Ammonia-oxidising archaea living at low pH: Insights from comparative genomics

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Significance statement

Acidic soils represent 30% of terrestrial environments. Nitrification in such soils is a crucial process contributing to fertilizer loss and greenhouse gas formation on a global scale. Members of the genus "*Candidatus* Nitrosotalea" are the only cultivated acidophilic archaeal nitrifiers and are widespread and abundant in acidic soils. In this study, we performed comparative genomics, including a previously published genome and two newly determined genomes from cultured *Ca.* Nitrosotalea species as well as one almost complete metagenome-assembled genome of a *Ca.* Nitrosotalea species from an acidic peatland, to better understand pH adaptation in this genus. Furthermore, expression of genes of interest was determined in culture and *in situ.* We identified a core set of genes absent from other ammonia-oxidizing archaea that are expressed in *Ca.* Nitrosotalea members in nature and during laboratory cultivation. Some *Ca.* Nitrosotalea core genes that have been horizontally transferred from acidophiles are predicted to have functions related to pH adaptation, suggesting that acquisition of genes from other acidophilic community members contributes to the competitive success of the *Ca.* Nitrosotalea lineage in acidic environments.

Abstract

Obligate acidophilic members of the thaumarchaeotal genus *Candidatus* Nitrosotalea play an important role in nitrification in acidic soils, but their evolutionary and physiological adaptations to acidic environments are still poorly understood, with only a single member of this genus (*Ca.* N. devanaterra) having its genome sequenced. In this study, we sequenced the genomes of two additional cultured *Ca.* Nitrosotalea strains, extracted an almost complete *Ca.* Nitrosotalea metagenome-assembled genome from an acidic fen, and performed comparative genomics of the four *Ca.* Nitrosotalea genomes with 19 other archaeal ammonia oxidiser genomes. Average nucleotide and amino acid identities revealed that the four *Ca.* Nitrosotalea strains represent separate species within the genus. The four *Ca.* Nitrosotalea genomes contained a core set of 103 orthologous gene families absent from all other ammonia-oxidizing archaea and, for most of these gene families, expression could be demonstrated in laboratory culture or the environment via proteomic or metatranscriptomic analyses, respectively. Phylogenetic analyses indicated that four of these core gene families were acquired by the *Ca.* Nitrosotalea common ancestor via horizontal gene transfer from acidophilic representatives of Euryarchaeota. We hypothesize that gene exchange with these acidophiles contributed to the competitive success of the *Ca.* Nitrosotalea lineage in acidic environments.

Introduction

Nitrification, the oxidation of ammonia to nitrate via nitrite, is a central process within the terrestrial nitrogen cycle, determining the form of inorganic nitrogen available to plants, decreasing nitrogen fertiliser utilisation efficiency and contributing to atmospheric and groundwater pollution by nitrous oxide and nitrate, respectively (Robertson and Vitousek, 2009). Nitrification in soil is generally limited by the initial oxidation of ammonia to nitrite, in which archaeal ammonia oxidisers play a significant role (e.g. Lu *et al*., 2015; Hink *et al*., 2016). Net rates of nitrification do not show a strong correlation with soil pH and some of the highest rates are found in acidic soils (pH<5) (Booth *et al*., 2005), which comprise approximately 30% of all soils (von Uexküll and Mutert, 1995). Surveys of 16S rRNA and ammonia monooxygenase subunit A (*amoA*) genes demonstrate that ammonia oxidising archaea (AOA) are distributed globally in soils, with pH being an important driver of both community composition and adaptation (Gubry-Rangin *et al*., 2011; Gubry-Rangin *et al*., 2015; Vico Oton *et al.*, 2016).

Genome-wide prediction of the functional adaptation of ammonia oxidising Thaumarchaeota to low pH has thus far been limited to the genome of the soil isolate *Candidatus* Nitrosotalea devanaterra (Lehtovirta-Morley *et al*., 2016). This genomic analysis identified potential mechanisms for substrate acquisition and pH homeostasis in acidic environments that present potential constraints for ammonia oxidisers. The concentration of NH_3, the most likely substrate for bacterial and archaeal ammonia monooxygenases, is significantly reduced at pH values below 7, as the pK_a for the $NH_4^+ \leftrightarrows NH_3$ equilibrium is 9.25. Decreasing pH also moves the $NO_2^- \leftrightarrows HNO_2$ equilibrium towards inhibitory nitrous acid, which is highly reactive, with breakdown products that can cause extensive cellular damage. In addition, growth at low pH requires mechanisms for pH homeostasis, to maintain the transmembrane proton gradient required for ATP production and normal function of cellular processes. Genome and cell membrane analyses indicated that such mechanisms might exist in *Ca*. N. devanaterra, including cation uptake, cytoplasmic buffering, and a cell membrane composition distinct from that of neutrophilic AOA (Lehtovirta-Morley *et al.*, 2016). In addition, Amt-type NH_4^+ transporters are predicted to be encoded by all sequenced AOA genomes (including *Ca*. N. devanaterra) and are distinct from Rh-type NH_3 transporters found in some ammonia oxidising bacteria (AOB) (Offre *et al.*, 2014; Lehtovirta-Morley *et al.*, 2016). Ammonia or ammonium is required for both energy generation and nitrogen assimilation by ammonia oxidisers, and the preference of *Ca*. N. devanaterra (and other AOA) for transporting NH_4^+ may contribute to its ability to grow in acidic environments containing limiting concentrations of NH_3. In this context, it is interesting to note that the recently isolated acid-adapted (growth in the range of pH 5 – 7.5) gammaproteobacterial

AOB *Ca.* Nitrosoglobus terrae does not encode known transporters for NH_4^+ or NH_3 and might thus rely on passive diffusion of ammonia through its membrane for assimilation (Hayatsu *et al*. 2017).

