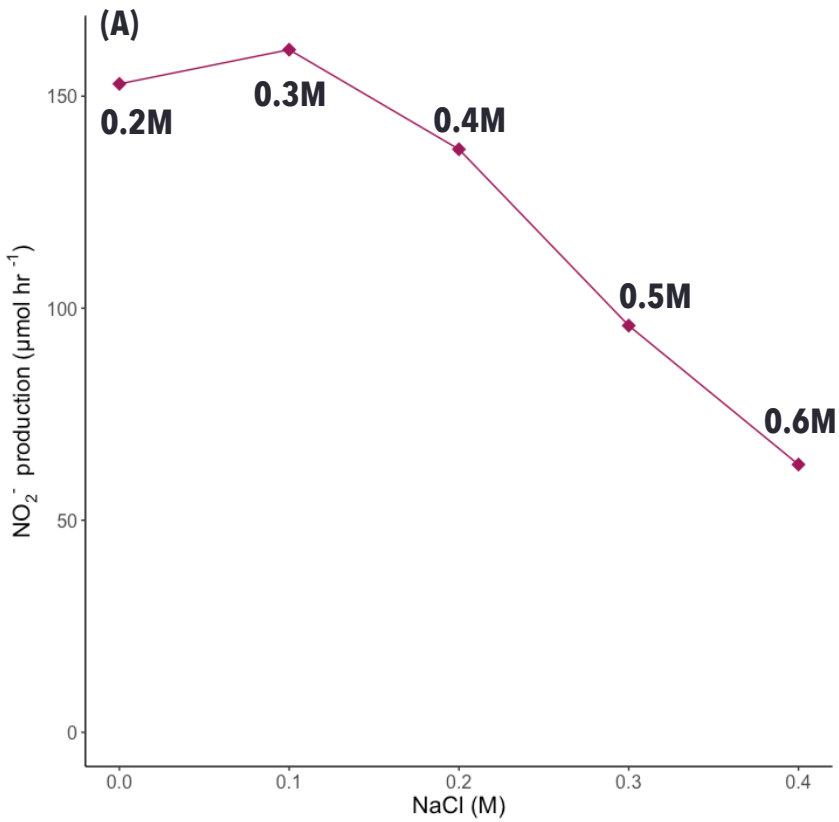
Table 7: Qualitative growth of *Nitrosomonas sp.* CSM during dilution series. Each table depicts the results of the colorimetric reaction from the dilution of CSM from the enrichment culture initiated in December 2023. Note that the 4th dilution attempt was after the sequencing of the suspected isolate and contains bolded text to delineate the progression difference between the enrichment growing at the two conditions. Plus (+) symbols indicate a positive reaction for the detection of nitrite production. Minus (-) symbols indicate a negative reaction for the detection of nitrite production.

	Relative Abundance (%)	Completeness	Contamination
Nitrosomonas sp. CSM	42.27	92.13	0.11
Marinobacter sp.	4.25	99.66	0.88
Maricaulis sp.	3.49	94.18	0.09
Unclassified Bacteria	1.45	12.57	0.01
Marinobacter sp.	8.16	77.51	19.1
Marinobacter sp.	7.55	37.88	0.71
Marinobacter sp.	7.44	40.28	1.99
Halomonas sp.	4.9	99.98	0.59
Unmapped	20.5	-	-

Table 8: Relative abundance of microorganisms within the lab enrichment culture metagenome including *Nitrosomonas sp.* CSM. Each row corresponds to an individual MAG that the enrichment reads were binned into.

Phylum, *class*	Organism name	Gene Accession / ID Number
Archaea		
Nanoarchaeota	*Nanoarchaeum equitans*	WP_011153211
Crenarchaeota	*Aeropyrum pernix*	WP_010866940
	Caldivirga maquilingensis	WP_012186938
	Cenarchaeum symbiosum	DP000238
	Hyperthermus butylicus	WP_011821951
	Ignicoccus hospitalis	WP_011998716
	Pyrobaculum aerophilum	WP_011007478
	Staphylothermus marinus	WP_011839461
	Sulfolobus acidocaldarius	Q4J8L5
Euryarchaeota		
Archaeoglobi	*Archaeoglobus fulgidus*	WP_010878657
Halobacteria	*Haloarcula marismortui*	YP_137567
	Halobacterium salinarum	BAA13179
Methanobacteria	*Methanobrevibacter smithii*	YP_001273012
	Methanosphaera stadtmanae	WP_011406719
	Methanothermobacter thermautotrophicus	WP_010876590
Methanococci	*Methanocaldococcus jannaschii*	WP_010869717
	Methanococcus maripaludis	NP_988160
Methanomicrobia	*Methanococcoides burtonii*	WP_011499307
	Methanothrix thermoacetophila	WP_011696758
	Methanosarcina acetivorans	NP_619022
	Methanosarcina barkeri	AAZ69362
	Methanosarcina mazei	NP_632808
	Methanospirillum hungatei	WP_011448759
Methanopyri	*Methanopyrus kandleri*	WP_011019381
Thermococci	*Pyrococcus horikoshii*	WP_010886041
	Thermococcus kodakarensis	YP_184011
Thermoplasmata	*Ferroplasma acidarmanus*	ZP_01708868
	Picrophilus torridus	WP_011177295
Bacteria		
Bacteroidetes	*Bacteroides fragilis*	BAD49478
	Porphyromonas gingivalis	WP_004583476
Chlamydiae	*Chlamydia trachomatis*	NP_219809
	Chlamydophila pneumoniae	WP_010882742
	Protochlamydia amoebophila	WP_011176222
Deinococcus-Thermus	*Deinococcus geothermalis*	YP_605514
	Deinococcus radiodurans	NP_294419
	Thermus thermophilus	BAA09869
Bacillota		
Bacilli	*Enterococcus faecalis*	WP_002363905
	Enterococcus hirae	2BL2_A
	Streptococcus pneumoniae	NP_345779
Clostridia	*Caloramator fervidus*	ABD18895
	Clostridium perfringens	NP_562558
	Thermoanaerobacter ethanolicus	EAO65277
Mollicutes	*Acholeplasma laidlawii*	WP_012243102
Fusobacteria	*Fusobacterium nucleatum*	NP_602556
Proteobacteria		
Alpha	*Stappia aggregata*	EAV41135
Beta	*Nitrosomonas halophila Nm1*	WP_090415413
	Nitrosomonas sp. ANs5	WP_090415413
Gamma	*Beggiatoa* sp. PS	ZP_01999677
	Nitrosococcus oceani	WP_002809002
	Nitrosococcus wardiae	WP_134356153
	Nitrosococcus wastsonii	WP_013220046
	Nitrosococcus halophilus	WP_013031854
Delta	*Anaeromyxobacter dehalogenans*	YP_464409
	Geobacter uraniumreducens	WP_011937361
Spirochaetes	*Borrelia burgdorferi*	B7J127
	Treponema pallidum	WP_010881878
Thermotogae	*Thermotoga neapolitana*	WP_010881878
	Thermotoga sp. RQ2	EDQ30134
Eukaryota		
Fungi	*Saccharomyces cerevisiae*	P25515
Mycetozoa	*Dictyostelium discoideum*	P54642
Metazoa	*Drosophila melanogaster*	P23380
	Bos taurus	P23956
Viridiplantae	*Arabidopsis thaliana*	P59229

Table 9: Gene accession numbers for the V-type ATPase sequences included in the phylogenetic tree of Figure 8.


