Single cell genomic and transcriptomic evidence for the use of alternative nitrogen substrates by anammox bacteria

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Abstract

Anaerobic ammonium oxidation (anammox) contributes substantially to ocean nitrogen loss, particularly in anoxic marine zones (AMZs). Ammonium is scarce in AMZs, raising the hypothesis that organic nitrogen compounds may be ammonium sources for anammox.

Biochemical measurements suggest that the organic compounds urea and cyanate can support anammox in AMZs. However, it is unclear if anammox bacteria degrade these compounds to ammonium themselves, or rely on other organisms for this process. Genes for urea degradation have not been found in anammox bacteria, and genomic evidence for cyanate use for anammox is limited to a cyanase gene recovered from the sediment bacterium Candidatus Scalindua profunda. Here, analysis of Ca. Scalindua single amplified genomes from the Eastern Tropical North Pacific AMZ revealed genes for urea degradation and transport, as well as for cyanate degradation. Urease and cyanase genes were transcribed, along with anammox genes, in the AMZ core where anammox rates peaked. Homologs of these genes were also detected in metagenomic datasets from major AMZs in the Eastern Tropical South Pacific and Arabian Sea. These results suggest that anammox bacteria from different ocean regions can directly access organic nitrogen substrates. Future studies should assess if and under what environmental conditions these substrates contribute to the ammonium budget for anammox.

Keywords: Candidatus Scalindua, urease, cyanase, ammonium, oxygen minimum zone, nitrogen cycle
Introduction

Anaerobic ammonium oxidation (anammox) plays a major role in aquatic nutrient cycling. In this microbial process, autotrophic bacteria oxidize ammonium with nitrite, producing energy for CO₂ fixation and cellular growth, and N₂ as an end product. Anammox is therefore a nitrogen sink, converting bioavailable nitrogen to a gaseous form unavailable to most organisms. Anammox is particularly important in anoxic marine zones (AMZs) (Thamdrup et al., 2006; Dalsgaard et al., 2012; Ulloa et al. 2012). In the major AMZs of the Eastern Tropical Pacific and Arabian Sea, dissolved oxygen is below detection (<10 nM), nitrite is abundant (often > 5 μM), and anammox combined with heterotrophic denitrification drive nitrogen loss, with ~20-40% of ocean nitrogen loss occurring in AMZs (Codispoti et al., 2001; Sabine et al., 2004; Thamdrup et al., 2012; Tiano et al., 2014; Ganesh et al., 2015). In these systems, anammox bacteria increase in abundance at anoxic depths, where ammonium is supplied by the mineralization of organic matter, yet concentrations are low and turnover and competition for this resource are high (Woebken et al., 2008; Kalvelage et al., 2013). Under these conditions, anammox bacteria may be under pressure to use alternative substrates as ammonium sources, or potentially to use energy and biomass production pathways other than anammox. Indeed, in experimental studies of anammox in AMZs, the direct use of organics as a source of ammonium by anammox bacteria was proposed as an explanation for higher anammox rates in incubations with ¹⁵N-nitrite compared to those with ¹⁵N-ammonium, as the former would integrate N₂ production from anammox based on all ammonium sources (Nicholls et al., 2007; De Brabandere et al., 2014). However, the metabolic versatility of marine anammox bacteria remains largely unknown. This is due in part to limited genomic characterizations of anammox bacteria from diverse marine habitats, including AMZs.
Anammox has thus far been described only in bacteria of the Order Brocadiales in the phylum Planctomycetes. This Order occurs globally in natural and man-made environments in both fresh and saltwater. No Brocadiales bacteria have yet been isolated in pure culture. The known diversity of this group is distributed across the *Candidatus* genera Brocadia, Kuenenia, Anammoxoglobus, Jettenia, and Scalindua. Of these, *Ca. Scalindua* is the dominant genus in ocean habitats, including sediments and AMZs (Woebken *et al.*, 2008; Villanueva *et al.*, 2014), but has also been found in freshwater (Sonthiphand *et al.*, 2014). Insight into the genomic potential of *Ca. Scalindua* is based on metagenomic contigs of *Ca. S. profunda* from marine sediment (van de Vossenberg *et al.*, 2013), *Ca. S. brodae* from a wastewater plant (Speth *et al.*, 2015), and *Ca. S. rubra* from a marine brine pool (Speth *et al.*, 2017). These large genomes (>4000 genes; ~4-5.2 Mbp) contain many genes absent from characterized genomes of other anammox genera, but also vary in gene content among species. For example, of these three species, only *Ca. S. rubra* contains genes for gas vesicle biosynthesis, presumably as an adaptation for regulating position in brine pools. However, genomes of *Ca. Scalindua* cells from AMZs have not yet been reported. Our knowledge of gene content in *Ca. Scalindua* from AMZs is based on recruitment of meta-omic sequences to non-AMZ genomes, e.g., of *Ca. Scalindua profunda* (van de Vossenberg *et al.*, 2013; Ganesh *et al.*, 2015; Luke *et al.*, 2016). We therefore have limited understanding of how anammox bacteria may be adapted to AMZ conditions.

The waste product urea (CO(NH$_2$)$_2$) and its breakdown product cyanate (OCN$^-$) are potential alternative substrates for anammox bacteria. Urea is ubiquitous in ocean waters, originating from microbial degradation of dissolved organic matter and nitrogenous waste from microbes and
animals (Zehr and Ward, 2002). Diverse microorganisms produce urease enzymes that
hydrolyze urea to ammonia and CO₂, potentially to aid pH regulation or to acquire ammonia for
biomass production or energy generation (Konieczna et al., 2012). Ureases have even been
found in aerobic ammonia-oxidizing bacteria (Burton and Prosser, 2001) and archaea (Hallam et
al., 2006; Qin et al., 2014) as well as nitrite-oxidizing bacteria (Koch et al., 2015), suggesting
that organic nitrogen plays a role in nitrification. Indeed, certain aerobic ammonia-oxidizing
bacteria not only oxidize ammonia derived from urea, but also assimilate the CO₂ resulting from
urease activity (Marsh et al., 2005). Recently, anammox bacteria from the Eastern Tropical
South Pacific (ETSP) AMZ were shown to produce N₂ from added urea, but only after a lag of
1.5 days (Babbin et al., 2017). This was interpreted as evidence that anammox bacteria do not
degradate urea directly but instead rely on the urealytic activity of other organisms or on abiotic
urea degradation to supply ammonium. An inability of anammox bacteria to directly degrade
urea is supported by the absence of urease-encoding genes (ure) in available anammox genomes.

