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Ecophysiology of an Ammonia-Oxidizing Archaeon Adapted to Low-Salinity Habitats

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Abstract Ammonia oxidation in marine and terrestrial ecosystems plays a pivotal role in the cycling of nitrogen and carbon. Recent discoveries have shown that ammonia-oxidizing archaea (AOA) are both abundant and diverse in these systems, yet very little is known about their physiology. Here we report a physiological analysis of a novel low-salinity-type AOA enriched from the San Francisco Bay estuary, *Candidatus* Nitrosoarchaeum limnia strain SFB1. *N. limnia* has a slower growth rate than *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* EN76, the only pure AOA isolates described to date, but the growth rate is comparable to the growth of marine AOA enrichment cultures. The growth rate only slightly decreased when *N. limnia* was grown under lower-oxygen conditions (5.5 % oxygen in the headspace). Although *N. limnia* was capable of growth at 75 % of seawater salinity, there was a longer lag time, incomplete oxidation of ammonia to nitrite, and slower overall growth rate. Allylthiourea (ATU) only partially inhibited growth and ammonia oxidation by *N. limnia* at concentrations known to completely inhibit bacterial ammonia oxidation. Using electron microscopy, we confirmed the presence of flagella as suggested by various flagellar biosynthesis genes in the *N. limnia* genome. We demonstrate that *N. limnia* is representative of a low-salinity estuarine AOA ecotype and that more than 85 % of its proteins have highest identity to other coastal and estuarine metagenomic sequences. Our findings further highlight the physiology of *N. limnia* and help explain its ecological adaptation to low-salinity niches.

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Introduction

The recent discovery of ammonia-oxidizing archaea (AOA) capable of converting ammonia to nitrite has raised many questions about their physiology and ecology relative to ammonia-oxidizing bacteria (AOB). AOA are found in many environments, including ocean waters, estuaries, salt marshes, sediments, soils, hot springs, hydrothermal vents, caves, corals and sponges, wastewater treatment plants, groundwater, lakes, and rivers. AOA outnumber AOB in many of these environments, often by orders of magnitude (based on quantitative polymerase chain reaction (qPCR) estimates) (e.g., [1] and references therein).

Phylogenetic analysis suggests that different AOA sequence types are found in different habitats; for example, most soil sequences are phylogenetically distinct from marine water column sequences. AOA phylogeny and abundance are often correlated with specific environmental variables (e.g., [2, 3] and references within). A broad-scale biogeographical survey of over 8,000 archaeal ammonia monooxygenase (*amoA*) sequences from GenBank confirmed that different habitats contain distinct AOA populations, providing strong evidence for niche partitioning [Biller et al., in revision].

Physiological studies on AOA cultures are required to further test functional differences between distinct populations of AOA. Such studies have the potential to highlight specific traits selected for different habitats. For instance,

Nitrosopumilus maritimus has a remarkably high affinity for ammonia and appears to be adapted to life under extreme nutrient limitation [4]. *N. maritimus* may therefore be able to outcompete AOB in environments with low ammonia concentrations.

Here, we describe the physiology of a novel, low-salinity-type AOA enriched from the San Francisco Bay estuary, *Candidatus* Nitrosoarchaeum limnia strain SFB1. The *N. limnia* genome revealed features that may be crucial for fitness in the estuarine environment, including a substantial suite of genes for flagellar biosynthesis and chemotaxis and the presence of a large mechanosensitive channel protein involved in protection against osmotic stress [5]. Our aim here was to use the physiological profile of this organism to better understand its potential niche distribution in the environment.

Results and Discussion

Description of the Enrichment

The *N. limnia* enrichment culture was initiated from sediments in San Francisco Bay at site SU001S in the northern part of the estuary [5, 6]. At the time of sampling, salinity was 7.9, temperature was 21.6°C, oxygen was 6.4 mg/L, and sediment C/N ratio was 15.8 (data courtesy of the San Francisco Bay Regional Monitoring Program). Nutrient concentrations in the bottom water just above the sediments were 2 μM ammonia, 14 μM nitrate, and 0.9 μM nitrite. After nearly 4 years of growth, catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) showed that Archaea (Arch915 probe, [7]) accounted for approximately 84 % of cells in the enrichment and the remaining 16 % were Bacteria (Eub338 I–III probes,[8, 9]). The bacterial community contained organisms related to the alpha- and beta-proteobacteria in the Rhodospirillaceae, Methylophilaceae, and Hyphomonadaceae families (based on partial 16S rRNA gene sequences). The *N. limnia* genome was assembled using a combination of single-cell and metagenomic sequence data and then annotated and compared to other AOA genomes [5].