While the genome of *Ca*. N. devanaterra has allowed the generation of hypotheses regarding mechanisms facilitating its unique physiology, the absence of further acidophilic archaeal ammonia oxidiser genomes made it difficult to confirm these findings. The aim of this study was to gain a greater understanding of the function and origin of the genes potentially involved in acidophilic adaptation in the *Ca.* genus Nitrosotalea through comparative genomics by including three newly determined genomes from this genus, and to learn whether these genes are expressed under natural and/or cultivation conditions. Specifically, this study aimed to reveal (1) the (compositional) similarity of genomes within *Ca.* Nitrosotalea and with those of other AOA; (2) the size and predicted function of the *Ca.* Nitrosotalea core genome compared to that of other AOA genera; (3) whether the *Ca.* Nitrosotalea core genome is expressed; and (4) the evolutionary origin(s) of gene families that comprise the *Ca.* Nitrosotalea core genome.

Results

Expanded genomic representation of Candidatus *Nitrosotalea*

In this study, the genomes from two cultured strains of *Ca.* Nitrosotalea, strain Nd2 (Lehtovirta-Morley *et al.*, 2014) and strain CS (Jung *et al.*, 2014), were sequenced. In addition, a *Ca.* Nitrosotalea metagenome-assembled genome (strain SbT1) was recovered from an acidic fen, anaerobic, stable isotope probing experiment (Pester *et al.*, 2010; Hausmann *et al*., 2016; and see SI.1 and Figure S1).

Molecular evidence for four Candidatus *Nitrosotalea species*

The 16S ribosomal RNA gene sequences of the four *Ca.* Nitrosotalea strains exceed 99% nucleotide identity to one another and are thus not useful for elucidating precise taxonomic relationships among these strains (Yarza *et al*., 2014). Genomes of the four strains were therefore compared by determining average amino acid and nucleotide identities (AAI and ANI) (Figure 1). AAI between the four genomes were 79 - 83% with >80% of genes aligned, suggesting they represent different species of the same genus (Luo *et al*., 2014). Likewise, ANI values between the four genomes were 78% - 83%, far below the proposed species delineation boundaries of 95 - 97% (Goris *et al*., 2007; Varghese *et al*., 2015). Therefore, we propose that each of the four analysed strains represents a separate species within the *Ca.* Nitrosotalea genus.

Phylogenomic relationship of Candidatus *Nitrosotalea with other AOA*

In single gene trees based on 16S rRNA (Figure S2a) and *amoA* (Figure S2b) genes, the four *Ca.* Nitrosotalea species formed a monophyletic sister group to group 1.1a Thaumarchaeota (*Ca.* Nitrosopumilus, *Ca.* Nitrosoarchaeum and *Ca.* Nitrosotenuis), consistent with previous placements of this genus (Lehtovirta-Morley *et al*., 2011, Pester *et al*., 2011, Vico Oton *et al*., 2016). For more refined analyses, two concatenated sets of marker genes (a “universal” marker set consisting of 34 genes (Parks *et al*., 2015) and a set of 198 single-copy genes that are phylogenetically congruent among all AOA, see Table SI.2.1) were also used to infer the phylogenetic relationship of the four *Ca.* Nitrosotalea species with other fully sequenced AOA genomes. Again, *Ca.* Nitrosotalea was consistently recovered as a monophyletic sister group to group 1.1a, distinct from 1.1b taxa (*Nitrososphaera*, *Ca*. Nitrosocosmicus) (Bayesian P>0.999 and bootstrap support = 1) (Figures 2 and S2c). The only major disagreement between these two trees is in the branching order of lineages represented by *Ca.* Cenarchaeum symbiosum and *Ca.* Nitrosopelagicus brevis (Figures 2 and S2c). As both lineages are currently represented by a single member, addition of sister taxa to these two relatively long-branch taxa may help resolve the disagreement.

Thaumarchaeota and Candidatus *Nitrosotalea core genomes*

The quality of the sampled genomes and the phylogenetic breadth of groups used for comparison strongly influence core genome analyses. Genome completeness, open reading frame predictions, and AAI-based grouping of genomes were therefore considered *a priori* in the description of core genomes. Genome completeness, assessed by two methods, was high (>92%) for all 23 AOA genomes (Table 1) but many contain pathway gaps, likely to be artefacts of different gene-calling approaches. Gene-calling was therefore repeated for all AOA genomes using Prodigal (Hyatt *et al.*, 2010) and resulted in a much larger core genome shared by all 23 AOA (640 and 743 using gene calls from GenBank and *de novo* gene calls made by Prodigal, respectively). Prodigal gene calls were thus used in subsequent analyses. AAI between genomes varied extensively within and between groups (Figure 1). The range of proposed genus-level cut-offs for AAI (60–80%; Luo *et al*., 2014) is inconsistent with the currently used AOA taxonomy. For instance, at 60% AAI *Ca*. Nitrosotenuis, *Ca*. Nitrosopumilus, *Ca*. Nitrosopelagicus and *Ca*. Nitrosoarchaeum would form a single genus, at 70% AAI, *Nitrososphaera* would be split into two genera, while *Ca*. Nitrosopumilus and *Ca*. Nitrosoarchaeum would form a single genus. At 80% *Nitrososphaera, Ca*. Nitrosopumilus and *Ca*. Nitrosotenuis would split into multiple genera. This information was considered when comparing genus-specific core gene sets (see below).