In contrast to urea, cyanate addition stimulated N₂ production by anammox without a lag phase
in incubations of AMZ water (Babbin et al., 2017). This suggests that AMZ anammox bacteria
might use cyanate directly, presumably though conversion to ammonia and CO₂ by a cyanase
enzyme, with the resulting ammonium used for anammox. Although absent from draft genomes
of other Ca. Scalindua species (Speth et al., 2017), a putative cyanase-encoding gene (cynS) is
present in the metagenome of Ca. S. profunda from sediment (van de Vossenberg et al., 2013),
and sequences related to this gene were detected in AMZ metagenomes and metatranscriptomes
(Babbin et al., 2017). However, data conclusively linking cyanases to anammox bacterial
genomes from AMZs are not yet available, and it is therefore unknown if these bacteria might
also rely on other microbes for cyanate degradation, as has been shown for certain aerobic ammonia oxidizers (Palatinszky et al., 2015).

Here, we explored the metabolic properties of *Ca*. Scalindua sp. from a marine AMZ, testing the hypothesis that these bacteria have the potential for directly catabolizing organic nitrogen substrates as ammonium sources for anammox. We explored this hypothesis using genomes of *Ca*. Scalindua cells from the Eastern Tropical North Pacific (ETNP) AMZ off Mexico. These genomes were then analyzed in conjunction with ETNP chemical concentration, anammox rate, and metatranscriptome data from a cruise in 2014. The results provide insight into the genetic basis for environmental variation and adaptation in this globally important lineage.

**Materials and Methods**

*Sample collection*

Samples for single amplified genome (SAG) analysis were collected in 2013 from station 6T (18° 54.0N, 104° 54.0W; Figure S1) in the ETNP AMZ during the Oxygen Minimum Zone Microbial Biogeochemistry Expedition (OMZoMBiE) cruise (*R/V New Horizon*; 13-28 June). Seawater for cell sorting and SAG sequencing was collected from the secondary nitrite maximum (125 m) and AMZ core (300 m) using Niskin bottles on a rosette containing a Conductivity-Temperature-Depth profiler (Sea-Bird SBE 911plus). From each depth, triplicate 1 ml samples of bulk seawater (no pre-filtration) were aliquoted into sterile cryovials and 100 µl of a glycerol TE stock solution (20 ml 100X TE pH 8.0, 60 ml deionized water, 100 ml glycerol) was added to each vial. The vials were then mixed and frozen at -80°C.
Samples for metatranscriptome analysis and measurements of anammox rates were collected from the ETNP AMZ during a second OMZoMBiE cruise, in 2014 (R/V New Horizon; 10 May – 8 June, 2014). Water was collected at six stations spanning a coastal to offshore gradient (Figure S1). Stations and depths sampled for metatranscriptomics (n=21) are in Table S1. Eight of the 21 metatranscriptome datasets were generated in this study; the remainder were generated in two prior studies (Padilla et al., 2016; Garcia-Robledo et al., 2017) and re-analyzed here. Seawater was collected by Niskin with microbial biomass then collected by in-line filtration of seawater (~1.5-2.5 L) through a glass fiber disc prefilter (GF/A, 47 mm, 1.6 µm pore-size, Whatman) and a primary collection filter (Sterivex™, 0.22 µm pore-size, Millipore) using a peristaltic pump. Sterivex™ filters were filled with RNA stabilizing buffer (25 mM sodium citrate, 10 mM EDTA, 5.3M ammonium sulfate, pH 5.2), flash-frozen in liquid nitrogen, and stored at -80°C. Approximately 15-45 min elapsed (depending on depth) between capture in the Niskin and arrival on deck; approximately 20 min elapsed between water retrieval from the Niskin and fixation of filters in buffer.

Ammonium concentrations were determined fluorometrically aboard ship using the orthophaldialdehyde method (Holmes et al., 1999), with a detection limit of 10 nM. Samples for measuring nitrite concentrations were collected in acid-cleaned HDPE bottles and stored frozen until spectrophotometric measurement using the Griess method (Grasshoff et al., 1983) with a Westco SmartChem 200 (Unity Scientific). On a cruise to the study area in 2017 (R/V Oceanus cruise OC1705), urea concentrations were determined fluorometrically following Mulvenna and Savidge (1992) with a 5 cm cuvette. This method has a detection limit of 45 nM.
Anammox rates were measured for 14 of the 21 water samples from which metatranscriptomes were generated (Figures 1, S2, Table S1). Water was sampled directly from the Niskin and transferred to 250 ml glass bottles without pre-filtration. Bottles were overflowed (three volume equivalents) and sealed without bubbles with deoxygenated butyl rubber stoppers to minimize oxygen contamination (following De Brabandere et al., 2014). Within 6 hours of collection, each bottle was amended with 5 µM $^{15}$NH$_4^+$, and purged with helium for ~20 min. With a slight overpressure, water was dispensed into 12 ml exetainers (Labco, Lampeter, Ceredigion, UK), which were immediately capped with deoxygenated lids. Headspaces of 2 ml were introduced into each exetainer and flushed twice with helium, with shaking between flushings. Exetainers were then incubated in the dark at in situ temperature (13°C) for 24 hours. For each sample, triplicate exetainers were preserved with 100 µl of 50% (w/v) ZnCl$_2$ at the start of the incubation and again after 24 hours.

Production of $^{14}$N$^{15}$N and $^{15}$N$^{15}$N was determined on a gas chromatography isotope ratio mass spectrometer (GC-IRMS) as in Dalsgaard et al. (2012). Rates of N$_2$ production by anammox were calculated as in Thamdrup and Dalsgaard (2002) from the slope of the linear regression of $^{14}$N$^{15}$N with time. T-tests were applied in all cases to determine whether rates were significantly different from zero (p<0.05).

SAG generation and taxonomic screening

SAGs were generated from individual bacterial cells according to the Department of Energy Joint Genome Institute workflow following Rinke et al. (2013, 2014) with minor modifications.
Cells were sorted on a BD Influx (BD Biosciences) and treated with Ready-Lyse lysozyme (Epicentre; 5U/μL final conc.) for 15 min at room temperature prior to adding lysis solution. Whole genomes were amplified by multiple displacement amplification (MDA) using the REPLI-g Single Cell Kit (Qiagen), with final reaction volumes of 2 L and termination after 6 hours. The taxonomic identity of each SAG was determined by PCR amplification and Sanger sequencing of a ~470 bp region of the 16S rRNA gene using primers 926wF (5’-AAACTYAAAKGAATTGRCGG- 3’) and 1392R (5’-ACGGGCGGTGTGTRC- 3’) for archaea and bacteria. Recovered sequences (average length: 423 bp) were classified using MOTHUR's 'classify_seq' against the Greengenes database, with the probability of correct assignment to a taxonomic group calculated using the naïve Bayesian classifier method (Wang et al., 2007).

SAG sequencing

20 SAGs classified with high confidence as belonging to the genus *Ca. Scalindua* were selected for genome sequencing. These included 9 and 11 SAGs from 125 m and 300 m, respectively. Indexed DNA sequencing libraries were prepared using the Nextera XT DNA Library Prep kit (Illumina, San Diego, CA, USA) following manufacturer instructions, pooled, and sequenced on an Illumina MiSeq using a v2-500 cycle (paired end 250x250 bp) kit.