Low-Salinity Estuarine Ecotype

From nearly 2,000 AOA *amoA* sequences from coastal sediments, lakes, and rivers, six distinct ecotypes were identified with strong defining signals from salinity and habitat setting (Biller et al., in revision): (1) high-salinity estuary sites, (2) low-salinity estuary sites, (3) high-salinity surf zone sites, (4) low-salinity surf zone sites, (5) low-salinity lake sites, and (6) high-salinity sites within estuaries, salt marshes, and heathland pools. These ecotypes were based on AdaptML [10] analyses, which define ecologically meaningful phylogenetic groups using an evolutionary hidden-Markov model that identifies populations as groups of related strains sharing a common projected habitat. Almost all of the *amoA* sequences (> 94 %) in the sediments that *N. limnia* was enriched from grouped within the low-salinity estuarine ecotype. The *N. limnia amoA* gene is nearly identical (>99 %) to these sediment sequences and the culture is therefore assumed to be an environmentally relevant representative of this low-salinity estuarine ecotype. Interestingly, quantitative PCR showed that AOA were ~30 times more abundant than AOB in this low-salinity region of the estuary [11], suggesting that the low-salinity estuarine AOA ecotype is able to outcompete AOB in these settings.

Habitat Distribution of *N. limnia*-Like Proteins

The biogeography of the *N. limnia* ecotype was further explored by comparing all predicted proteins identified in the *N. limnia* genome [5] to predicted proteins from metagenomic studies across a wide range of environments. Predicted *N. limnia* proteins were queried against "All Metagenomic ORF peptides" using Blastp in CAMERA [12]. Blastp hits to each *N. limnia* protein were then plotted by habitat type (based on sample metadata provided by CAMERA) (Fig. 1). Over 70 % of the *N. limnia* proteins were most similar to coastally derived sequences (mangroves, estuaries, lagoons, coastal oceans) and another 15 % of the proteins were most similar to freshwater-derived sequences. Of the proteins with a high sequence identity (≥90 %) to *N. limnia*, 92 % were from the coastal ocean, mangroves, and estuaries. Among the proteins involved in ammonia oxidation, carbon cycling, and phosphorus acquisition, 82 % were most similar to coastal and freshwater sequences. Only 11 % of all *N. limnia* proteins were most similar to open ocean-derived sequences, including hypothetical proteins, transcription and translation proteins, radical SAM proteins (involved in biosynthetic pathways with radical-based mechanisms), and others. This is further evidence that open ocean marine AOA are genetically distinct from coastal/estuarine AOA, which likely translates into physiological differences as well. Less than 1 % of the *N. limnia* proteins were most similar to soil-derived sequences, which may in part be due to high sequence divergence between marine/coastal AOA and soil AOA, as well as low representation of soil sequences in the CAMERA database.

Physiology of *N. limnia*

N. limnia enrichment cultures grew chemoautotrophically by aerobic ammonia oxidation to nitrite (Fig. 2). Ammonia concentrations leveled off during late exponential phase and never