Predicted genes from the 23 AOA genomes (including the four *Ca.* Nitrosotalea genomes) were clustered into 11,655 orthologous gene families using OrthoMCL (Li *et al.,* 2003), of which 4868 gene families were unique to single taxa and 743 were found in all genomes, forming a thaumarchaeotal core genome (SI.1 and Table SI.2.1). As expected, this number is lower than the 860 core genome gene families of Thaumarchaeota recently reported by Kerou *et al.* (2016) reflecting our inclusion of more genomes and use of different cut-off values and algorithms. Of the 743 gene families of the thaumarchaeotal core genome determined in this study, 697 were also retrieved by Kerou *et al.* (Table SI.2.2).

Ca. Nitrosotalea genes were present in 2902 gene families, almost half of which (1363) were common to all four *Ca.* Nitrosotalea genomes. The *Ca.* Nitrosotalea-specific core genome comprised 103 orthologous gene families restricted within the Thaumarchaeota to *Ca.* Nitrosotalea (SI.1 and Table SI.2.3). This was lower than the respective *Nitrososphaera*-specific core genome, whether including *Ca*. N. gargensis (331 gene families) or excluding it (333 gene families) due to low shared AAI with other members of this genus. Their larger core genome likely reflects the greater genome size of genus *Nitrososphaera* members. The *Ca.* Nitrosotalea-specific core is, however, much larger than that of group 1.1a AOA (Table SI.2.1), contrasting with only 10, 23, and 40 gene families for the *Ca.* Nitrosopumilus-specific core, the *Ca.* Nitrosoarchaeum-specific core, and the combined *Ca.* Nitrosopumilus/*Ca.* Nitrosoarchaeum-specific core, respectively. To account for sampling bias in core-genome definitions, AAI was used to select combinations of four dissimilar *Ca.* Nitrosopumilus/ *Ca.* Nitrosoarchaeum genomes to mimic the diversity of *Ca.* Nitrosotalea. This resulted in a maximum of 28 *Ca.* Nitrosopumilus/*Ca.* Nitrosoarchaeum-specific core gene families.

Origin of gene families in the Candidatus *Nitrosotalea-specific core genome*

The potential evolutionary origin of the 103 orthologous gene families (comprising 420 genes) identified as the "*Ca.* Nitrosotalea-specific core" was examined based on phylogenetic tree topology-based inference. Of these 103 gene families, 7 shared homology with gene families present in non-AOA microbes but not in other AOA; 38 showed little or no homology (<30% amino acid identity) to any other gene families in other AOA or to any other sequences in the NCBI GenBank nr protein database; 12 returned only one to three low scoring (30-45% amino acid identity) hits in Blast-based searches, preventing further phylogenetic analysis; and 46 shared homology with other gene families in Thaumarchaeota (>30% amino acid similarity between members of each gene family). These 46 gene families were nevertheless inferred to be *Ca.* Nitrosotalea-specific in the OrthoMCL-based approach because the pairwise similarity and connectivity between the members of each gene family was insufficient to assign them confidently to common orthologous groups with other AOA (Table 2). In manually examined phylogenetic trees (not shown), all but one of this subset of 46 *Ca.* Nitrosotalea-specific core gene families branch with other AOA. Phylogenetic reconstructions for eight gene families (one with homology in other AOAs and seven absent from other AOAs), suggested HGT events involving a common ancestor of the *Ca.* Nitrosotalea (Table 2, Figures S3-S10). Five of these HGT events affected gene families of potential importance for the acidophilic lifestyle of these AOA by playing a putative role in metal transport, detoxification, or protection from stress. Four of the gene families share a common ancestor with acidophilic archaea (Table 2).

Expression of the Candidatus *Nitrosotalea core genome*

Proteomics and metatranscriptomics were used to assess which *Ca.* Nitrosotalea-specific core genes are expressed. Proteomic analysis of *Ca.* Nitrosotalea strains Nd2 and CS, cultivated at optimal pH, identified 65% (1227 proteins) and 13% (308 proteins) of all predicted proteins, respectively (Tables SI.2.1 and SI.2.4). This confirmed expression of 62 of the 103 *Ca.* Nitrosotalea core gene families, 4 of which were horizontally acquired by a common ancestor of *Ca.* Nitrosotalea (Tables 2 and SI.2.1). Metatranscriptomics data from the acidic fen, that the *Ca.* Nitrosotalea strain SbT1 was assembled from, confirmed transcription of 79 of the 103 *Ca.* Nitrosotalea-specific core gene families, including 7 of the gene families that were acquired via HGT, 4 of which were also identified through proteomics (Tables 2, SI.2.1 and SI.2.5).

Discussion

The four acidophilic thaumarchaeotal strains investigated in this study consistently form a monophyletic group branching as a sister clade to the Group I.1a Thaumarchaeota in phylogenetic trees based on *amoA* gene, 16S rRNA gene, and two concatenated gene sets (Figures 2 and S2). ANI and AAI values clearly illustrate that the four strains are separate species. Due to their phylogeny and their high AAI values among each other, we assigned the four species to the *Ca.* genus Nitrosotalea and propose the following names for them: *Ca.* Nitrosotalea bavarica SbT1, *Ca.* Nitrosotalea okcheonensis CS, and *Ca.* Nitrosotalea sinensis Nd2.