RNA extraction and cDNA sequencing

RNA was extracted from Sterivex™ filters as in Ganesh *et al.* (2015) using a modification of the mirVana™ miRNA Isolation kit (Ambion). Filter cartridges were thawed on ice, and RNA stabilizing buffer was expelled by syringe from each cartridge and discarded. Cells were lysed by
adding Lysis buffer and miRNA Homogenate Additive (Ambion). Following vortexing and incubation on ice, lysates were transferred to RNAase-free tubes and RNA extracted by acid phenol:chloroform according to the kit. The TURBO DNA-free™ kit (Ambion) was used to remove DNA and the extract purified using the RNeasy MinElute Cleanup Kit (Qiagen). RNA was prepared for sequencing using the ScriptSeq™ v2 RNA-Seq Library preparation kit (Epicentre). cDNA was synthesized from fragmented total RNA (rRNA not removed) using reverse transcriptase and amplified and barcoded using ScriptSeq™ Index PCR Primers (Epicenter) to generate single-indexed libraries. cDNA libraries were pooled and sequenced on an Illumina MiSeq using a v2-500 cycle (paired end 250x250 bp) kit.

*SAG assembly, quality control, and sequence analysis*

Illumina reads were filtered for quality using a Phred score cutoff of 25 and trimmed using TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). High quality paired reads were merged using FLASH (Magoc and Salzberg, 2011). Quality-trimmed merged and unmerged reads were combined and assembled using the SPAdes assembler (Bankevich *et al.*, 2012) with k-mer sizes of 21,33,55,77,99,127, and the single-cell (-sc) option. Coding sequences were predicted using GeneMark.hmm (Lukashin and Borodovsky, 1998), and 16S rRNA gene sequences were identified using RNAmmer (Lagesen *et al.*, 2007), both using default parameters. Percentage of contamination and genome completeness were assessed based on detecting lineage-specific marker genes using CheckM (Parks *et al.*, 2015).

Full-length (>1500 bp) 16S rRNA gene sequences were detected on 8 SAGs. These sequences were imported into the ARB environment (Ludwig *et al.*, 2004) and placed within the ARB.
backbone tree using the parsimony tool. Brocadiales-associated 16S rRNA genes from Woebken et al. (2008) and Galan et al. (2009) were imported for comparative purposes to assign SAG 16S rRNA genes to previously reported sub-clades of marine Ca. Scalindua. Additional sequences from Schmid et al. (2003), representing species-level Candidatus Scalindua designations, were also included as outgroups to the ‘Arabian Sea’ sequence cluster (see Results below). Sequence alignments were created using the automated aligner, then manually curated when needed. To assess the 16S rRNA gene phylogeny using only informative positions, a mask was created based on the curated alignment, and used for construction of Neighbor-Joining (with Feldstein correction), Maximum Likelihood (with LG substitution model), and Parsimony trees with 1000 bootstraps for all models.

All SAG-associated assemblies generated from MDA products were analyzed using Prokka (Seemann, 2014). The ‘faa’ files from this pipeline were used as queries for BLASTP searches against public and custom databases (described below). For visualization of gene order and synteny, contigs with features of interest were extracted from the ‘gbk’ files from Prokka. Contigs of interest were then imported into ‘EasyFig’ and compared to one another using BLASTN. The associated output figures were manually curated in Adobe Illustrator. The package T-REKs (Jorda and Kajava, 2009) was used to identify tandem repeats on contigs of interest.

Predicted amino acid sequences from 6 SAGs were used to create a composite SAG database for comparison against public databases using BLASTP and for use as a reference database for BLASTX-based analyses of metagenomes and metatranscriptomes (described below). Our goal
in creating this database was to capture the majority of functional gene content across the SAGs (related to one another at roughly the species level; see Results), rather than to resolve population-level variation among the SAGs. The 6 SAGs were chosen because they had relatively high completeness (28.2-50.0%), minimal contamination (<5%), and full-length 16S rRNA gene sequences. SAG sequences were clustered (using UCLUST and USEARCH commands) at 50% amino acid identity (AAI). Clustering yielded 2703 proteins, including 49 of the 54 universally conserved single-copy ribosomal proteins (Yutin et al., 2012). This result suggested that the composite database was ~91% complete (based only on universal marker gene recovery) and that a complete composite database would contain ~3000 protein-coding genes (50% AAI clusters), roughly consistent with genome size estimates based on individual SAGs (extrapolated using estimated genome completeness and counts of detected genes per SAG; Table 1).

A custom database of protein sequences from anammox taxa was created for comparison to the SAG gene set. Anammox-associated genomes were identified in NCBI using the search term ‘Brocadiales’. All amino acid sequences (n=50,272) from these genomes were downloaded and combined with amino acid sequences (n=4330) from Ca. Scalindua profunda (obtained according to (van de Vosseneng et al., 2013)), yielding a database of 54,602 proteins. SAG proteins were then queried against this database via BLASTP. A sequence was considered unique to the SAG set if this query did not return a significant match (bit score > 50) to a database sequence. Using this method, all urea-associated proteins (urease and accessory proteins) were identified as unique to the ETNP SAG set, along with 1803 other genes (1811
total unique). Of the other genes unique to ETNP SAGs, 1604 encoded hypothetical proteins.

Non-hypothetical, unique protein-coding genes are listed in Table S2.

To further assess relatedness among SAGs, average nucleotide identity (ANI) and average amino acid identity (AAI) between each pair of SAGs was calculated using the ani.rb and aai.rb scripts from the enve-omics toolkit (Rodriguez-R and Konstantinidis, 2016) with ani.rb cutoffs of 700 bp minimum alignment length and 70% minimum identity, and aai.rb cutoffs of bit score > 50 and 90% minimum alignment length (as a fraction of the shorter sequence). The get_homologues package (Contreras-Moreira and Vinuesa, 2013) was used to identify genes shared between each SAG and protein-coding sequences in the Ca. Scalindua profunda genome.

Metatranscriptome analysis

Metatranscriptomic reads were trimmed and merged as above for SAG sequences. Merged reads were compared to the SILVA rRNA database using BLASTN, and sequences with significant matches to rRNA genes were identified and removed. Non-rRNA reads were queried (BLASTX) against the composite database (see above). BLAST output was parsed to identify transcripts recruiting to SAGs with bit score >50 and AAI >95%. Counts of mapped transcripts per gene were normalized by gene length and sequencing depth, with final counts expressed as kilobase pairs of mapped transcripts per Megabase pairs sequenced.