(A)
0.2M
0.3M
0.4M
0.5M
0.6M
150
100
50
0
0.0
0.1
0.2
0.3
0.4
NaCl (M)
NO2- production (µmol hr-1)

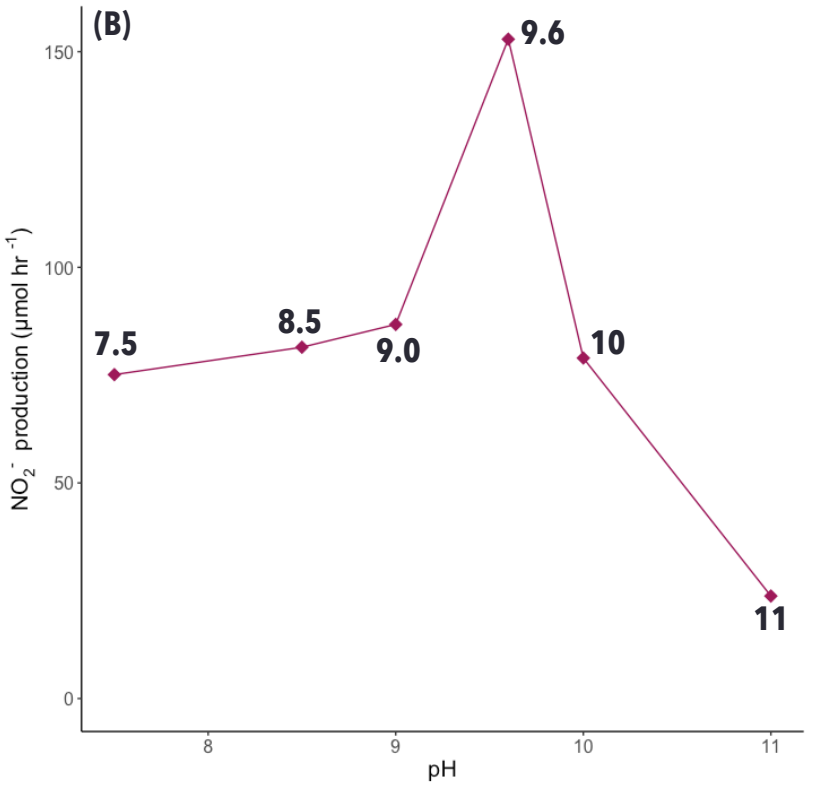

(B)
7.5
8.5
9.0
9.6
10
11
150
100
50
0
8
9
10
11
pH
NO2- production (µmol hr-1)

Figure 1: Growth of *Nitrosomonas sp.* ANs5 along a salinity (A) and pH (B) gradient. The media base contained 200 mM [Na^+] through carbonate salts for both experiments. (A) 100 mM NaCl additions were made to each batch medium while keeping the pH constant at 9.6. The amount of added NaCl is shown in the x-axis; total [Na^+] is shown in bold at each data point. (B) The pH was modified between each batch from the media base by 10% HCl or 5M NaOH, the point at pH 9.6 represents the 0 mM NaCl condition in (A). The total [Na^+] and final pH of the media are denoted above the points. Cultures were transferred from parent cultures at mid-exponential phase at 10% across all pH and salinity conditions.

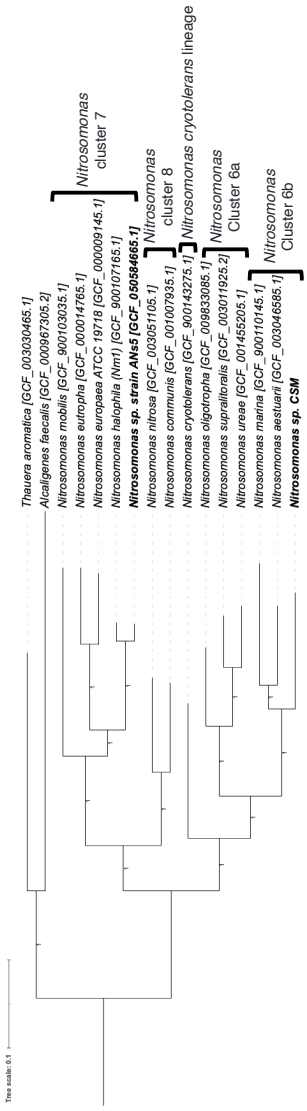


Figure 2: The phylogenomic relationship of reference *Nitrosomonas* species and newly identified alkalitolerant species ANs5 and MAG *Nitrosomonas. sp.* CSM. Analysis includes the proteobacterial marker set from GToTree that generated an approximate maximum likelihood tree with FastTree2 v2.1.11 and was visualized with iTOL (v7.2). Local bootstrap support values are indicated below the branches. The phylogenomic tree is rooted based on the outgroup represented by *Betaproteobacteria* outside of the genus *Nitrosomonas*. The NCBI refseq accession tags are provided. Strain names in bold are new *Nitrosomonas* species.

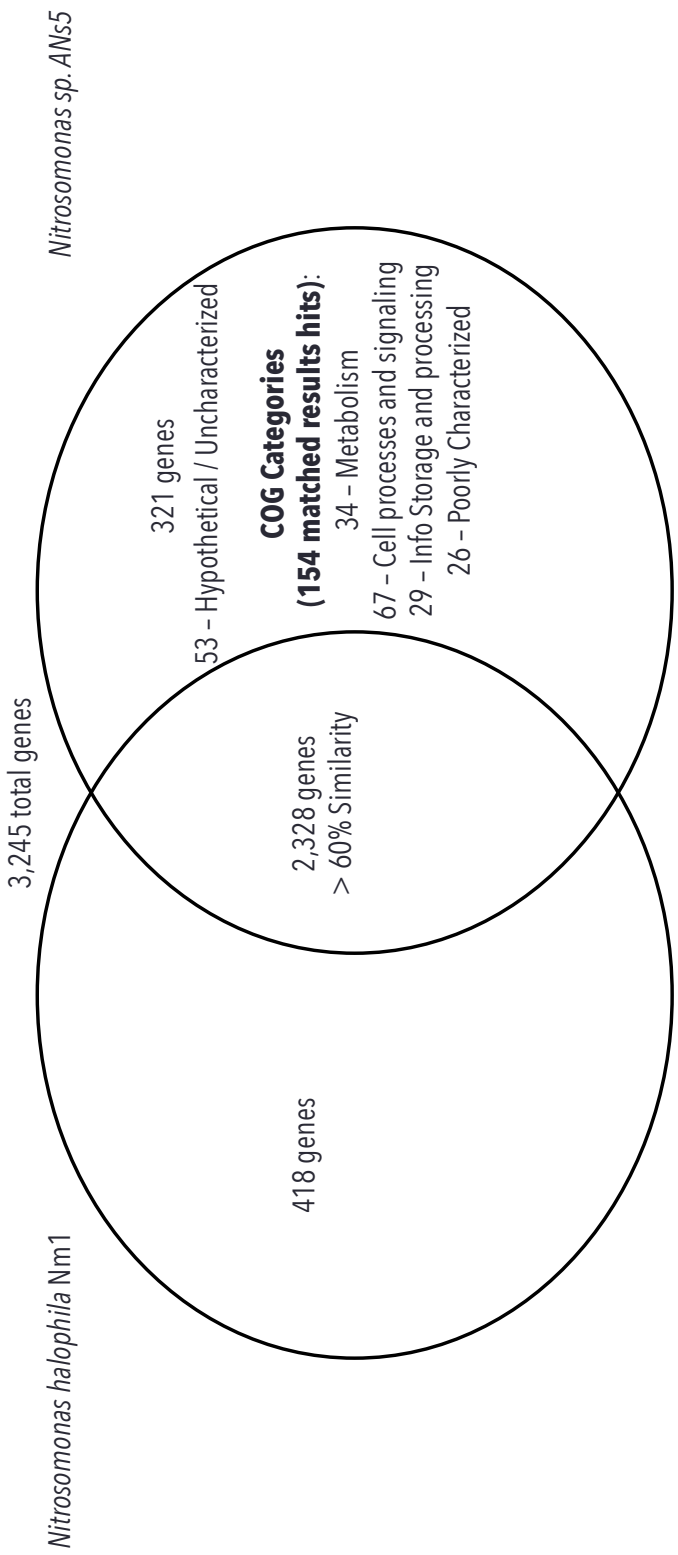


Figure 3: Breakdown of unique genes in *Nitrosomonas sp.* ANs5 by COG categories when compared to *N. halophila* Nm1.