As *Ca.* Nitrosotalea occupies a unique low pH niche among cultivated Thaumarchaeota, the genomes of the four *Ca.* Nitrosotalea species were mined for gene families that they share to the exclusion of all other Thaumarchaeota, to identify candidate gene families that may be associated with their acidophilic lifestyle. In total 103 gene families were assigned to this "*Ca.* Nitrosotalea-specific core", almost half of which (50) are novel or too divergent for confident determination of function or origin. Another 46 families share homology with other

Thaumarchaeota genes, despite being assigned to the *Ca.* Nitrosotalea specific core group. This is not surprising, given that a graph-based orthologue definition will separate genes with homology if they are sufficiently different from one other, but strongly connected internally. It is possible that some of the gene families shared between all *Ca.* Nitrosotalea and other thaumarchaeotes facilitate life at low pH, with specific protein evolutionary adaptation, as previously observed for the ammonia monooxygenase protein (Macqueen and Gubry-Rangin, 2016).

Careful phylogenetic analysis of the *Ca.* Nitrosotalea-specific core gene families provided strong evidence that eight were affected by HGT events. For six of these gene families, *Ca.* Nitrosotalea genes clustered with homologues from non-AOA archaeal phyla while two clustered with homologues from bacteria (Figures S3-S10). Interestingly, four HGT events (OGs 2531, 2888, 2932, 2933) occurred with members of the *Thermoplasmatales* and in one case possibly also with acidophilic *Crenarchaeota*. The *Thermoplasmatales* is an order within the *Euryarchaeota* that comprises acidophiles growing preferentially below pH 2 and that also encompasses members of the genus *Picrophilus* growing at around pH 0, representing the most acidophilic microbes described (Huber & Stetter 2006). For seven of the eight horizontally exchanged gene families, expression in culture and/or *in situ* could be confirmed (Table 2), suggesting their functional importance.

A previously published analysis of the *Ca.* N. devanaterra Nd1 genome identified 51 candidate genes of importance for its acidophilic lifestyle (Lehtovirta-Morley *et al.*, 2016). The analyses presented here now shows that a surprisingly low number (n=10) of these genes are also present in all three newly determined *Ca.* Nitrosotalea genomes and absent in all other non-acidophilic AOA (Tables 3 and SI.2.6). This is unlikely a result from lack of genome closure, as estimated genome completeness for all four *Ca.* Nitrosotalea was high

(96.6-100%). Therefore, the *Ca.* Nitrosotalea-specific core genome likely lacks some of the typical mechanisms of pH homeostasis described in acidophiles (Baker-Austin *et al.,* 2007). For example, the *kdp* potassium transporter (EC 3.6.3.12) of *N. devanaterra* is found in only two of the four *Ca*. Nitrosotalea genomes. This is unexpected as potassium is considered a critically important solute in extreme acidophiles, responsible for generating the reverse membrane potential, and its absence implies that representatives of this genus either have a novel unrecognised mechanism for potassium uptake or use other cations to generate a reverse membrane potential. In contrast to a previous hypothesis (Lehtovirta-Morley *et al*., 2016), the carbonic anhydrase (EC 4.2.1.1) of *Ca*. N. devanaterra Nd1 is not suitable for intracellular consumption of protons as it, like the respective homologues in other Thaumarchaeota, has an N-terminal signal peptide, indicating an extracellular localization (Kerou *et al.* 2016). These γ-class carbonic anhydrase (CA) homologs likely facilitate carbon transfer into the cell by converting bicarbonate to CO_2, which can subsequently diffuse through the cell membrane. At an intracellular pH of 7, CO_2 will be rehydrated to bicarbonate and used for carbon fixation. As members of the *Ca.* Nitrosotalea thrive in very low pH soils containing much more CO_2 than bicarbonate, extracellular carbonic anhydrases are not necessary. Consistently, two of the four *Ca.* Nitrosotalea species (*Ca*. N. okcheonensis CS and *Ca*. N. sinensis Nd2) do not encode this enzyme.

Interestingly, a specific subset of gene families thought to play a role in adaptation to low pH in *Ca.* N. devanaterra Nd1 are exclusively shared among AOA between the four *Ca.* Nitrosotalea genomes and *Ca.* Nitrosocosmicus oleophilus MY3, an AOA that can grow between pH 5.5 and 8.5 (Jung *et al*., 2014) (Table 3). For instance, all five genomes encode electroneutral CPA1-type (cation/proton antiporter) Na^+/H^+ antiporters (TC 2.A.36) that were postulated to be involved in pH homeostasis in *Ca*. Nitrosotalea. In contrast, all neutrophilic AOA, including *Ca.* N. oleophilus MY3, possess electrogenic CPA2-type Na^+/H^+

exchangers (TC 2.A.37) (Padan *et al.,* 2005) which are absent in genus *Ca.* Nitrosotalea (OG0030, OG0824). It has been previously demonstrated that CPA1-type transporters export protons and are downregulated at alkaline pH (Călinescu *et al.,* 2014), while CPA2-type transporters are downregulated at acidic pH (Alkoby *et al.,* 2014), although it is not clear whether this distinction applies to all CPA1- and CPA2-type exchangers. Likewise, subunits of the membrane-bound domain and central and peripheral stalks of the A-type ATP synthase of *Ca.* Nitrosotalea and *Ca.* N. oleophilus MY3 (EC 3.6.3.14) were dissimilar (<30% AA identity) to other AOA. The functional implications of this divergence are currently unknown. In contrast, the cytoplasmic domain (A_1) (*atpAB*) of the ATP synthase is conserved in all AOA, including *Ca.* Nitrosotalea genomes. The direction of proton transport by A-type ATP synthase is reversible (Grüber *et al.*, 2014), and thus the modified ATP synthase may be involved in proton extrusion in the genus *Ca*. Nitrosotalea. This would necessitate a dual role in ATP synthesis and proton export since the A-type ATP synthase is the only ATP synthase encoded in the genomes of *Ca.* Nitrosotalea.