Screening of AMZ metagenomes and metatranscriptomes

Publically available metagenomes and metatranscriptomes from the ETSP AMZ and metagenomes from the Arabian Sea and Saanich Inlet AMZs were screened for close homologs
of SAG ure and cyn genes (Table 2). Sequences first were compared to the SILVA rRNA database using BLASTN, and sequences identified as rRNA genes/transcripts were removed. Remaining reads were compared using BLASTX against a database of the urease and cyanase-encoding genes recovered from the ETNP SAGs, using match thresholds of bit score >50 and AAI >95%. To test for the presence of SAG urease genes in the ETNP community, we screened a deeply sequenced (HiSeq) metagenome from 200 meters at station 6T against a 39,476 bp ure-containing SAG contig using BLASTN. This metagenome was trimmed and cleaned as in (Padilla et al., 2017) and binned using MetaBat (Kang et al., 2015). Genes recovered on the assembled contigs of the aforementioned metagenome were also compared by BLASTX against a custom database containing the SAG urease genes and 2870 ureC genes available in NCBI’s protein database (as of 01-03-2018), using the match parameters described above. The taxonomic identities of top matching database entries were used to estimate the taxonomic richness of ureases in the OMZ community. We did not screen for Ca. Scalindua-like cynS genes, as these have been previously reported in Eastern Pacific AMZ meta-omic datasets (Babbin et al. 2017).

All sequence data generated in this study are in NCBI under BioProject PRJNA407229.

Results and Discussion

Hydrographic conditions and anammox activity in the ETNP AMZ

Figure 1 shows data from the three most extensively sampled ETNP stations, including two near-shore stations (6T, 7T) and a station (3T) farther offshore; data from three additional stations are in Figure S2, with station coordinates in Table S1. At all stations, anoxic conditions were
observed from depths of ~70-100 meters. Nitrite concentration was near the detection limit in the surface layer, but increased directly below the oxic-anoxic interface to 3-5 μM, a characteristic feature of AMZs. Ammonium concentrations were generally in the low nanomolar range (20-50 nM) (Figures 1, S2). Neither cyanate nor urea was measured on the 2014 cruise. However, in samples from a 2017 cruise along the same transect, urea concentration was below the detection limit within the AMZ, but above detection in the mixed layer, reaching maxima of ~100 nM (data not shown).

Anammox rates varied over depth and with proximity to shore. Measured rates were highest in the upper AMZ, increasing sharply below the oxic-anoxic interface with near-maximal rates reached only 10–20 m deeper (Figures 1, S2). Rates were lowest and confined to a narrow depth range at station 3T farthest from shore (up to 1 nM N₂ d⁻¹), with rates increasing to 5.5 nM N₂ d⁻¹ at the near-shore sites (6T, 7T; Figure 1). This is consistent with analyses in the ETSP AMZ off Chile and Peru, which showed anammox rates to be highest in shelf waters and to diminish with distance from shore, strongly correlating with organic matter export (Kalvelage et al., 2013). Our measured rates are similar to those measured previously at sites close to ours in the ETNP (Babbin et al., 2014, Ganesh et al., 2015) and at other open ocean AMZ sites (Lam and Kuypers, 2011).

Genomic evidence for organic nitrogen utilization in ETNP Ca. Scalindua

Ca. Scalindua bacteria in AMZs may contain metabolic features distinct from those of anammox bacteria in other environments. We explored this potential by analyzing 20 SAGs from two anoxic depths at station 6T in the ETNP. All 20 SAGs were classified with high confidence
(Probability score = 100.0, Naïve Bayesian classifier) as *Ca. Scalindua* sp. based on PCR-amplified 16S rRNA gene fragments.

As is common in single-cell analysis (Rinke *et al.*, 2013, Thrash *et al.*, 2014), estimated genome completeness and strain heterogeneity varied considerably among the SAGs (range: 0-50% and 0-100%, respectively; average: 27.4% and 33.0%). However, contamination was consistently low (average: 2.0%) and in some cases non-detectable, with moderate levels (>5%) in only two SAGs (Table 1). Based on genome completeness and total recovered sequence length, estimated genome size averaged 2.6 Mbp (range: 2.0-3.8 Mbp). The SAG with the highest estimated completeness (50%, SAG N22) contained 1637 protein-coding genes, suggesting a total gene count (~3300) smaller than that of *Ca. Scalindua* genomes of other species (>4000 genes). On average, regions homologous between SAGs shared 94.1% ANI (standard deviation: 2.7) and homologous open reading frames shared 85.3% AAI (standard deviation: 3.8) among SAGs and 73% AAI with homologs from *Ca. Scalindua profunda*, a sediment anammox bacterium with a near complete (>90%) genome. Analysis of diverse bacteria shows that strains of the same species generally share >94% ANI (Konstantinidis and Tiedje, 2005), whereas the AAI value observed here falls at the lower end of the estimated species boundary (Rodríguez and Konstantinidis, 2014). Full-length (>1500 bp) 16S rRNA genes were identified in 8 SAGs (1 from 125 m, 7 from 300 m), shared 98-100% ANI, and were identical or nearly identical to the 16S rRNA gene fragments obtained from all SAGs by PCR-based screening (Figure S3). These 8 full-length 16S rRNA sequences clustered in a monophyletic sub-clade of *Ca. Scalindua* referred to as the Arabian Sea cluster and were nearly identical to clones primarily from the AMZ in the Arabian Sea (Woebken *et al.*, 2008), but more distantly related to a cluster of *Ca. Scalindua*
sequences from the ETSP AMZ off Peru and Chile (Figure 2). Together, these data suggest high relatedness among the analyzed cells, which cluster within a Ca. Scalindua clade distributed widely across diverse AMZs.

Protein-coding genes in the SAGs, hereafter referred to as ETNP Ca. Scalindua, were compared against a database of amino acid sequences from all available anammox-associated genomes. Genes diagnostic of the anammox process encoding hydrazine synthase (HZS), hydrazine oxidase/dehydrogenase (HZO), and cd1 nitrite:nitric oxide oxidoreductase (NirS) were found in 5, 8, and 6 of the 20 SAGs, respectively (Table 1), confirming the metabolic role of these bacteria. Genes encoding octahaem hydroxylamine oxidoreductases (HAO) and ammonium transporters (Amt), both of which are observed in multiple copies in anammox genomes, were identified in 16 and 15 of the SAGs, respectively. Amino acid sequences of HZS, HZO, NirS, HAO, and two of the three Amt proteins displayed highest scoring matches to homologs from other anammox bacteria when queried (BLASTP) against the NCBI nr database. Together, the recovery of multiple genes of anammox central metabolism and the shared ancestry of these genes with other Brocadiales identifies the SAGs as members of the AMZ anammox community.

Comparative analyses revealed 1811 non-redundant genes (out of 14,610 total (redundant/non-redundant) across the SAGs) that did not have a significant (bit score > 50, BLASTP) match to a protein sequence in the custom database, and therefore may be unique to the ETNP Ca. Scalindua group. This “unique” gene set is dominated by uncharacterized hypothetical proteins (1569 of 1811, 86%), consistent with high proportions of uncharacterized lineage-specific genes in other anammox genomes (Speth et al., 2017). A total of 206 non-redundant proteins displayed
significant matches to the COG database via BLASTP, while 36 displayed an identifiable protein
domain structure but did not display significant similarity to the COG database (Table S2).