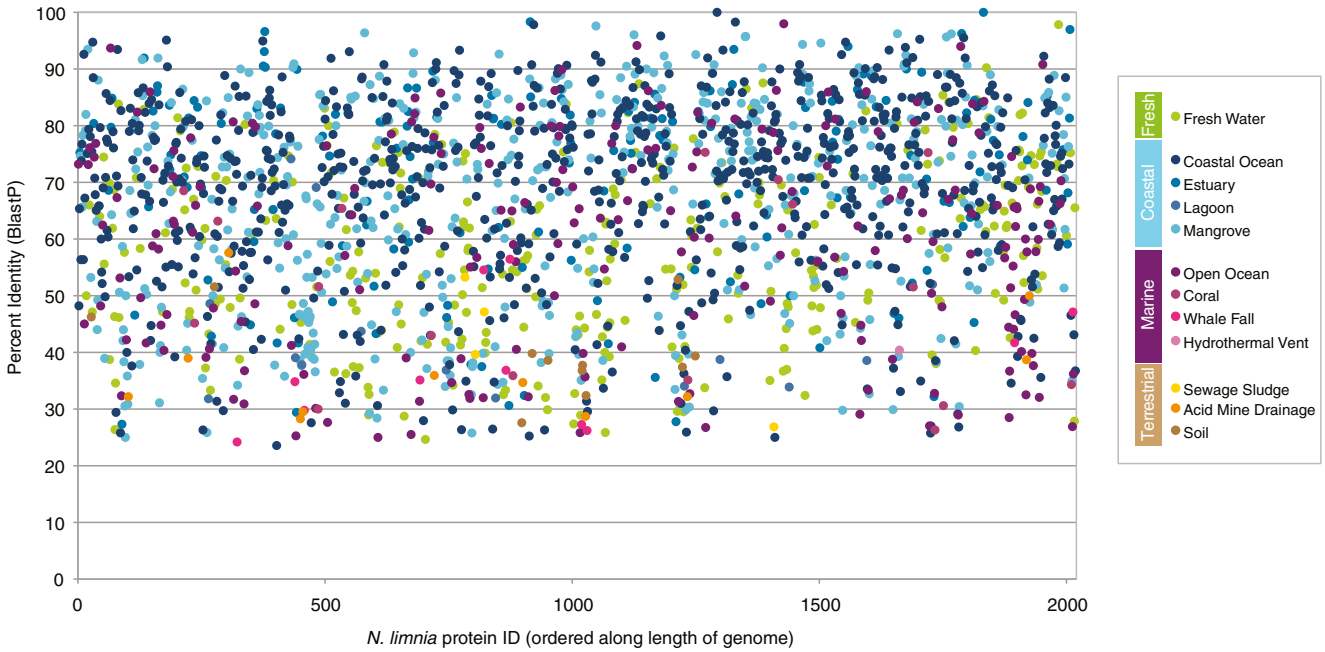

Figure 1 Comparison of proteins predicted in the *N. limnia* genome to predicted proteins from metagenomic studies across different habitats (Blastp analysis of predicted *N. limnia* proteins against all metagenomic ORF peptides in CAMERA database). Sequence hits are color-coded by habitats from which the samples were collected. Predicted *N. limnia* proteins are ordered along the length of the genome (*x*-axis)

decreased below ~100 μM, while nitrite concentrations increased to ~450 μM as expected based on stoichiometric ammonia oxidation. The extra ammonia present in the cultures was likely produced through mineralization of residual organic nitrogen in the culture. Similar anomalous ammonia concentrations have also been reported in the enrichment cultures of the obligate acidophilic AOA, *Nitrosotalea devanaterra* [13]. The incomplete ammonia removal in the *N.*

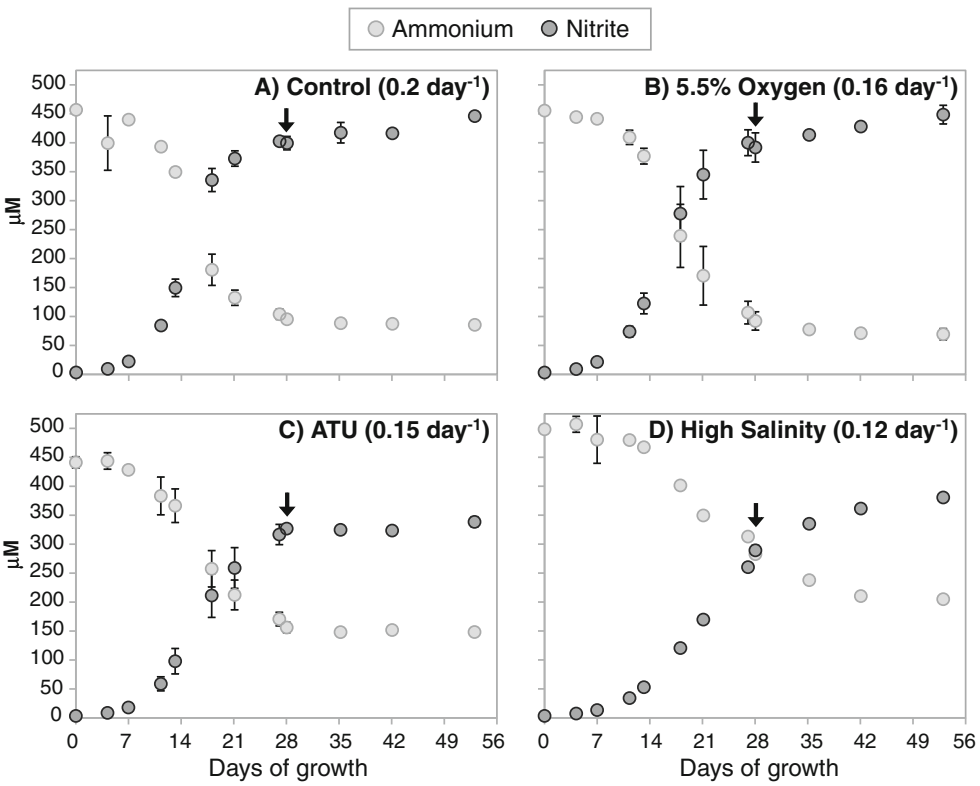

Figure 2 Ammonia oxidation by *N. limnia* enrichment cultures under different growth conditions: **a** standard culture conditions, **b** low oxygen concentration (5.5 %), **c** addition of allylthiourea, an ammonia oxidation inhibitor (172 μM ATU), and **d** high salinity (75 % of seawater salinity). The growth rate is shown in *parentheses* (calculated from the slope of the nitrite production measurements during exponential growth). *Error bars* represent the standard deviation of ammonium and nitrite measurements from triplicate cultures for each treatment (when not visible, error bars are smaller than the *symbol*). Abiotic culture blanks showed no significant change in ammonium or nitrite concentrations. The *black arrow* in each plot represents the time point when the cultures were harvested for RNA extraction

limnia cultures may be due to inhibition of the organisms through (1) buildup of nitrite or (2) ammonia oxidation-induced decreases in pH in the weakly buffered media. Nitrite inhibition and pH changes were not investigated in this study.