Ca. *Nitrosotalea core gene families acquired through horizontal gene transfer*

Five of the *Ca.* Nitrosotalea-specific core gene families affected by HGT might play important roles in the acidophilic lifestyle of these Thaumarchaeota (Table 2), suggesting that horizontal gene acquisition was important for their adaptation to low pH environments. Firstly, one of the gene families exchanged with members of the *Thermoplasmatales* is a Na^+/solute symporter (OG 2888) (Lehtovirta-Morley *et al.,* 2016) and is present in many bacterial and archaeal acidophiles. Characterised members of the Na^+/solute symporter family (TC 2.A.21) take up a wide range of organic solutes, including amino acids, sugars and mono- and dicarboxylates (Jung, 2002; Groeneveld *et al.,* 2010). Amino acid alignment suggests that the Na^+/solute symporters of *Ca*. Nitrosotalea lack the sodium binding site (data not shown) and the phylogenetic placement of the four *Ca*. Nitrosotalea species transporters with characterized homologues consistently recovers a robust relationship to *mctP* of *R. leguminosarum*, a proton-coupled monocarboxylic acid symporter (Figure S11; Jung, 2002). This implies proton- rather than sodium-coupled symport. Uptake of organic compounds seems paradoxical because the three cultivated *Ca.* Nitrosotalea strains grow autotrophically in inorganic media. However, there is evidence for stimulation of *Ca*. Nitrosotalea growth by some organic acids, e.g., oxaloacetate (Lehtovirta-Morley *et al.,* 2014). While we can only speculate on the function of this protein, its conservation in *Ca*. Nitrosotalea core genome, its consistent presence in other archaeal acidophiles, and its absence from all other AOA makes it a strong candidate for future characterisation and determination of the substrate specificity together with its role in acidophily.

Two more genes of the horizontally transferred *Ca*. Nitrosotalea-specific core encode metal transporters (OG 2531 and OG 2924). While OG2531 is a member of the Zinc-Iron Permease (ZIP) family and can be annotated with high confidence as a Zn^{2+} importer, OG 2924 is a member of the divalent cation transporter NRAMP (TC 2.A.55) family found in many acidophiles for which substrate predictions are not possible without experiments. The gene families OG2531 and OG2924 were horizontally exchanged with members of the *Thermoplasmatales* and Woesearchaeota, respectively. We postulate that these metal transporters provide adaptation for metal uptake under low pH conditions, where the bioavailability of metals is strongly increased (Violante *et al.*, 2010) and transporters with different properties (e.g., a lower affinity) might be beneficial. Interestingly, all other genome-sequenced AOA also encode a ZIP transporter (not closely related and likely replaced by the laterally acquired ZIP in *Ca*. Nitrosotalea, data not shown). In most AOA, this transporter is located immediately adjacent to the multicopper oxidase 1 (MCO 1), which has recently been hypothesized as an interesting candidate for thaumarchaeotal hydroxylamine oxidation (Kerou *et al.* 2016). Interestingly however, MCO 1 is absent from all four *Ca.* Nitrosotalea species.

A fourth gene that has been laterally exchanged between *Ca*. Nitrosotalea and other archaea, belonging to the *Methanosarcinales*, is a FKBP-type peptidyl-prolyl cis-trans isomerase gene (OG 2943) encoding a folding chaperone for proteins containing proline residues. While classified within the *Ca*. Nitrosotalea-specific core, distantly related FKBP-type peptidyl-prolyl cis-trans isomerases are also found in neutrophilic AOA, indicating that not all folding chaperones are confined to AOA with an acidophilic lifestyle. Although homologues of OG 2943 have not been linked specifically to acidophily in other organisms, chaperones in general are prevalent in acidophilic genomes and up-regulated during pH down-shift (Baker-Austin and Dopson, 2007).

Finally, a FMN-dependent NADH-azoreductase (EC 1.7.1.6) (OG 2912) that has been exchanged with members of the recently proposed bacterial candidate phylum “Dependentiae” (Yeoh, *et al*., 2015) is present in all analysed *Ca*. Nitrosotalea species and may function in detoxification of reactive nitrogen compounds (Nakanishi *et al.*, 2001). Diazo compounds may be formed by reaction between amine side groups with reactive nitrogen (e.g., nitrous acid, hydroxylamine), which is particularly important at low pH, although they have been reported to occur rarely naturally (Nawrat *et al.*, 2011).