Of the unique classifiable sequences, we focused on those that allowed us to explore the
hypothesis of alternative nitrogen substrate use. In contrast to all characterized genomes of
anammox bacteria, ETNP Ca. Scalindua SAGs contain genes for hydrolysis and transport of urea
(Table 1, Figure 3A). In three SAGs (G15, M13, N19), we identified contigs containing ureC
encoding the alpha subunit of urease, the nickel (Ni)-containing enzyme that facilitates cleavage
of urea into ammonia and carbamate (Mobley et al., 1995), with the carbamate then
spontaneously forming ammonia and carbon dioxide. The ETNP Ca. Scalindua ureC encodes
conserved catalytic site residues present in enzymatically verified UreC of urease-positive
bacteria (Figure S4) and is directly downstream of genes for the non-catalytic gamma and beta
urease subunits and directly upstream of genes encoding urease accessory proteins UreEFG
required for assembly and activation of the apoprotein (Figure 3). This gene order is nearly
identical to that observed in enzymatically verified urease-positive bacteria (e.g., Proteus
mirabilis; (Pearson et al., 2008). Studies using ure knockout mutants indicate that UreE is likely
the Ni donor, while UreF and UreG are chaperones enabling Ni donation from UreE (Mobley et
al., 1995). The ureD gene, which encodes a fourth subunit whose function is unclear but is
required for urease assembly in P. mirabilis, was identified downstream of ureG on one of the
ureC-containing contigs, and on separate, smaller contigs in other SAGs. Genes encoding high
affinity ABC-type urea transporters (urtCDE, Figure 3) are also present on the ure-containing
contigs of SAGs N19 and M13. The urease-associated genes (ure and urt) show ≥98% ANI
among SAGs, with the vast majority of mutations at the third codon position. The sequences
from one SAG (G15) are nearly identical to those from an assembled metagenome contig from the AMZ core (JGI Scaffold in Figure 3A), confirming the presence of these genes in community data from the site. However, SAG-affiliated ure genes (ureC) were at low proportional abundance (1 of 42, ~3%) in the total pool of ure genes present in the metagenome (Table S3). Overall, the dominant ureC variants were most closely related (based on BLASTX) to those of an alphaproteobacterium (Sphingorhabdus flavimaris; 24 of 42 ureC fragments), suggesting that other organisms in the OMZ may compete with ETNP Ca. Scalindua for urea.

UreC of ETNP Ca. Scalindua does not display a close phylogenetic affiliation with that of other lithotrophic organisms, including the ammonia-oxidizing Thaumarchaea and nitrite-oxidizing bacteria (e.g., Nitrospira). Rather, Ca. Scalindua UreC is most closely related (70% AAI) to UreC of a facultatively anaerobic marine Bacteroidetes bacterium (Raineyella antarctica; Pikuta et al., 2016) (Figures 4A, S4). None of the urease-encoding contigs recovered from the SAGs contain marker genes typically used to assess phylogenies (e.g., 16S rRNA gene). However, the largest of these contigs (contig 1 from SAG M13; Figure 3A) contains a gene encoding the glycolysis protein glyceraldehyde 3-phosphate dehydrogenase (GspA). GspA is conserved among representative genomes from most anammox genera, and phylogenetic analysis placed the M13 GspA in a highly supported clade with that of other Brocadiales, including other Ca. Scalindua species (Figure S5). Several other genes on this contig also display highest similarity (BLASTP) to anammox-associated Brocadiales. These include two genes encoding XerC, an enzyme mediating site-specific recombination, a process potentially associated with horizontal gene transfer. Tandem repeat sequences, which are often affiliated with recombination, were identified on this contig in five protein-coding genes, all >1000 bp from the ure genes. Taken
together, these data link a potential for urea utilization to ETNP Ca. Scalindua and, given the absence of these genes from other Ca. Scalindua species, raise the possibility that this function was acquired horizontally from a non-anammox organism. The potential for horizontal transfer is supported by the high ANI (≥98%) among ure genes from different SAGs, potentially reflecting recent transfer or strong selection pressure.

The SAG data also support the hypothesis, proposed by Babbin *et al.* (2017), that Ca. Scalindua in AMZs can use cyanate as an ammonium source. Five of the SAGs (Table 1) contain the cynS gene putatively encoding cyanate hydratase (cyanase). Cyanases cleave cyanate to carbamate (H$_2$NCOO$^-$) and carbon dioxide and occur in diverse non-anammox bacterial and eukaryotic lineages (Rocap *et al.*, 2003; Kamennaya *et al.*, 2008; Kamennaya and Post, 2011), as cyanate is a common by-product of urea degradation and amino acid metabolism. The SAG cynS sequences share 94.5-98.9% ANI and, in two of the SAGs, are present on >9 kbp contigs with conserved synteny (Figure 3B). The SAG cynS is most closely affiliated (82% AAI) with that of the only other cynS sequence linked to an anammox bacterium, Ca. Scalindua profunda from sediment. Both Ca. Scalindua CynS sequences cluster in a monophyletic clade with those of aerobic nitrite-oxidizing bacteria (*Nitrospina*) common in the oxycline of AMZ regions (Figure 4B) (Zaikova *et al.*, 2010, Fussel *et al.*, 2012). These SAG results link CynS to Ca. Scalindua in AMZs, supporting the work of Babbin *et al.* (2017) showing that cyanate stimulates AMZ anammox.

**Transcription of alternative nitrogen acquisition pathways by ETNP Ca. Scalindua**

Metatranscriptomics confirmed the activity of key genes of Ca. Scalindua nitrogen-based energy metabolism (Tables S4-S6). We first estimated ETNP Ca. Scalindua’s contribution to
community transcription by querying metatranscriptome datasets from 5 ETNP sites and multiple depths against a composite SAG amino acid database using a match threshold of >95% AAI (above the average between-SAG AAI of 85%). The composite database contained sequences from 6 of the most complete SAGs, representing an estimated ~90% of all homologs shared among the SAG set. This analysis showed that the representation of ETNP Ca. Scalindua transcripts increased dramatically from the base of the oxycline into the AMZ (Figures 1B,1E,1H, S2), likely due to an increase in the number of Ca. Scalindua bacteria along this gradient (Ganesh et al., 2015). This is consistent with the increase in anammox rates into the AMZ, although the depth of highest transcript representation was below that of highest rates and below the nitrite maximum (Figures 1C-D).