The growth rate (calculated from the slope of the nitrite production measurements during exponential growth) of *N. limnia* was 0.2 day^{-1}, corresponding to a doubling time of 3.4 days. *N. limnia* grew at comparable rates to AOA enrichments from the marine water column (0.15–0.17 day^{-1} [14]), but growth was much slower than that of *N. maritimus* (0.65 day^{-1} [4]), *Nitrosocaldus yellowstonii* (0.8 day^{-1} [15]), and enrichments from marine sediments (0.57–0.65 day^{-1} [16]). Interestingly, the generation time of *Nitrososphaera viennensis* EN76 was ~23 days when grown autotrophically but increased substantially to ~45 h when supplemented with pyruvate [17]. Varying culture conditions (e.g., addition of organic carbon, gentle shaking, optimization of temperature and pH) may increase the growth rate and decrease the lag time of *N. limnia*, as seen for *N. viennensis* EN76 [17].

When *N. limnia* was grown under low oxygen availability (average of 5.5 % oxygen in the headspace during the experiment, approximately equivalent to 67 μM), there was only a slight change in the growth rate (0.16 day^{-1}) relative to the control culture (Fig. 2; Fig. S1 of the "Electronic Supplementary Material"). Interestingly, in a separate experiment, no growth was observed after 64 days at 2.4 % oxygen (~29 μM), although growth did occur at intermediate oxygen concentrations of 4.9 % (~59 μM; data not shown). The precise lower limit of oxygen concentration (between 29 and 59 μM) that would support the growth of *N. limnia* is unclear at this time, but the data suggest some capacity to survive under low oxygen conditions. *N. maritimus* showed growth at oxygen concentrations as low as 112 μM [18], but the lower limit that inhibited growth was not reported.

Although the *N. limnia* genotype is most often found in low-salinity environments (based on *amoA* gene surveys), it can also be found in environments with higher salinity (e.g., salt marshes) or environments that experience high salinity at some points during the year. For example, in San Pablo Bay within the San Francisco Bay estuary, salinity fluctuates seasonally depending on the volume of freshwater riverine flow relative to the marine surge. *N. limnia* was enriched at 25 % of seawater salinity and is also capable of growth in freshwater media (data not shown). To test whether *N. limnia* is tolerant of higher salinity, we grew the enrichment culture at 75 % of seawater salinity (salinities commonly seen in estuaries). *N. limnia* grew at high salinity, yet there was a longer lag time, incomplete oxidation of ammonia to nitrite, and slower overall growth rate (0.12 day^{-1}; Fig. 2). Thus, it appears that *N. limnia* is capable of surviving at more marine salinities, presumably an advantageous trait for an estuarine organism, but with hindered growth that may make it susceptible to being outcompeted by other ammonia oxidizers.

In the presence of 172 μM allylthiourea (ATU), a known inhibitor of bacterial ammonia oxidation, the growth rate of *N. limnia* decreased to 0.15 day^{-1} and there was incomplete oxidation of ammonia to nitrite (i.e., nitrite concentrations leveled off at ~330 μM rather than increasing to ~450 μM as seen in the control culture; Fig. 2). Ammonia oxidation by the moderately thermophilic AOA, *Nitrososphaera gargensis*, was also partially inhibited at 100 μM ATU [19]. In marine water column AOA enrichment cultures, ammonia oxidation was partially inhibited at 86 μM ATU and completely inhibited at 860 μM ATU [14]. Variable levels of inhibition of ammonia oxidation were observed in marine water column nitrification rate assays in the presence of 86 μM ATU [20]. ATU concentrations in the range of 86–100 μM are known to completely inhibit AOB [21, 22], and yet AOA appear to be much less susceptible to the inhibitory effects. This may be due to structural (or functional) differences in the ammonia monooxygenase enzyme between AOA and AOB since ATU acts directly on this protein. The use of ATU to inhibit nitrification in marine and terrestrial studies should be reconsidered.

Nitrous Oxide Production

Nitrous oxide (N_2O) is a highly potent greenhouse gas and is currently the most important contributor to stratospheric ozone depletion [23]. N_2O concentrations in the atmosphere from natural and anthropogenic sources may be rising at unprecedented rates [24]. Ammonia oxidizers are one of the primary contributors to natural N_2O emissions ([25] and references therein). Under well-oxygenated conditions, AOB produce N_2O at low rates as a by-product of ammonia oxidation. At low oxygen concentrations, AOB reduce nitrite to N_2O via nitrite reductase (Nir) and nitric oxide reductase (Nor) proteins in a process called nitrifier denitrification [26]. Most AOB studied to date have genes coding for the copper-containing form of nitrite reductase (*nirK*) [27].

It appears that many AOA also contain *nirK* genes, providing a potential pathway for N_2O production in AOA. While absent in *Cenarchaeum symbiosum*, archaeal *nirK* genes have been identified in *N. maritimus*, as well as in soils, oceans, stream biofilms, and lakes [28–31] [32]. Walker et al., [31] proposed that *nirK* might play a role in electron transfer in *N. maritimus*. One *nirK* gene (Nlim_1007) was identified in the *N. limnia* genome, based on analysis of the conserved metal-binding residues within multicopper oxidases, as described by Bartossek et al., [30]. The *N. limnia nirK* sequence shares only 83 and 85 % identity to the two *N. maritimus nirK* sequences at the amino acid level. Using degenerate PCR primers targeting archaeal *nirK* genes, a number of *N. limnia*-like *nirK* genes

were recently detected in sediments from North San Francisco Bay [32].