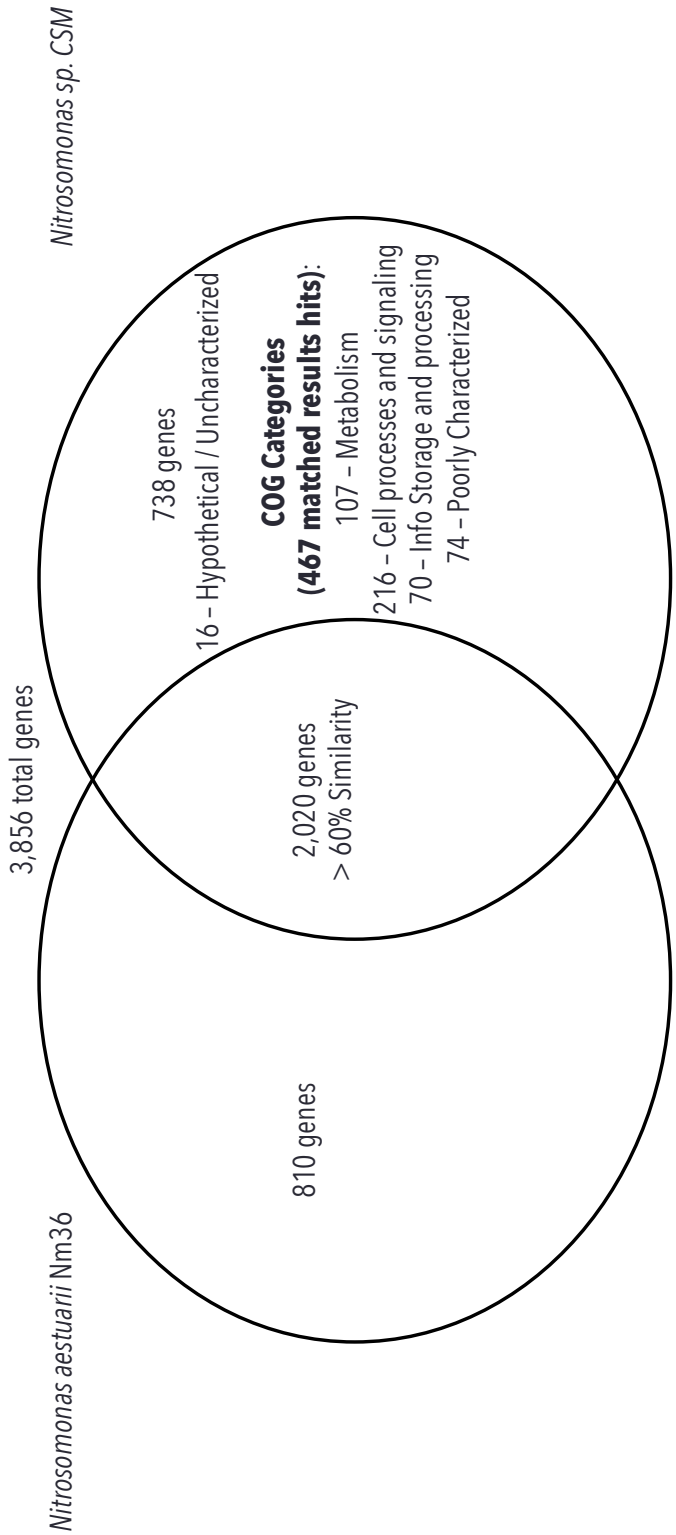
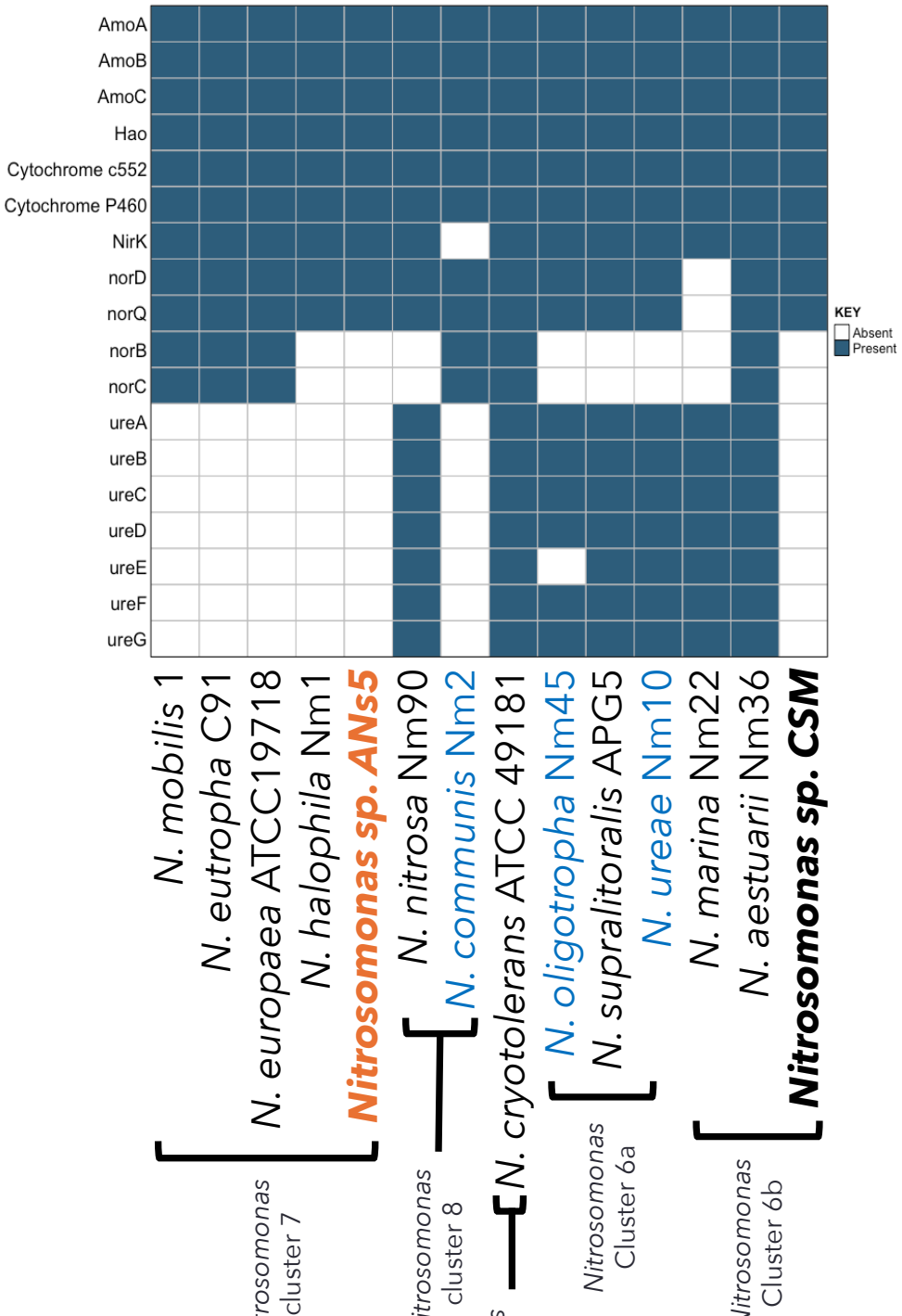