Transcripts encoding the putative urease and cyanase of ETNP Ca. Scalindua were detected throughout the study area, increasing in representation with depth in a pattern roughly paralleling that of the total ETNP Ca. Scalindua transcript pool (Figures 1C,1F,1I, S2). An analysis of metatranscriptome data from the AMZ core (200 m) at station 6 indicated that over half of all detected ureC transcripts in the AMZ community were most similar to those recovered on ETNP Ca. Scalindua SAGs (Table S6). Transcripts encoding ABC-type urea transporters (Urt) were also detected (Tables S4-S5), peaking in proportional representation at 200 meters at Station 6 where urease transcripts were also most abundant (data not shown). In general, urease transcripts were less common than cyanase transcripts. Indeed, cyanases were among the top 40 most transcribed Ca. Scalindua genes at stations with high anammox rates (e.g., station 6T, Figure S6). However, both urease and cyanase transcripts were far less abundant than those encoding genes diagnostic of anammox, mainly HZS and HZO, which were commonly among
the top 5 most transcribed ETNP Ca. Scalindua proteins (Figures S6, S7). CO₂ fixation in anammox bacteria occurs through the Wood-Ljungdahl pathway, with electrons donated from the oxidation of nitrite to nitrate. Genes diagnostic of this pathway and of nitrite oxidation, notably *acsA* encoding acetyl coA synthase and *narG* encoding nitrate/nitrite oxidoreductase respectively, were consistently observed among the Ca. Scalindua transcripts, albeit at low levels (Figure S7).

The transcript data also provide insight into the importance of other nitrogen-containing compounds in AMZ anammox. Nitric oxide (NO) is a key intermediate in anammox, having been shown in *Ca. Kuenenia stuttgartiensis* to be condensed with ammonium to form hydrazine (Kartal *et al.*, 2011). Under non-limiting nitrogen conditions in batch reactors, NirS-type cytochrome cd-1 containing nitrite reductase is implicated as the major route to NO and is among the most highly expressed proteins. However, while we identified NirS homologs in the SAGs, NirS transcripts were far less abundant than those encoding other anammox proteins, such as HZS and HZO (Figures 5, S7). A similar result was observed in a prior study that used the *Ca. S. profunda* genome to recruit metatranscriptome data from the ETSP AMZ (van de Vossenberg *et al.*, 2013). Interestingly, biochemical investigations of octahaem cytochrome c-containing hydroxylamine/hydrazine oxidoreductase (OCC-HAO) proteins from *Ca. Kuenenia stuttgartiensis* revealed that one protein (kustc1061) produced NO through the oxidation of hydroxylamine (Maalcke *et al.*, 2014). This protein belongs to a subclade of OCC-HAO proteins named ‘HZO cluster 2a’ and is phylogenetically distinguishable from other OCC-HAO/HZO homologs (Schmid *et al.*, 2008). ETNP SAGs also contain a homolog belonging to HZO cluster 2a (B14_Prokka_00643 in Figure 5A) and transcripts encoding this protein were consistently
more abundant (up to 10-50 times) than those encoding NirS (Figure 5C). A second HAO homolog, belonging to ‘cluster 3’, has also been predicted to produce NO from nitrite, although this function is not experimentally verified. This homolog (B14_00071 in Figure 5) was detected in the SAGs and transcribed at a level comparable to that of ‘HZO cluster 2a’ (Figure 5C). Interestingly, recent work on an anammox bacterium from activated sludge (*Ca. Brocadia sinica*) demonstrated that in the absence of canonical enzymes of NO production (*nirS, nirK*), nitrite was reduced to hydroxylamine, potentially by an OCC-HAO protein (although this remains untested), and the resulting hydroxylamine was coupled with ammonium for hydrazine and ultimately N₂ production (Oshiki *et al.*, 2016). Our results raise the possibility that hydroxylamine is also a critical intermediate in anammox bacteria from open ocean AMZs. However, the exact role of this substrate and that of associated OCC-HAO proteins in both NO-dependent and independent pathways of ETNP *Ca. Scalindua* remains speculative.

*Evidence for alternative nitrogen use pathways by anammox bacteria in other AMZs*

We screened other AMZ datasets to determine if the potential for urea and cyanase use by anammox bacteria is widespread (Table 2). All screened AMZ datasets contain high numbers of sequences matching (>95% AAI) genes in our composite SAG database (data not shown). Of these, sequences closely related to the urease and cyanase-encoding genes from ETNP *Ca. Scalindua* were identified in metagenomes and metatranscriptomes from the ETSP AMZ off Chile. These genes were not detected in the ETSP oxycline (Table 2), consistent with the low abundance of *Ca. Scalindua* at non-AMZ depths in this region (Stewart *et al.*, 2012). ETNP *Ca. Scalindua*-like urease genes were also detected in a metagenome from the core of the Arabian Sea AMZ (Luke *et al.*, 2016), although cyanase genes were not found in this dataset. Close
relatives of urease and cyanase genes were not detected in a metatranscriptome from a seasonally
anoxic coastal AMZ in Saanich Inlet. These results suggest that the potential for urea and
cyanate use for anammox exists in some AMZs, including the major AMZs of the ETSP and
Arabian Sea where high anammox rates have been recorded (Galan et al., 2009, Ward et al.,
2009, Bulow et al., 2010). Also, the failure to detect these genes in some sites (e.g., Saanich
Inlet) is not definitive evidence of their absence, as several factors may preclude detection,
including the depth of sequence coverage, the proportional abundance of anammox-cells,
variability in the timing and depths of sample collection, and potential sequence divergence
across systems. Further genomic analyses of anammox populations across systems, and perhaps
at finer spatial and temporal scales of resolution, will help identify the evolutionary and
environmental controls determining the distribution of urease and cyanase genes in marine
anammox bacteria.

Conclusions
This study provides evidence that anammox bacteria have the potential to degrade both urea and
cyanate. At this time, however, it is not possible to determine the exact biochemical role that
ureases and cyanases play in ETNP Ca. Scalindua. It is possible that the ammonium liberated by
these enzymes is used as an energy substrate for anammox, and therefore contributes to nitrogen
loss through N₂ production. Alternatively, it is possible that these enzymes serve other
functions. Urea and cyanate are common products of protein degradation, and high intracellular
concentrations of these substrates may be detrimental to cellular processes. Ureases and
cyanases may therefore serve in detoxification, or potentially to cycle ammonium into anabolic
pathways. Thus, these enzymes may play a role in the biological retention or recycling, rather than loss, of valuable nitrogen. However, the detection and transcription of urea transporter genes indicates that ETNP Ca. Scalindua likely consumes this organic substrate from the environment, suggesting that urease activity in this organism is not linked exclusively to nitrogen recycling. Furthermore, even recycling will decrease the assimilatory demand for exogenous ammonium and thereby indirectly increase its availability for dinitrogen production.