Santoro et al. [33] recently showed for the first time that AOA are capable of producing N_2O, using physiological and isotopic studies of marine AOA enrichment cultures. *N. maritimus* has also been shown to produce N_2O [18], the first direct evidence for N_2O production in a pure culture of AOA. This culture-based evidence, along with environmental data showing a correlation of high AOA abundance with high nitrification rates [20] and N_2O concentrations [18] in the upper ocean, suggests that AOA likely contribute a significant portion of oceanic N_2O production [33].

We measured N_2O gas production from *N. limnia* enrichment cultures (Fig. 3). N_2O concentrations increased more than 3.5-fold over the 53-day experiment. N_2O production occurred concurrently with ammonia oxidation (Figs. 2 and 3), and the amount of N_2O produced was positively correlated with growth rates under the different culture conditions (r=0.73). The N_2O yield for archaeal ammonia oxidation ranged from 0.35 to 0.61 nmol N_2O–N produced per μmol NO_2^- produced across the different culture conditions. These yields are comparable to the N_2O yields observed within marine AOA enrichments (average of 0.41 nmol N_2O–N produced per μmol NO_2^- produced) [33] and *N. maritimus* (0.002–0.026 % based on the ratio of N_2O/NH_4^+) [18]. Interestingly, N_2O concentrations were higher in the low-oxygen treatment, increasing by 12.8-fold over the experiment. Increased N_2O production under low oxygen availability has also been reported for *N. maritimus* [18] and AOB [34, 35]. Because the enrichment culture contains approximately 10–20 % bacteria, production of N_2O specifically by *N. limnia* cannot be confirmed. If *N. limnia* does indeed produce N_2O, it would suggest that AOA may be a significant source of N_2O production in low-salinity environments such as the northern San Francisco Bay estuary where AOA are the dominant ammonia oxidizers [11].

Expression of Genes Putatively Involved in Ammonia Oxidation

In addition to the proteins traditionally expected to be involved in archaeal ammonia oxidation, it has been suggested that nitrite reductase may be directly involved in energy production from ammonia oxidation [31]. The expression of *N. limnia amoA* and *nirK* genes was evaluated under each culture condition: control, 5.5 % oxygen, high salinity, and in the presence of the nitrification inhibitor ATU (Fig. 4). RNA was extracted from the cultures at the end of exponential phase and converted to cDNA, and quantitative PCR was used to estimate mRNA copy numbers. There was a strong correlation between transcript copy numbers of *amoA* and *nirK* across the different culture conditions (r=0.996). Transcript copy numbers of *nirK* were on average 15 times higher than of *amoA*. This may be due to differences in the qPCR assay conditions (e.g., reaction efficiency) or to different biological mechanisms acting on each gene (e.g., transcript stability and turnover). High levels of

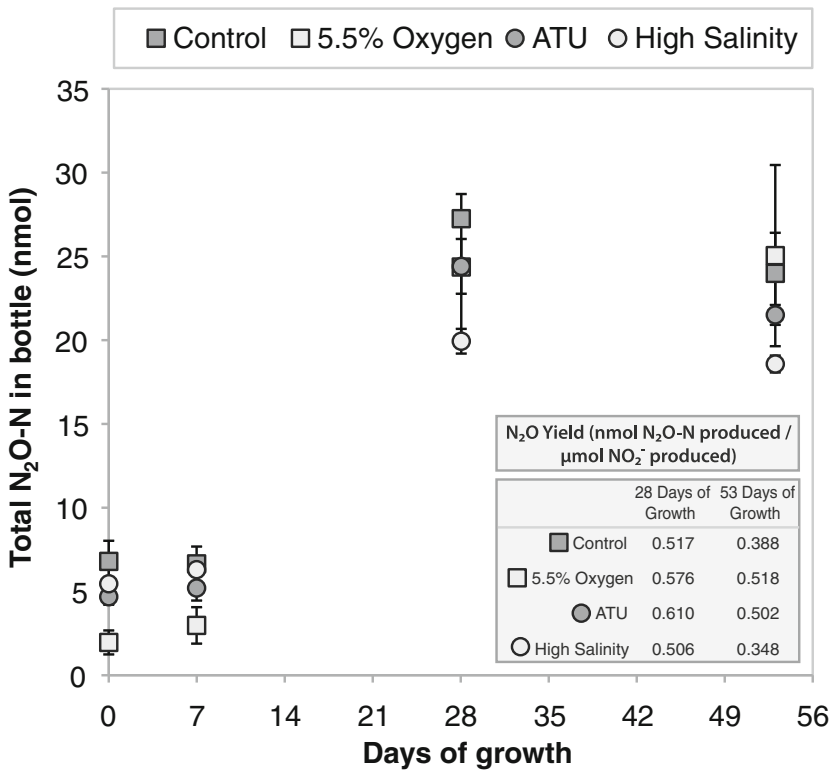

Figure 3 Nitrous oxide gas production from *N. limnia* enrichment cultures grown under different conditions. *Error bars* represent the standard deviation of nitrous oxide concentrations from triplicate cultures for each treatment (when not visible, *error bars* are smaller than the *symbol*). The *inset* shows the N_2O yield under each culture condition (nmol N_2O-N produced/μmol NO_2^- produced) after 28 and 53 days of growth. Abiotic culture blanks showed no significant change in nitrous oxide concentrations