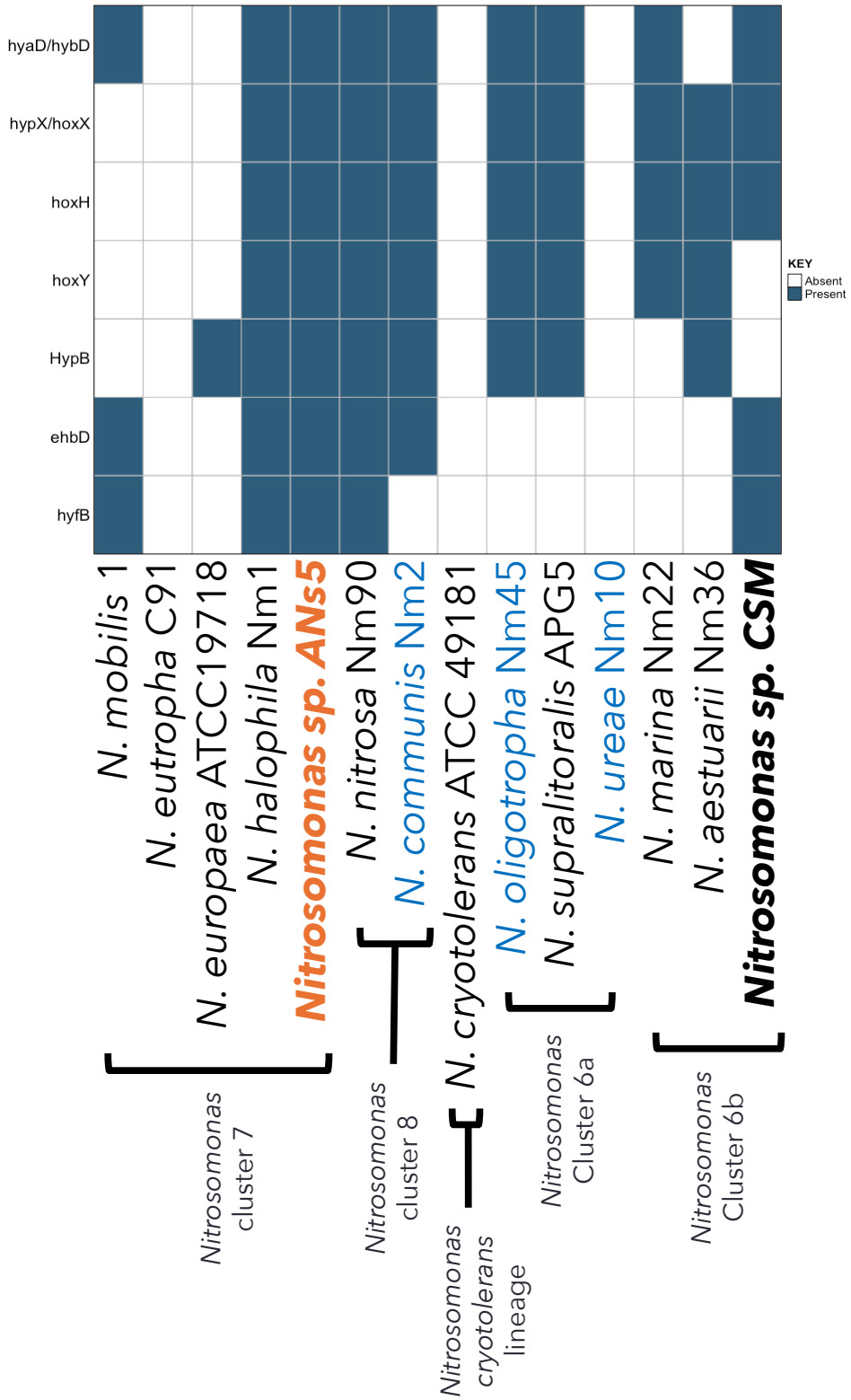
Figure 4: Breakdown of unique genes in MAG *Nitrosomonas sp.* CSM by COG categories when compared to *N. aestuarii* Nm36.

Figure 5 (A-F): Inventory of ammonia oxidation (A) and alternative metabolic genes (B), potential genetic adaptations to salt and pH of *Nitrosomonas* ammonia-oxidizing bacteria (C-E), and cytochrome oxidases (F). Organisms highlighted in blue text are considered freshwater, those in black text are considered halotolerant, those in orange are low-salt tolerant natronophiles based on salt requirement, range, and optima (Tables 1 and 2). Bolded names are new species. AOB are organized on the x-axis to match the order of the phylogenomic tree with clustering designations as in Figure 3.