The consumption of organic nitrogen by aerobic ammonia-oxidizing microorganisms has gained much attention, notably as urease potential occurs in ubiquitous ammonia-oxidizing Thaumarchaeota (Alonso-Saez et al., 2012). Aerobic nitrite-oxidizing bacteria of the genus *Nitrospira* have also been shown to produce ammonia from urea, thereby sustaining co-occurring ammonia-oxidizers that provide nitrite to *Nitrospira* (Koch et al., 2015). However, the contribution of urea to anaerobic pathways of lithotrophic ammonium consumption remains unclear. Indeed, these results are the first report of urease genes in any anammox-capable lineage. Furthermore, while cyanase genes had been reported in anammox bacteria from non-AMZ environments and cyanate shown to support anammox activity in AMZs (Babbin et al., 2017), cyanase genes had not yet been definitively linked to genomes of AMZ anammox bacteria, for example by being found co-localized on a metagenome contig with definitive Ca. Scalindua signatures, or in a genome from a Ca. Scalindua cell/culture. The extent to which ammonium limitation in AMZs selects for organic nitrogen consumption by anammox bacteria remains uncertain, although our results suggest that urea and cyanate use may occur in populations from different AMZs.
Overall, the results expand our knowledge about the metabolic capacity of anammox bacteria and predict mechanisms by which these widespread organisms might supplement direct consumption of free ammonium. Few urea and cyanate measurements have been made for AMZs and data from other regions suggest that concentrations rarely exceed nanomolar levels in the open ocean (Solomon et al., 2010; Widner et al., 2017). While urea and cyanate concentrations were not comprehensively surveyed in this study, our preliminary measurements, coupled with previous measurements of cyanate in Eastern Pacific AMZs (Widner et al., 2016, 2017), suggest levels comparable (urea) or likely lower (cyanate) than those of ammonium. However, it is possible that turnover of these substrates is rapid, particularly at times or depths of lower ammonium levels. Counts of cyanase transcripts in this study were higher than those for urease, suggesting a potential greater role for cyanate as an alternative ammonium source. We caution, however, that the transcript data provide no temporal resolution and may be a poor proxy for actual substrate turnover. Indeed, our knowledge of the temporal variability in AMZ inorganic and organic substrate availability, and in rates of coupled microbial metabolisms, remains limited. Our data suggest only that the potential for direct cyanate and urease use exists for AMZ anammox bacteria. Future experiments should assess the environmental conditions that constrain the use of different ammonium sources. Such experiments should also determine what proportion of the urea and cyanate pools consumed by anammox bacteria are indeed lost through anammox, versus lost through detoxification or incorporation into new biomass. Urea and cyanate have gathered increasing attention within the context of aerobic nitrification, and recent data have indicated that marine populations of aerobic nitrifiers can oxidize ammonia at vanishingly low oxygen levels (nM) that are well within the range under which anammox occurs.
(Bristow et al., 2016). Thus, an important question becomes to what extent periodic oxygenation of the anoxic core, and shoaling of the oxycline, change the dynamics of thaumarchaeal ammonium consumption and anammox, and the extent that use of alternative substrates by either group is stimulated or inhibited by these events. Resolving such questions may improve models estimating the role of diverse nitrogen consumption pathways in bulk nitrogen and carbon budgets under AMZ expansion, providing refinements for global marine nutrient cycling.

Acknowledgments

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The authors declare no financial, commercial, or personal conflict of interest involving the publication of this work.

Supplementary Information

Supplementary information is available at The ISME Journal website.
References


Widner B, Mordy CW, Mulholland MR. (2017) Cyanate distribution and uptake above and within the Eastern Tropical South Pacific oxygen deficient zone. *Limnol Oceanogr*


Table Legends

Tables 1 and 2 are uploaded as Excel documents.

Table 1. ETNP Ca. Scalindua single amplified genome (SAG) statistics.

Table 2. Detection of ETNP Ca. Scalindua urease and cyanase genes in diverse AMZ datasets.

Figure Legends

Figure 1. Anammox rates and representation of ETNP Ca. Scalindua transcripts relative to dissolved oxygen, ammonium, and nitrite concentrations at three ETNP stations: 6 (A-C), 7 (D-F), and 3 (G-I). The first column displays dissolved oxygen (black line, μM), nitrite (red circles and line, μM), and ammonium (green circle and line, nM). An ammonium profile at station 6 showed concentrations consistently above 140 nM, which is inconsistent with all other stations sampled and with AMZ literature to date. We therefore interpret this signal as potential contamination and have excluded these data. The second column displays anammox rates (purple line and circles) and the cumulative contribution of all transcripts recruiting to ETNP Ca. Scalindua (orange circle and lines, kbp/Mbp). Purple crosses denote non-significant rates. Transcript representation is calculated as length-corrected kilobase pairs of transcripts mapping (via BLASTX, with bit score > 50 and AAI > 95%) to a composite ETNP Ca. Scalindua SAG database, per Megabase pairs sequenced. The third column designates the activity and distribution of cyanate hydratase (cynS) and urease (ureC) transcripts associated with ETNP Ca. Scalindua. For all rows, the y-axis indicates water column depth. SAG samples were collected from 125 and 300 m at station 6 in 2013.
Figure 2. 16S rRNA gene-based phylogenetic placement of 8 ETNP Ca. Scalindua-related SAGs from two anoxic depths in the ETNP AMZ. Full length (>1500 bp) 16S rRNA genes were identified in SAGs using RNAmmer, and characterized phylogenetically relative to database sequences. The phylogeny was estimated using maximum likelihood in ARB with boot strap values based on Neighbor joining, maximum likelihood, and maximum parsimony using 1000, 100, and 1000 boot-strap re-samplings, respectively. Maximum likelihood support values are based on approximate likelihood Bayesian ratios (‘abayes’).

Figure 3. Gene order and synteny of putative urease (A) and cyanase (B)-encoding contigs identified in SAGs and a metagenomic assembly (JGI Scaffold). Grey shading indicates nucleotide similarity based on BLASTN using the default settings in EasyFig.

Figure 4. Maximum likelihood-based phylogeny of the ureC gene encoding the urease alpha subunit (A) and the cynS gene encoding cyanase (B). Representative sequences recovered from ETNP Ca. Scalindua SAGs are highlighted in red, relative to homologs identified as best matches in BLASTP queries of the SAG sequences against the NCBI nr database (black). For both trees, support values are based on approximate likelihood Bayesian ratios (‘abayes’), and trees were constructed using the maximum likelihood method.

Figure 5. Phylogeny and transcription of genes potentially mediating nitric oxide formation in anammox. (A) Octahem cytochrome c hydroxylamine/hydrazine oxidoreductase (HAO/HZO) phylogeny. Clade nomenclatures are based on a previous phylogenetic assessment of HAO/HZO proteins (Schmid et al. 2008). Clades with an asterisk indicate subgroups that are new based on the current work. (B) Cytochrome cd-1 containing nitrite reductase (NirS) phylogeny. Both trees were constructed using maximum likelihood, with support values based on approximate
likelihood Bayesian ratios (‘abayes’). Proportional abundance of transcripts encoding the hypothesized hydrazine-oxidizing HZO (Panels 1, 4, 7), the hypothesized nitrite-reducing Hao (Panels 2, 5, 8), and the hydroxylamine oxidizing/NO forming HAO and cytochrome cd-1 containing NirS (panels 3, 6, and 9) at ETNP stations 6 (top row), 7 (middle row), and 3 (bottom row). All scales display activity in kbp/Mbp sequenced.