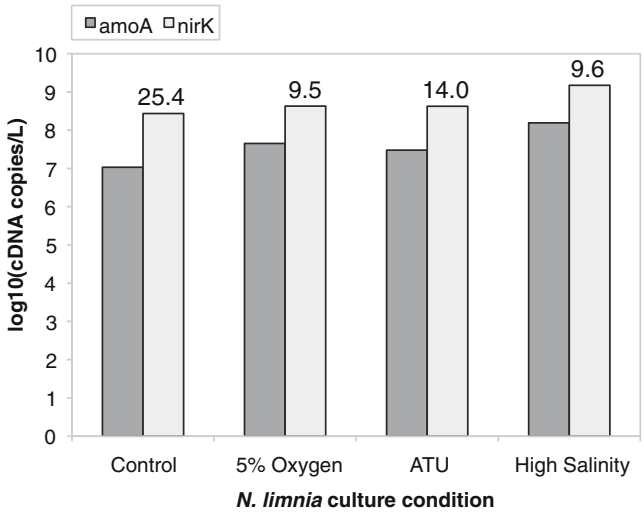

Figure 4 Abundance of expressed archaeal *amoA* (*dark gray*) and archaeal *nirK* (*light gray*) genes per liter of *N. limnia* enrichment culture under different growth conditions, based on qPCR analysis of cDNA. The ratio of *nirK*/*amoA* cDNA copies is shown *above the bars*. Filters from replicate cultures were pooled prior to RNA extraction

nirK expression were also detected in Georgia coastal samples [36] and in the water column in Monterey Bay [32]. Expression of *nirK* was also seen in soils, although expression did not seem to correlate with N_2O production [30].

Motility

The *N. limnia* genome contains over 38 genes associated with motility, including those required for Flp pilus assembly, flagellar assembly, and chemotaxis [5]. More than 50 % of the motility genes identified in the genome were found on one gene cassette (Fig. 5) containing 13 genes associated with chemotaxis, followed by eight genes associated with archaeal flagella. The other seven non-motility genes within the cassette have no annotated function; however, five genes contained signal peptide or transmembrane domains (identified by InterProScan [37]). It is possible that these proteins are also involved in motility or chemotaxis.

The presence of flagella was confirmed by transmission electron microscopy of *N. limnia* (Fig. 6). Because >85 % of the cells in the enrichment are AOA and the majority of cells viewed by transmission electron microscopy (TEM) had uniform morphology with flagella, these data support the notion that *N. limnia* has a flagellum. The cells appeared as straight rods with an average diameter of 0.24 μm and length of 0.77 μm (range 0.19–0.27×0.55–1.00 μm). *N. limnia* cells were similar in size and shape to *N. maritimus* (0.17–0.22×0.5–0.9 μm) [38].

Among the cultivated AOA and the available AOA genome sequences, motility appears to be a distinctive trait of *N. limnia*. *N. limnia* may use motility as an adaptive response to varying substrate and/or oxygen concentrations in the surface sediments of the estuary. Interestingly, in the Blastp comparison against all metagenomic proteins (Fig. 1), all of the *N. limnia* flagellar proteins, both pilus proteins, and 54 % of the chemotaxis proteins were most similar to sequences from mangroves. We anticipate that motile AOA will also be discovered in other sedimentary environments.

Genetic Potential for Phosphorus Uptake

The *N. limnia* genome contains genes for phosphorus acquisition using the high-affinity inorganic phosphate-specific transporter (Pst) system. The Pst system [39, 40] includes proteins that transfer inorganic phosphorus through the inner membrane (PstA and PstC; Nlim_0923 and Nlim_0924), an ATPase that energizes transport (PstB; Nlim_0922), a periplasmic inorganic phosphorus-binding protein (PstS; Nlim_0927), and an uptake regulator (PhoU; Nlim_0921, Nlim_1362, Nlim_1441, and Nlim_0925). *N. limnia* does not appear to have the sensor or response regulator proteins in the Pst system (PhoR and PhoB). *N. limnia* may also have the ability to use the low-affinity inorganic phosphate-specific transporter (Pit) system. The Pit system consists of a single membrane protein (putatively Nlim_0661 or Nlim_0662) and is the preferential transport system when inorganic phosphorus is in excess [39, 41]. Unlike *N. maritimus* [31], the *N. limnia* genome has no genes for transport of organic phosphorus. Although the northern region of San Francisco Bay where *N. limnia* was isolated has lower phosphate concentrations (2.7 μM on average) than the rest of the estuary [42], phosphorus does not limit phytoplankton productivity [43, 44]. Thus, *N. limnia* may simply not require the utilization of organic phosphorus because inorganic phosphorus is in excess.