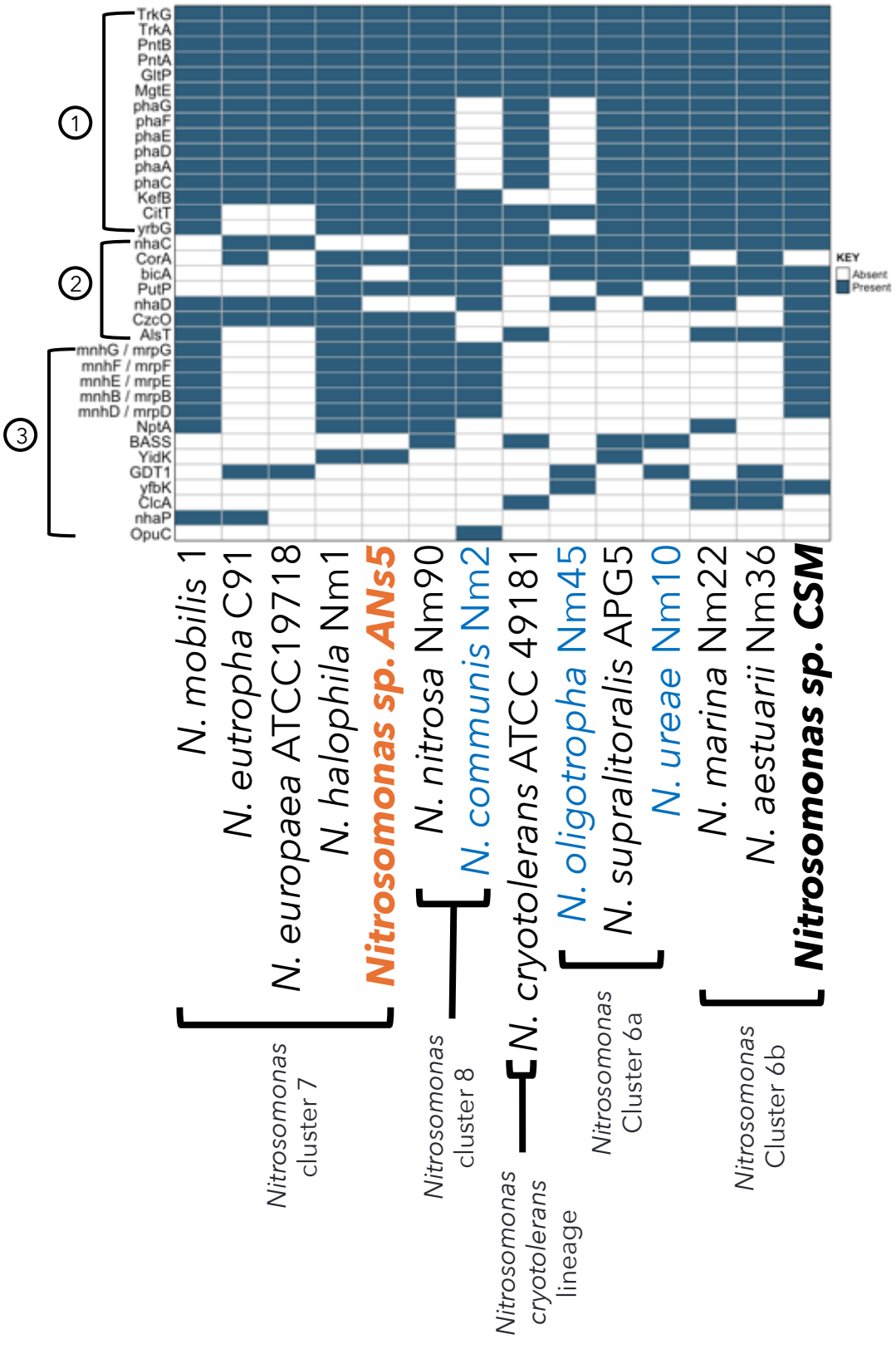
(A) Nitrogen


AmoA
AmoB
AmoC
Hao
Cytochrome c552
Cytochrome P460
NirK
norD
norQ
norB
norC
ureA
ureB
ureC
ureD
ureE
ureF
ureG
KEY
Absent
Present
N. mobilis 1
N. eutropha C91
N. europaea ATCC19718
N. halophila Nm1
Nitrosomonas sp. ANs5
N. nitrosa Nm90
N. communis Nm2
N. cryotolerans ATCC 49181
N. oligotropha Nm45
N. supralitoralis APG5
N. ureae Nm10
N. marina Nm22
N. aestuarii Nm36
Nitrosomonas sp. CSM
Nitrosomonas cluster 7
Nitrosomonas cluster 8
Nitrosomonas cryotolerans lineage
Nitrosomonas Cluster 6a
Nitrosomonas Cluster 6b