Supplementary Table and Figure Legends

**Table S1.** ETNP stations and depths sampled for metatranscriptome sequencing.

**Table S2.** Annotated, non-hypothetical proteins detected in ETNP Ca. Scalindua SAGs but not in any other available anammox-associated genomes (i.e., unique proteins). The table only shows unique proteins with an assigned annotation (based on similarity to homologs in the COG database).

**Table S3.** Taxonomic affiliation of *ureC* gene fragments recovered in AMZ metagenomes from station 6. Taxonomy is estimated by the identity of top BLASTX matches in a composite database of *ureC* genes from NCBI-nr and ETNP Ca. Scalindua SAGS.

**Table S4.** Length- and sequencing depth-normalized transcript distributions for genes associated with ETNP Ca. Scalindua.

**Table S5.** Annotations of genes associated with ETNP Ca. Scalindua (same genes as in Table S4).
**Table S6.** Taxonomic affiliation of *ureC* gene transcript fragments recovered in an AMZ metatranscriptome from 200 meters depth at station 6. Taxonomy is estimated by the identity of top BLASTX matches in a composite database of *ureC* genes from NCBI-nr and ETNP *Ca.* Scalindua SAGs.

**Figure S1.** Map of study area, showing locations of stations sampled in 2014 and identified in Figure 1 (main text). Samples for SAG analysis were collected from station 6T in 2013. Exact coordinates can be found in Table S1.

**Figure S2.** Anammox rates and representation of ETNP *Ca.* Scalindua transcripts relative to dissolved oxygen, ammonium, and nitrite concentrations at three ETNP stations: 8 (A-C), 10 (D-F), and 4 (G-I). The first column displays dissolved oxygen (black line, μM), nitrite (red circles and line, μM), and ammonium (green circle and line, nM). The second column displays anammox rates (purple line and circles) and the cumulative contribution of all transcripts recruiting to ETNP *Ca.* Scalindua (orange circle and lines, kbp/Mbp). Purple crosses denote non-significant rates. Transcript representation is calculated as length-corrected kilobase pairs of transcripts mapping (via BLASTX, with bit score > 50 and AAI > 95%) to a composite ETNP *Ca.* Scalindua SAG database, per Megabase pairs sequenced. The third column designates the activity and distribution of cyanate hydratase (*cynS*) and urease (*ureC*) transcripts associated with ETNP *Ca.* Scalindua. For all rows, the y-axis indicates water column depth.

**Figure S3.** Phylogenetic approximation of PCR-amplified 16S rRNA genes generated from SAG template DNA (following multiple displacement amplification). Sequences were inserted into the
backbone tree based on Figure 2 using the parsimony tool in ARB, and hence represent a phylogenetic approximation.

**Figure S4.** Alignment of UreC amino acid sequences from characterized urease-positive organisms *Proteus mirabilis*, *Streptomyces* sp. NRLL and MJM, and from two taxa (*Kouleothrix aurantiaca* and *Raineyella antarctica*) identified as best BLASTP matches to the ETNP Ca. Scalindua SAG UreC (UreC from SAG N19_00589 as a representative). Conserved catalytic site histidine and cysteine residues are noted by green and yellow arrows, respectively. The alignment was produced using clustalW and visualized with mView (https://www.ebi.ac.uk).

**Figure S5.** Phylogenetic approximation of SAG-associated glyceraldehyde 3-phosphate dehydrogenase, GspA (red). Sequences include GspA from a large ure and urt-containing contig from SAG M13 (see Figure 3A), and GspA from a smaller contig from SAG N22. Purple sequences were identified based on BLASTP against NCBI-nr. The phylogeny was estimated by Maximum likelihood with bootstrap support values based on the approximate Bayes method.

**Figure S6.** Top 40 most highly transcribed ETNP Ca. Scalindua genes observed at Station 6T (200 m, AMZ core).

**Figure S7.** Proportional abundance of transcripts encoding the hydrazine-producing (hydrazine synthase) and consuming (hydrazine oxidoreductase) enzymes, nitrite/nitrate oxidoreductases likely involved in nitrite oxidation, and acetyl coA synthase involved in the Wood-Ljungdahl pathway at ETNP stations 6 (A, D, G), 7 (B, E, H), and 3 (C, F, I).
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**Table 1. ETNP Ca. Scalindua single-cell amplified genome (SAG) statistics**

| Comp; genome completeness (%) estimated by CheckM |
| Cont; genome contamination (%) estimated by CheckM |
| Heter; strain heterogeneity index (range 1-100) estimated by CheckM |
| ORFs; number of protein-coding open reading frames recovered per SAG |
| Mbp; Mbp of contigs recovered per SAG |
| Est Gen; estimated genome size based on estimated completeness and Mbp of SAG; done only for SAGs with completeness >20% and contamination <5% |
| Ure; urease. UreA; gamma subunit, UreB; beta subunit, ureC; alpha subunit |
| Cyn; cyanate hydratase |
| Urease accessory proteins |
| Octa; Octahaem |
| Hzo; hydrazine oxidoreductase/dehydrogenase, S. profunda homolog scal03295 |
| Hsz; hydrazine synthase, S. profunda homolog scal01318 |
| Hao; hydroxylamine oxidoreductase, S. profunda Hao1 homolog 00421, S. profunda Hao2 homolog 01317, S. profunda Hao 3 homolog 02116, S. profunda homolog 4 04164 |
| Amt; ammonia transporter. S. profunda Amt1 homolog 00587, S. profunda Amt2 homolog 00591, S. profunda Amt3 homolog 00594 |
| NirS; cytochrome c nitrite reductase, S. profunda nirS homolog 02098 |
| Top BLASTP match to Brocadiales-affiliated homolog via queries against the SILVA database |
| Top BLASTP match to Brocadiales-affiliated homolog via queries against the NCBI-nr database + publically available Brocadiales databases |
| Top BLASTP match to non-Brocadiales via queries against the NCBI-nr database (i.e., homologs not detected in other Brocadiales) |
Table 2. Detection of ETNP Ca. Scalindua urease and cyanase genes in diverse AMZ datasets.

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<th>SRA identifier</th>
<th>DNA/RNA</th>
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<th>ureB</th>
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\(^1\) Total number of sequence fragments in dataset
Arabian Sea
Namibian coast
Eastern Tropical North Pacific, Mexico (This study)
Eastern Tropical South Pacific, Peru/Chile

Candidatus (Ca.) taxa

Arabian Sea
cluster

Namibian & Peruvian clusters

Jettenia, Brocadia, Anammoxoglobus

Nucleotide substitutions