Materials and Methods

Cultivation of Ammonia-Oxidizing Archaea

Enrichment cultures of *Candidatus* Nitrosoarchaeum limnia SFB1 were maintained as previously described [5]. Briefly, the cultivation media contained (per liter) 500 μM ammonium chloride, 1 mL selenite/tungstate solution, 1 mL vitamin solution [45], 1 mL sodium bicarbonate (1 M), 10 mL KH_2PO_4 (4 gL^{-1}), 1 mL trace metals [46], and 988 mL artificial seawater (containing 24.7 gL^{-1} $MgSO_4$, 2.9 gL^{-1} $CaCl_2$, 35.1 gL^{-1} NaCl, and 1.5 gL^{-1} KCl) diluted to ~25 % of seawater salinity.

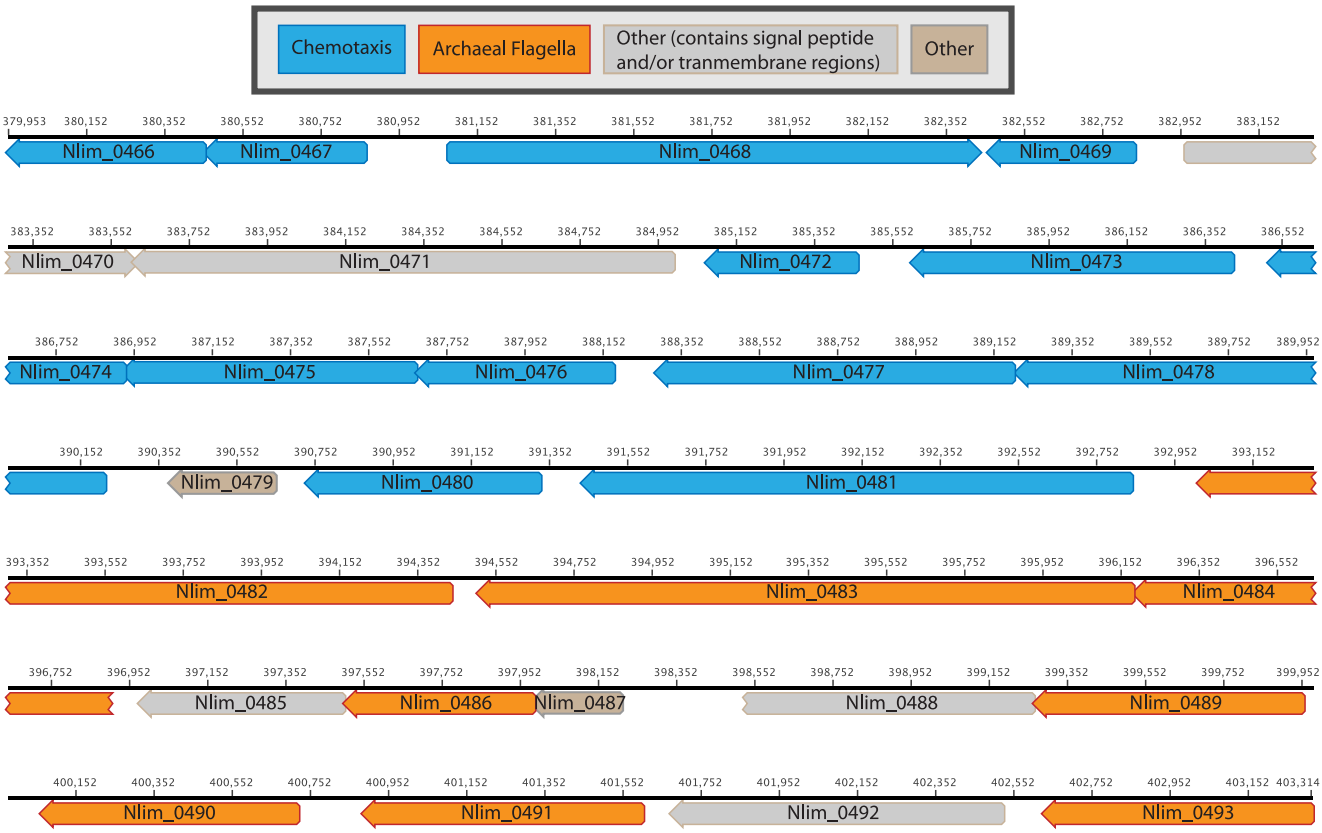

Figure 5 Motility gene cassette on the *N. limnia* genome. Chemotaxis-associated genes are colored *blue* and flagellar-associated genes are colored *orange*. Other genes with signal peptides or transmembrane regions annotated in the peptide sequence are colored *gray*

Fluorescence In Situ Hybridization

For in situ hybridization, cultures were fixed in 2 % formaldehyde and filtered onto 0.2-μm polycarbonate membranes (Millipore). CARD-FISH [47] was carried out with horseradish peroxidase-labeled probes Arch915 [7] and EUB338 I-III [8, 9]. Filters were treated with lysozyme or proteinase K for permeabilization of bacteria and archaea, respectively.