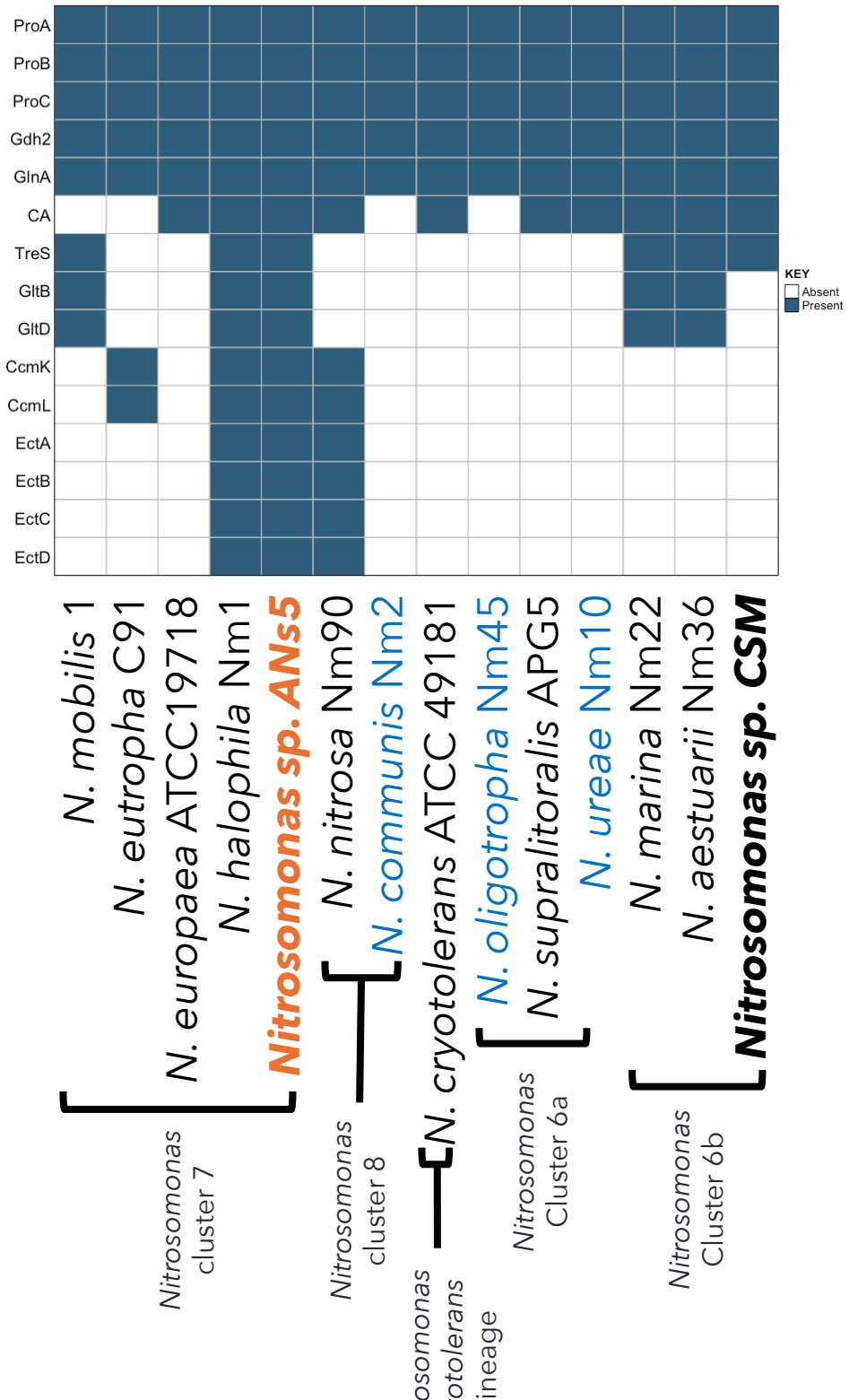
(B) Hydrogenase



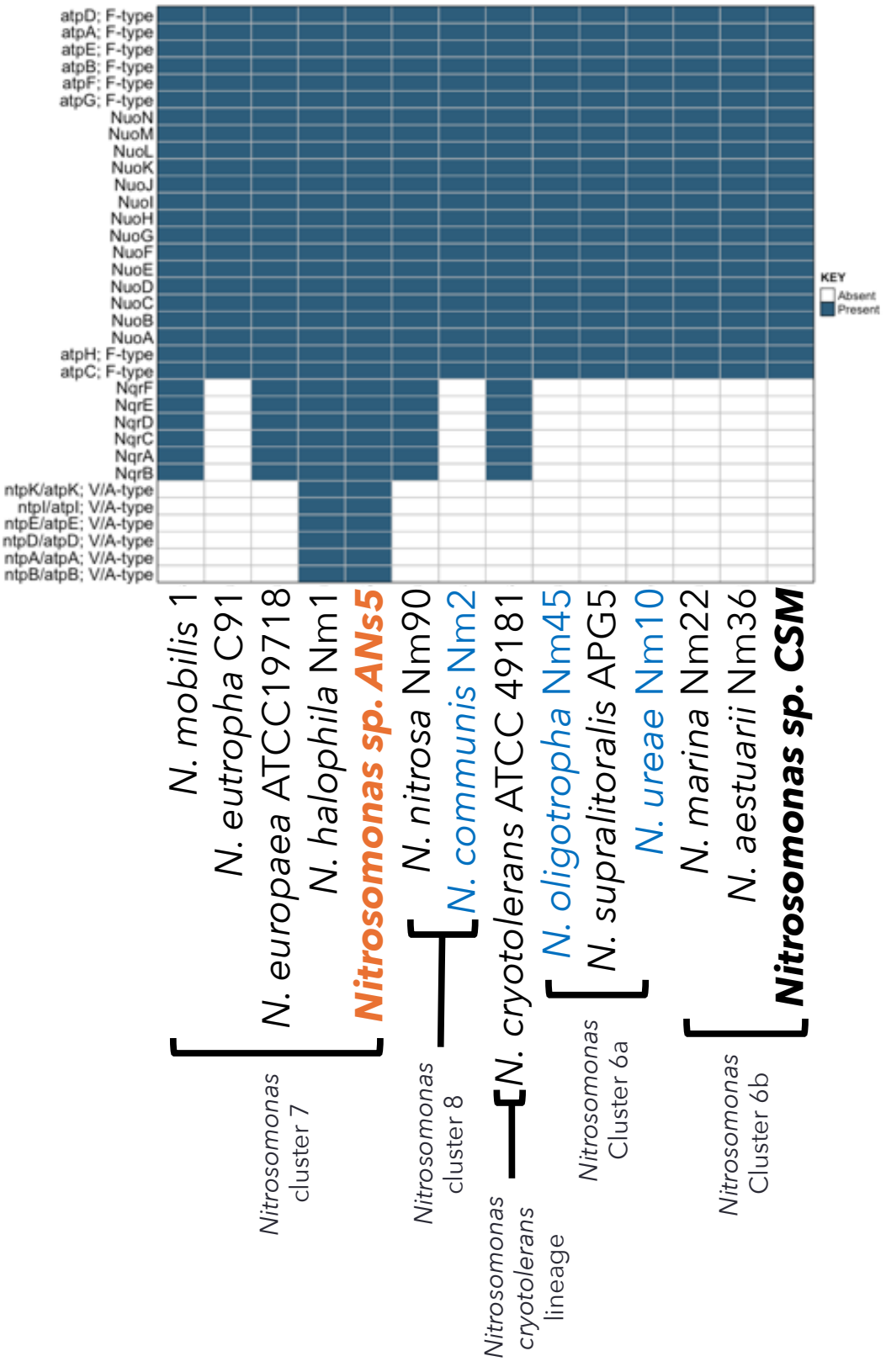
(C) Subset of transporter gene inventory involved in sodium or proton transport



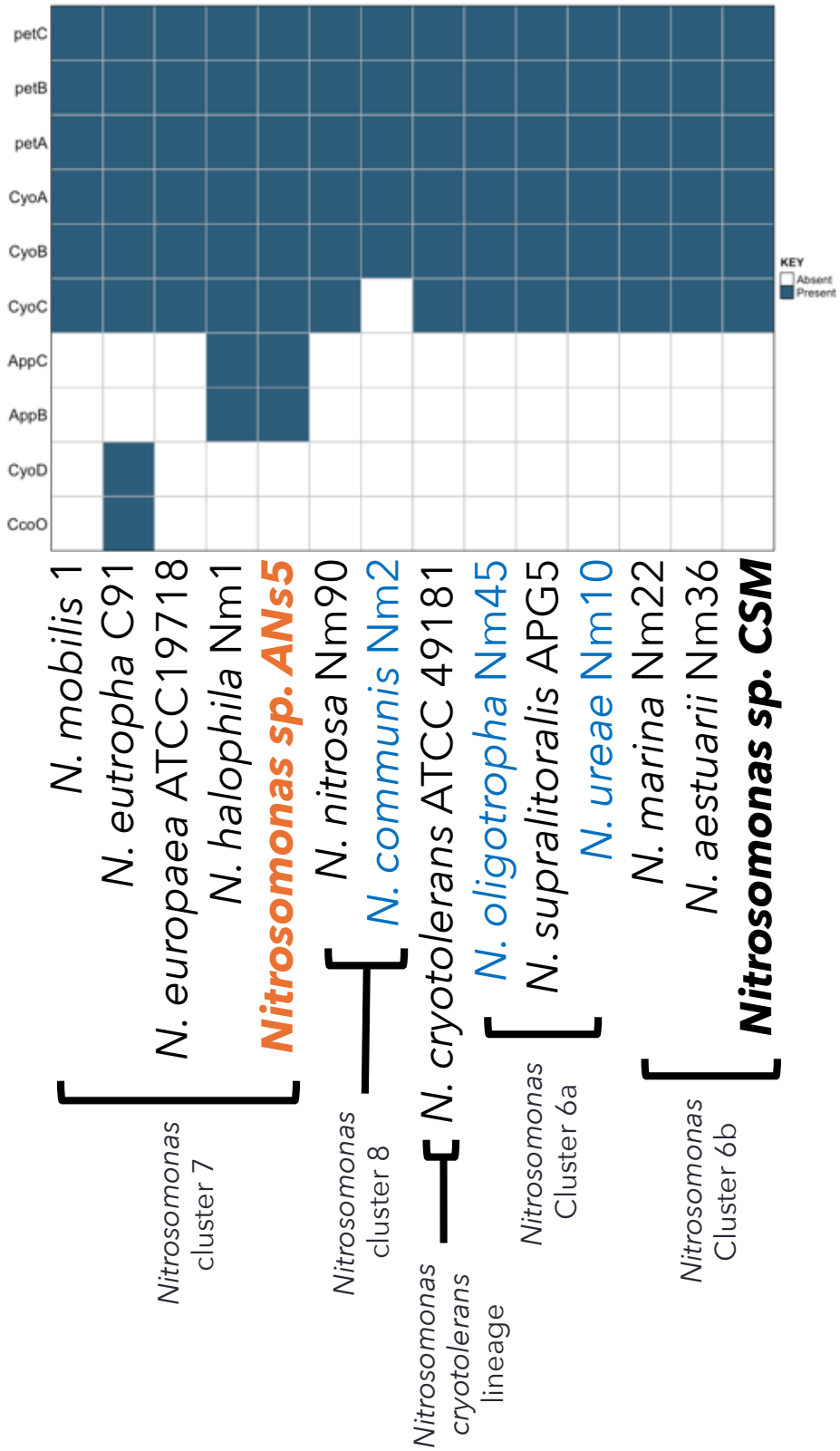
(D) Gene inventory of compatible solute biosynthesis and metabolism