Species-specific features of individual Ca. *Nitrosotalea genomes*

Several unexpected species-specific genes were observed in the newly determined *Ca.* Nitrosotalea genomes. For example, *Ca*. Nitrosotalea bavarica SbT1 harbours an archaeal (type III) RuBisCO (SCTHAUMv1_33063) implicated in CO_2 fixation, although other key Calvin cycle genes (e.g. phosphoribulokinase) are missing. This gene may function in the AMP salvage pathway as described for hot spring *Thaumarchaeota* (Beam *et al.,* 2014), particularly as another gene of the same pathway, encoding an AMP phosphorylase (SCTHAUMv1_33062), is located adjacent to the RuBisCO-encoding gene. In other archaea, e.g., *Pyrococcus furiosus*, excess AMP can be generated through saccharolytic activity, but *Ca*. N. bavarica SbT1 contains no ADP-dependent phosphofructokinase homologue or other recognisable ADP-dependent sugar kinases. As proposed for *Thermococcus kodakaraensis* (Sato *et al*., 2007), AMP may be produced instead through degradation of 5-phosphoribosyl 1-pyrophosphate (PRPP) by adenine phosphoribosyltransferase (SCTHAUM_10121), which is also encoded in other Thaumarchaeota. PRPP is produced in Thaumarchaeota by ribose-phosphate pyrophosphokinase (SCTHAUM_90122), as part of nucleotide biosynthesis, and can also spontaneously break down into ribose-1,5-BP. This could then be converted into ribulose 1,5-bisphosphate by ribose 1,5-bisphosphate isomerase of *Ca.* N. bavarica SbT1 (SCTHAUM_70401), providing a substrate for the RuBisCO, as demonstrated in the methanogenic archaeon *M. jannaschii* (Finn *et al.*, 2004), linking the pentose phosphate pathway and gluconeogenesis. Although *Ca.* N. bavarica SbT1 has the homologue of ribose-1,5-bisphosphate isomerase found in *M. jannaschii* (Mj0601) (Finn *et al.*, 2004), related proteins are also implicated in thiazole metabolism (Hwang *et al.*, 2014), and the function of the protein and the existence of this pathway in *Ca.* N. bavarica SbT1 needs to be verified experimentally, when a cultured member of this species becomes available.

Ni-Fe hydrogenase, gas vacuoles, genes for flagellar motility and chemotaxis, and phosphate utilisation genes are also encoded by some but not all genomes of *Ca*. Nitrosotalea (Table

SI.2.7), providing testable hypotheses for adaptations of *Ca.* Nitrosotalea strains to factors other than pH.

Interestingly, the *Ca.* N. okcheonensis CS genome has two *amoA* gene copies in contrast to all previously genome-sequenced AOA, which have a single *amoA* gene. One copy (NCS_11555) was found in the canonical arrangement *amoAxCB*, as in other *Ca.* Nitrosotalea genomes, and is transcribed during growth in batch culture (SI.1). The second copy (NCS_11033), which is located >400 kb upstream from the *amoAxCB* gene cluster, shares 95.5% DNA similarity with the first, but was not transcriptionally active under standard growth conditions (Figure S12). The local genomic region surrounding each *amoA* gene was confirmed by PCR amplification using primers designed to hybridize to adjacent ORFs. Multiple copies of the *amoCAB* operon can be found in AOB, and additional isolated copies of *amoC* can be found in both AOA and AOB (Spang *et al.*, 2012). In addition, two divergent copies of *amoB* were recently reported in the marine AOA *Ca.* N. piranensis D3C (Bayer *et al.*, 2016). The isolated *amoC* gene in *Nitrosomonas europaea* is not transcribed during growth, but only during a post-starvation stress response (Berube *et al.*, 2012). It is difficult to predict if, and under which conditions, the genomically isolated *amoA* gene of *Ca.* N. okcheonensis CS is transcribed, but its existence has immediate implications for molecular studies of AOA in the environment. The *amoA* gene is the most widely used marker for determining AOA and AOB diversity and abundance in environmental samples and the existence of two non-identical copies of this gene may lead to overestimation of AOA diversity and abundance, given the common assumption of one *amoA* gene per AOA genome (Trias *et al.*, 2012).

In conclusion, comparative genomics of four *Ca.* Nitrosotalea species enabled identification of a core set of gene families for this genus encompassing 103 gene families. Expression of the majority of these genes families was confirmed by proteomics under laboratory conditions and metatranscriptomics in an incubation experiment with acidic peat soil. Although the four analysed *Ca.* Nitrosotalea species all thrive at low pH, their genomic core excluded many gene families that were previously proposed to represent adaptations of *Ca.* N. devanaterra Nd1 to acidic environments (Lehtovirta-Morley *et al.*, 2016). Interestingly, some of the core genes with an inferred function for acidophily were clearly acquired by *Ca.* Nitrosotalea via horizontal gene transfer from other microbial groups, including the acidophilic *Thermoplasmatales*, demonstrating that adaptation of *Ca*. Nitrosotalea members to their low pH environment was facilitated by implementation of mechanisms having evolved in other microbes of these systems. It will be interesting to explore whether similar mechanisms for pH adaptation are also used by other (non-*Ca*. Nitrosotalea) thaumarchaeotal lineages that are abundant in acidic soils (Gubry-Rangin *et al.*, 2011), but for which no genome sequences are yet available.