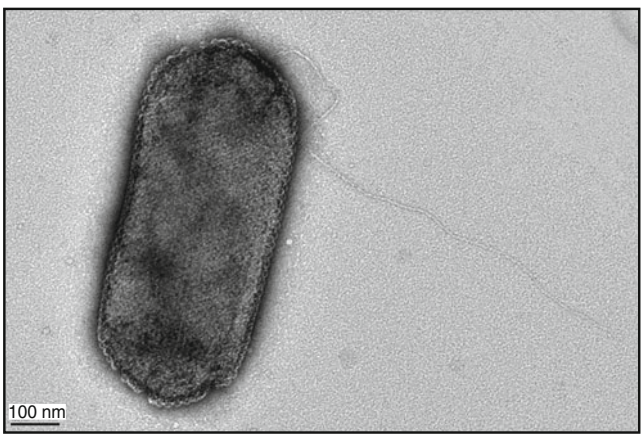

Figure 6 Transmission electron micrograph of a typical flagellated cell from the *N. limnia* enrichment

Physiology Experiments

AOA enrichment cultures were grown in sealed serum vials in the dark at room temperature. The AOA were grown under different treatment conditions: high-salinity media, varying oxygen concentrations, and with the addition of 172 μM ATU (an ammonia oxidation inhibitor). Each treatment included three identical replicates. The high-salinity media was made as described above using artificial seawater diluted to ~75 % of seawater salinity. The freshwater media was made as described earlier except that minimal salts were added instead of artificial seawater (1 gL^{-1} NaCl, 0.4 gL^{-1} $MgCl_2{\cdot}6H_2O$, 0.1 gL^{-1} $CaCl_2{\cdot}2H_2O$, and 0.5 gL^{-1} KCl, as described by [15]). The *N. limnia* enrichment culture was inoculated into the media at 1 % volume ratio.

Two milliliters of the culture was collected periodically for nutrient analysis and frozen at −20°C. Nitrite and ammonium concentrations were measured on a QuikChem 8000 Flow Injection Analyzer (Lachat Instruments) or SmartChem 200 Discrete Analyzer (WestCo Scientific Instruments). Three milliliters of headspace was sampled periodically to measure oxygen

and nitrous oxide gases using a Shimadzu 2014 Gas Chromatograph. Growth rates were estimated from the slope of the nitrite production measurements during exponential growth.

cDNA Synthesis and Transcript Quantification

After 29 days of growth, cells were harvested from the enrichment culture by filtration and immediately stored at −80°C. Total RNA was extracted using a mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions, except for the addition of a bead-beating step (10 min of vortexing at full speed). Filters from two to three replicate cultures for each treatment were pooled and extracted together. Genomic DNA was removed from the product using the Turbo DNA-free kit (Ambion) and confirmed by PCR. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers according to manufacturers' instructions, except for extending the reverse transcription step to 4 h. Archaeal *amoA* transcripts were quantified by qPCR using previously described methods [11]. Archaeal *nirK* transcripts were quantified using the primers AnirKa-58 F and AnirKa-579R as described in [32].

For both archaeal *amoA* and archaeal *nirK*, qPCR reactions were carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Plasmids containing cloned archaeal *amoA* or archaeal *nirK* PCR amplicons were used as standards. Standard curves spanned a range from 31 to 6.2×10^5 copies per reaction for the *amoA* assay and from 14 to 2.8×10^5 copies per reaction for the *nirK* assay. All sample and standard reactions were performed in triplicate and an average value was calculated. Melting curves were generated after each assay to check the specificity of amplification. qPCR assay efficiencies were 91 % for archaeal *amoA* and 71 % for archaeal *nirK*. Correlation coefficients (R^2) of the standard curves for both assays averaged >0.99.

Electron Microscopy

TEM was performed at the Cell Sciences Imaging Facility at Stanford University. For TEM with negative staining, 10-µl samples (unfixed or fixed in 4 % PFA with 2 % glutaraldehyde in 0.1 M Na cacodylate, pH 7.3) were spotted onto glow-discharged formvar-coated 100 mesh copper TEM grids and allowed to settle for 5 min. Grids were then stained with 1 % uranyl acetate for 2 min and dried overnight before visualization with a JEOL 1230 TEM, operated at 80 kV.

Fragment Recruitment

Protein sequences identified in the *N. limnia* genome [5] were blasted against the "All Metagenomic ORF peptides" database in CAMERA [12]. Blastp searches in CAMERA used the NCBI default blastall parameters with one database alignment per query. Blast hits to each *N. limnia* protein were then plotted by habitat type (based on sample metadata provided by CAMERA).

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