(E) Electron Transport Chain gene inventory



(F) Cytochrome oxidase



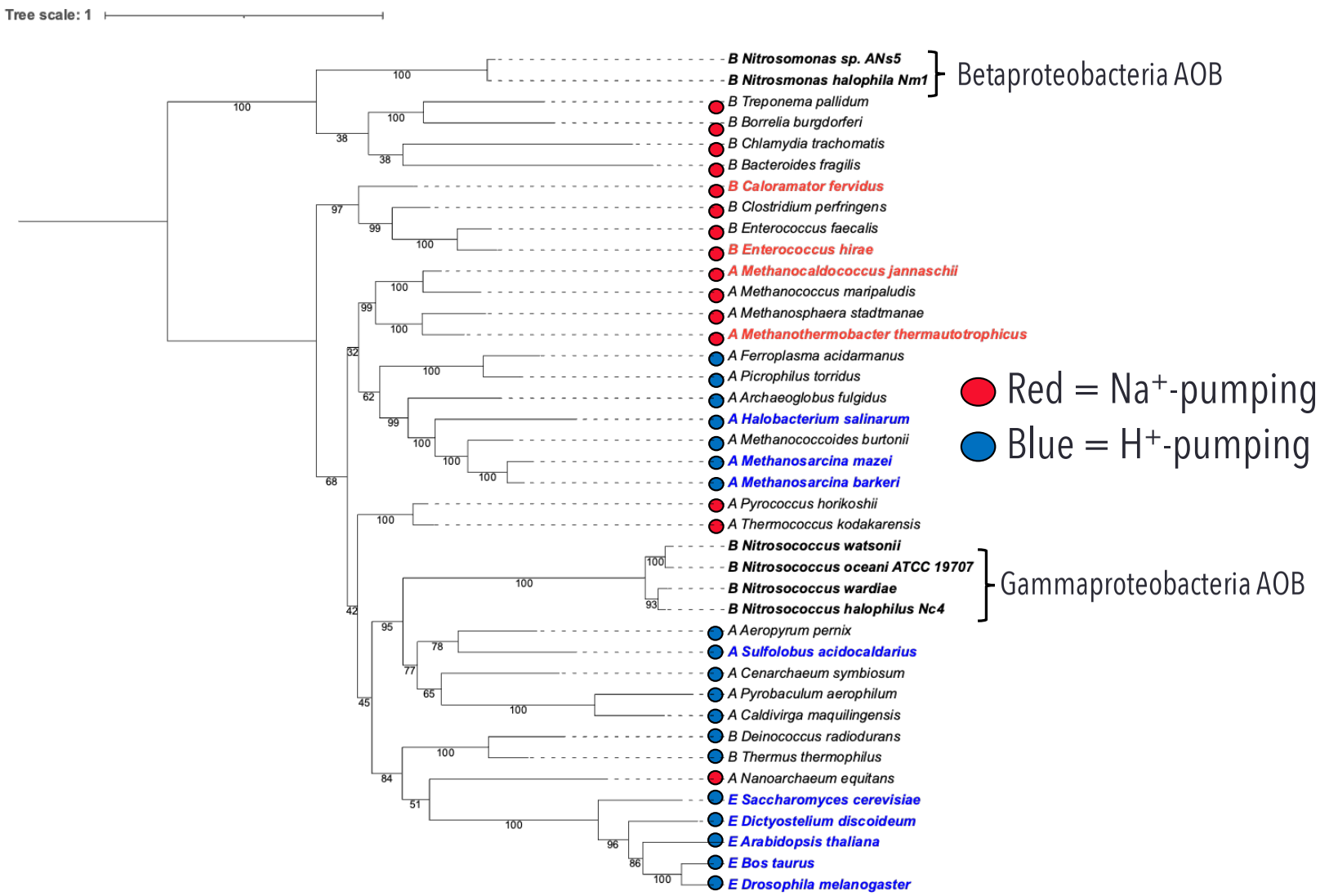

Tree scale: 1
B Nitrosomonas sp. ANs5
B Nitrosmonas halophila Nm1
Betaproteobacteria AOB
B Treponema pallidum
B Borrelia burgdorferi
B Chlamydia trachomatis
B Bacteroides fragilis
B Caloramator fervidus
B Clostridium perfringens
B Enterococcus faecalis
B Enterococcus hirae
A Methanocaldococcus jannaschii
A Methanococcus maripaludis
A Methanosphaera stadtmanae
A Methanothermobacter thermautotrophicus
A Ferroplasma acidarmanus
A Picrophilus torridus
A Archaeoglobus fulgidus
A Halobacterium salinarum
A Methanococcoides burtonii
A Methanosarcina mazei
A Methanosarcina barkeri
A Pyrococcus horikoshii
A Thermococcus kodakarensis
B Nitrosococcus watsonii
B Nitrosococcus oceani ATCC 19707
B Nitrosococcus wardiae
B Nitrosococcus halophilus Nc4
Gammaproteobacteria AOB
A Aeropyrum pernix
A Sulfolobus acidocaldarius
A Cenarchaeum symbiosum
A Pyrobaculum aerophilum
A Caldivirga maquilingensis
B Deinococcus radiodurans
B Thermus thermophilus
A Nanoarchaeum equitans
E Saccharomyces cerevisiae
E Dictyostelium discoideum
E Arabidopsis thaliana
E Bos taurus
E Drosophila melanogaster
Red = Na+-pumping
Blue = H+-pumping

Figure 6: Maximum likelihood phylogenetic tree of sodium-translocating and proton-translocating V-type ATPases in Archaea (A), Bacteria (B), and Eukarya (E). The phylogenetic tree was constructed based on the amino acid sequence alignment of alpha subunit of the V-type ATPases and fashioned after figure 5 in Mulkidjanian et al. 2008. Red colored circles indicate the ATPases whose c subunits contain all Na^+-ligands and are predicted to translocate sodium. ATPases whose c subunits are lacking one or more of the Na^+-ligands are predicted to translocate protons which are denoted by the blue circles. Organisms in the black bold are *Beta*- and *Gammaproteobacteria* clades of ammonia-oxidizing bacteria, those that have been experimentally characterized are bolded in red for the sodium-dependent enzymes and bolded in blue for the proton-dependent enzymes. The tree was midpoint rooted and numbers below the branches indicate the bootstrap probabilities. Accession numbers of the proteins used are provided in Table 9.