Experimental procedures

Thaumarchaeotal genomes

Genomes of four members of the genus *Ca.* Nitrosotalea, abundant in acidic soils, were compared in this study. Three of the genomes originated from pure cultures or enrichments: *Ca.* Nitrosotalea devanaterra Nd1 was isolated from a Scottish agricultural soil (pH 4.5) and its complete genome was recently sequenced (Lehtovirta-Morley *et al.*, 2016), *Ca.* Nitrosotalea sinensis Nd2 was isolated from a Chinese acidic paddy soil (pH 4.7) (Lehtovirta-Morley *et al.*, 2014) and *Ca.* Nitrosotalea okcheonensis CS was enriched from a Korean soil (pH 3.2) contaminated with acid mine drainage water (Jung *et al*., 2014). Details of the cultivation, DNA extraction, genome sequencing, and assembly are given in SI. In contrast, the genome of *Ca.* Nitrosotalea bavarica strain SbT1 was assembled and binned from a metagenomic dataset of the minerotrophic fen Schlöppnerbrunnen II (50°07’54.8’’ N, 11°52’51.8’’ E, 713 m above sea level, typical pH 4 - 5), located in the Fichtelgebirge Mountains in north-eastern Bavaria, Germany (Pester *et al.*, 2010; Hausmann *et al.*, 2016). For further details on this metagenomic experiment see SI.1 and Hausmann *et al.* (2016).

In addition to the four *Ca.* Nitrosotalea genomes, 19 other thaumarchaeotal genomes were compared (Table 1). For all 23 genomes, genome composition completeness was estimated using thaumarchaeotal-based arCOG markers (Table SI.2.8) and CheckM (Parks *et al.*, 2014), while genus and species assignments were evaluated using ANI and AAI (Konstantinidis and Tiedje, 2005) (see details in SI.1).

Comparative genomics

The core and flexible genomes of *Ca.* Nitrosotalea were identified using OrthoMCL (Li *et al.*, 2003), which uses a Markov Cluster algorithm to assign coding sequences to orthologous groups based on all-against-all BLASTp (Table SI.2.9). The core genome for AOA was defined as all orthologue groups for which all AOA had at least one coding sequence. Accordingly, the core genome of specific genera of AOA (e.g., *Ca.* Nitrosotalea) was defined as all orthologue groups for which all members had at least one coding sequence and no other AOA possessed a corresponding orthologue. The flexible genome was defined as orthologue groups which contained coding sequences from multiple AOA, but not from all AOA. Theoretical core genome and pangenome sizes were estimated (SI.1), while genomic synteny was calculated between all *Ca.* Nitrosotalea genomes (SI.1 and figure S13).

Origin of individual gene families comprising the Candidatus *Nitrosotalea core genome*

Gene families that comprised the core genome of *Ca.* Nitrosotalea were examined to identify possible origin scenarios. Each gene was used as a query in a blastp search against the Genbank nr protein database using default parameters, except returning up to 1,000 subjects for each query. All hits that matched at least one query over 70% of its length and with >30% identity was collected as "database homologues" (Table 1). From this set of database homologues, usearch (Edgar, 2016) was used to cluster database entries at 95% amino acid identity. Centroids were aligned using mafft (Katoh *et al*., 2002) and preliminary trees were constructed using FastTree (Price *et al*., 2009). Gene families were classified as "*Ca.* Nitrosotalea-specific" if no database entries outside the known *Ca.* Nitrosotalea were identified with blastp. Gene families were classified as "*Ca.* Nitrosotalea-specific with low AA-identity to non-AOA" if there were only one to three database matches at low identity (30-45% amino acid identity). The remainder of the gene families of the *Ca.* Nitrosotalea core genome were examined phylogenetically. If a gene family formed a clade with other Thaumarchaeota, it was assumed to be a divergent form of the homologue in other Thaumarchaeota. Phylogenetic trees were recalculated using RaxML (Stamatakis *et al*., 2015) for the remaining gene families, to verify the relationship of the *Ca.* Nitrosotalea gene family with its nearest phylogenetic neighbour(s), which were inferred to be the donor lineage of that gene family to a *Ca.* Nitrosotalea common ancestor.

Phylogenomic and phylogenetic approaches

Two independent phylogenomic approaches were implemented, maximum-likelihood on 198 phylogenetically congruent single-copy marker genes (Figure 2) or Bayesian-likelihood on 34 universal marker genes subset identified with CheckM (Figure S2c; Parks *et al.*, 2015). In addition, the 16S rRNA (Figure S2a) and *amoA* (Figure S2b) gene Bayesian phylogenies were performed as described in Gubry-Rangin *et al.* (2015). More details on these approaches can be found in SI.1.

Experimental validation of in silico *predictions*

To confirm the presence of two *amoA* genes (ORF11033 and 11555) in *Ca.* N. okcheonensis CS and generate qPCR standards for expression analysis, PCR primers were designed that hybridised at positions within adjacent predicted ORFs (11032/11034 and 11554/11556), with a further set of primers that hybridised within ORF11033 and ORF11555 to amplify mRNA transcripts (Table SI.2.10). Total RNA was extracted from cells harvested from 500 ml of exponentially growing culture using the RNeasy Mini Kit (Qiagen, Germany) and cDNA synthesised using the SuperScript First Strand synthesis system (Invitrogen, San Diego, CA) according to manufacturer's instructions. Concentrations of RNA and cDNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantitative real-time PCR experiments were carried out using a MiniOpticon real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and Opticon Monitor Software version 3.1 (Bio-Rad Laboratories, Hercules, CA). Thermal cycling parameters were 15 min at 95°C, followed by 40 cycles at 95°C for 20 s, 55°C for 20 s and 72°C for 20 s, with readings recorded after each cycle. A control 16S rRNA gene assay was also performed as described previously (Jung *et al*., 2014). PCR efficiency was 87 - 95% with r^2 values $\geqslant$0.99 for all assays.