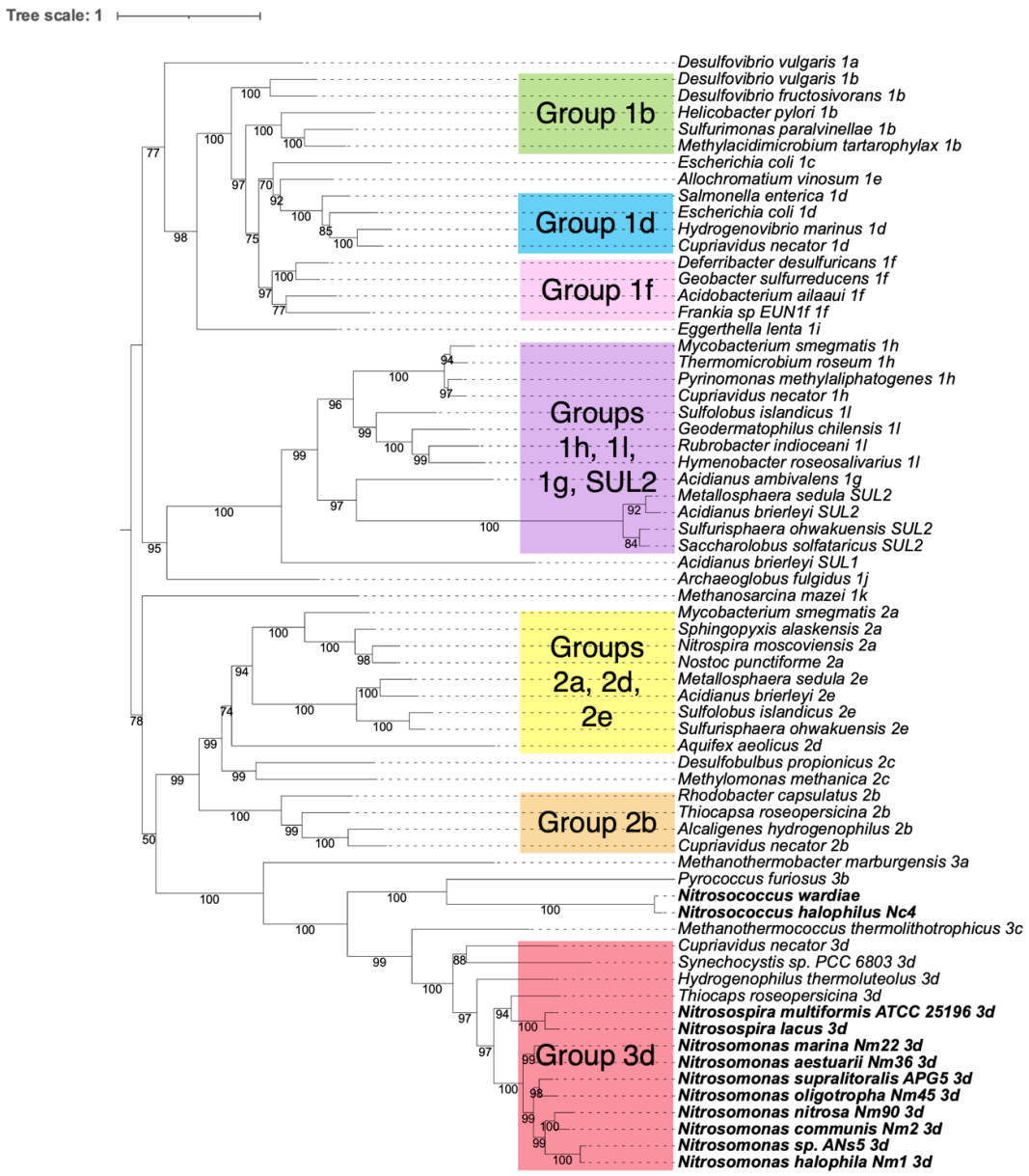


Figure 7: Phylogenetic tree of the [NiFe]-hydrogenase large subunit (*hoxH*) amino acid sequences showing key lineages after Jespersen et al. 2025. The tree is midpoint rooted with the bootstrap value below the branches. Black bolded names are highlighting the ammonia-oxidizing bacteria belonging to the genera *Nitrosomonas*, *Nitrosospira*, or *Nitrosococcus*.

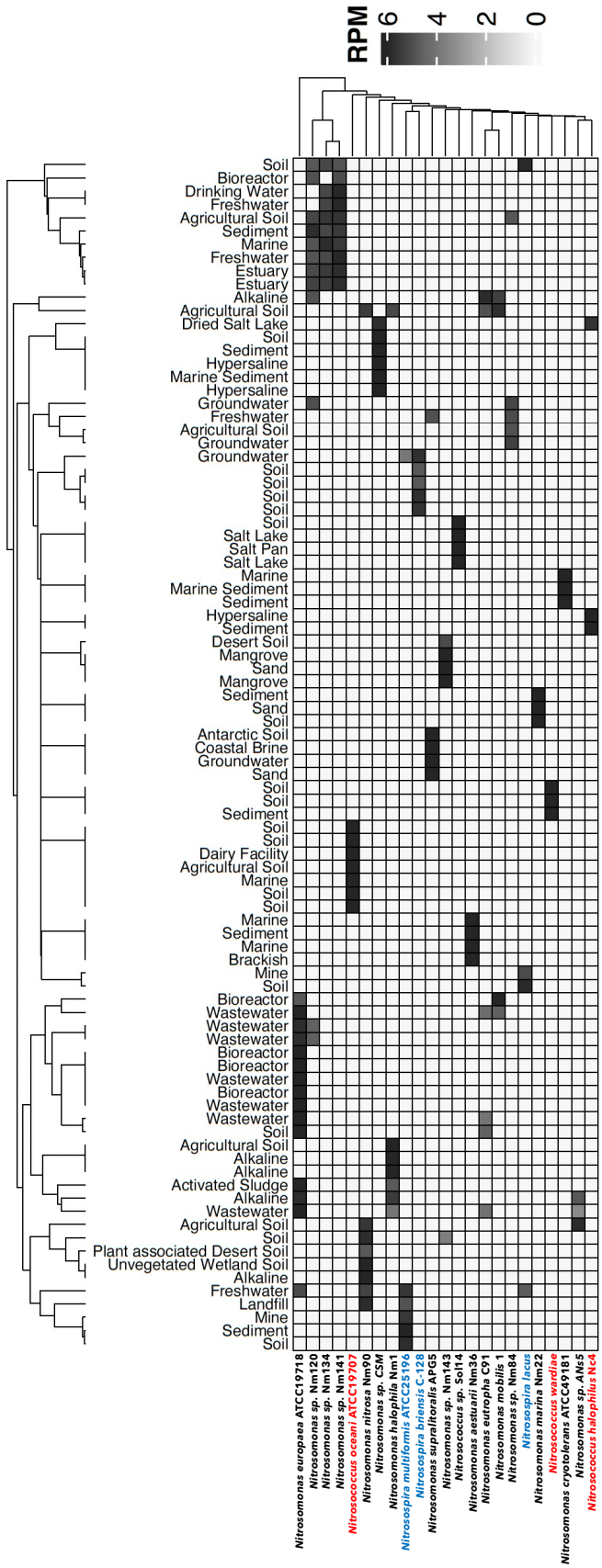

RPM
6
4
2
0
Soil
Bioreactor
Drinking Water
Freshwater
Agricultural Soil
Sediment
Marine
Freshwater
Estuary
Estuary
Alkaline
Agricultural Soil
Dried Salt Lake
Soil
Sediment
Hypersaline
Marine Sediment
Hypersaline
Groundwater
Freshwater
Agricultural Soil
Groundwater
Groundwater
Soil
Soil
Soil
Soil
Soil
Salt Lake
Salt Pan
Salt Lake
Marine
Marine Sediment
Sediment
Hypersaline
Sediment
Desert Soil
Mangrove
Sand
Mangrove
Sediment
Sand
Soil
Antarctic Soil
Coastal Brine
Groundwater
Sand
Soil
Soil
Sediment
Soil
Soil
Dairy Facility
Agricultural Soil
Marine
Soil
Soil
Marine
Sediment
Marine
Brackish
Mine
Soil
Bioreactor
Wastewater
Wastewater
Wastewater
Bioreactor
Bioreactor
Wastewater
Bioreactor
Wastewater
Wastewater
Soil
Agricultural Soil
Alkaline
Alkaline
Activated Sludge
Alkaline
Wastewater
Agricultural Soil
Soil
Plant associated Desert Soil
Unvegetated Wetland Soil
Alkaline
Freshwater
Landfill
Mine
Sediment
Soil
Nitrosomonas europaea ATCC19718
Nitrosomonas sp. Nm120
Nitrosomonas sp. Nm134
Nitrosomonas sp. Nm141
Nitrosococcus oceani ATCC19707
Nitrosomonas nitrosa Nm90
Nitrosomonas sp. CSM
Nitrosomonas halophila Nm1
Nitrosospira multiformis ATCC25196
Nitrosospira briensis C-128
Nitrosomonas supralitoralis APG5
Nitrosomonas sp. Nm143
Nitrosococcus sp. Sa114
Nitrosomonas aestuarii Nm36
Nitrosomonas eutropha C91
Nitrosomonas mobilis 1
Nitrosomonas sp. Nm84
Nitrosospira lacus
Nitrosomonas marina Nm22
Nitrosococcus wardiae
Nitrosomonas cryotolerans ATCC49181
Nitrosomonas sp. ANs5
Nitrosococcus halophilus Nc4

Figure 8: Relative abundance (reads per million, RPM) of ammonia-oxidizing bacteria of the *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira* genera across 90 globally distributed metagenomes. The environments and species were clustered by the distribution of the AOB with the ComplexHeatmap package (v2.21.2). The species names that are in blue are freshwater, red are halophilic, and black are halotolerant.

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Supplemental Files / Appendix

1) Text file with unique tags in *Nitrosomonas sp.* ANs5 from ANs5 vs *N. halophila* analysis
2) Text file with unique tags in *Nitrosomonas sp.* CSM from CSM vs *N. aestuarii* analysis