Genomic *in silico* predictions in *Ca.* Nitrosotalea strains were validated by analysing the proteomic profiles of two of the three cultured thaumarchaeotal strains after growth under optimal conditions (pH = 5.3 and 5.0 for *Ca.* N. sinensis Nd2 and *Ca.* N. okcheonensis CS, respectively). Cells from seven replicate cultures (1000-mL and 500-mL each for strains Nd2 and CS, respectively) were harvested individually by filtration and stored at -80°C upon protein extraction with denaturing SDS buffer and proteomic analysis by LC-MS (see SI.1). Genomic *in silico* predictions in *Ca.* Nitrosotalea strains were validated by metatranscriptomics analysis of samples from anoxic peat soil microcosms with or without amendments of several organic compounds (see Hausmann *et al.*, 2016 and SI.1).

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Tables

Table 1. AOA used for the comparative genome analysis.

Organism	**Source/accession number**	**Completeness (%)**	
		arCOG	**CheckM (1)**
Ca. Nitrosopumilus maritimus SCM1(2)	CP000866.1	100.00	100.0 (0.97)
Ca. Nitrosopumilus sp. SJ	NZ_AJVI00000000.1	97.50	96.12 (0)
Ca. Nitrosopumilus sp. AR1	CP003842.1	96.67	94.66 (0)
Ca. Nitrosopumilus sp. AR2	CP003843.1	97.50	97.09 (0)
Ca. Nitrosopumilus salaria BD31	NZ_AEXL00000000.2	92.50	92.39 (1.94)
Ca. Nitrosopumilus sp. D3C	CP010868.1	100.00	100.0 (0.97)
Ca. Nitrosopumilus sp. NF5	CP011070.1	100.00	100.0 (0)
Ca. Nitrosoarchaeum koreensis MY1	AFPU01000001.1	100.00	100.0 (0)
Ca. Nitrosoarchaeum limnia BG20	NZ_AHJG00000000.1	100.00	99.03 (5.83)
Ca. Nitrosoarchaeum limnia SFB1	CM001158.1	99.17	98.06 (0)
Ca. Nitrosopelagicus brevis CN25	NZ_CP007026.1	100.00	99.51 (0)
Ca. Cenarchaeum symbiosum A	DP000238.1	98.33	99.03 (0)
Ca. Nitrosotenuis uzonensis N4	NZ_CBTY000000000.1	100.00	100.0
Ca. Nitrosotenuis chungbukensis MY2	NZ_AVSQ00000000.1	98.33	99.03 (0.97)
Ca. Nitrosotenuis cloacae SAT1	CP011097.2	99.17	100.0
Ca. Nitrosotalea okcheonensis CS	ERS1465380	99.17	99.51 (0)
Ca. Nitrosotalea sinensis Nd2	ERS1465381	100.00	99.51 (0.97)
Ca. Nitrosotalea devanaterra Nd1(2)	ERS884509	100.00	98.54 (0)
Ca. Nitrosotalea bavarica SbT1	ERS1572876	98.33	96.60 (0.97)
Ca. Nitrosocosmicus oleophilus MY3	CP012850.1	99.17	98.06 (0.97)
Nitrososphaera viennensis EN76(2)	CP007536.1	100.00	100.0 (0.97)
Ca. Nitrososphaera evergladensis SR1(2)	CP007174.1	100.00	100.0 (2.91)
Ca. Nitrososphaera gargensis Ga9.2(2)	CP002408.1	100.00	100.0 (2.91)

(1) In addition to the genomic completeness, CheckM software predicts the level of genomic contamination (in brackets) as a proportion of multiple copies, in the genome of interest, of known conserved single-copy genes in closely related genomes.

(2) Closed genomes

Table 2. List of horizontally acquired genes identified in the "*Nitrosotalea*-specific core" gene set.

Orthologous group	**Predicted function**	**Genbank Accession for *Ca.* N. devanaterra**	**Present in other AOA**	**Database homologues (# used for phylogenies)(1)**	**Phylogenetically inferred gene exchange partner**	**Environment of gene exchange partner**	**Proteins detected in *Ca.* N. sinensis or *Ca.* N. okcheonensis cultures**	**Transcripts detected in *Ca.* N. bavarica metatranscriptome**
OG2531	Divalent heavy-metal cation transporter (zinc permease?)	CUR51883.1	No	55 (55)	Thermoplasmatales	Acidic soil	No	Yes
OG2888	Na^+/H^+ solute symporter	CUR52062.1	No	999 (462)	Thermoplasmatales or, Crenarchaeota	Acidic hot springs and acid mine drainage	No	Yes
OG2912	acpD | FMN-dependent NADH-azoreductase	CUR52158.1	No	340 (183)	Ca. Div. Dependentiae,	Terrestrial aquifer sediment	No	No
OG2924	mntH | NRAMP family Mn^{2+}/Fe^{2+} transporter	CUR51850.1	No	966 (477)	Woesarchaeota	Terrestrial aquifer sediment	No	Yes

OG2933	Coiled-coil motif protein	CUR52192.1	No	11 (11)	Thermoplasmatales	Acid mine drainage	Yes	Yes
OG2943	FKBP-type peptidyl-prolyl cis-trans isomerase	CUR51294.1	No	937 (513)	Methanosarcinales	Anaerobic environments	Yes	Yes
OG2932	Putative phage protein	CUR52193.1	No	7 (7)	Thermoplasmatales	Acid mine drainage	Yes	Yes
OG2113	Unknown (pentapeptide repeat containing protein)	CUR51439.1	Yes	1652 (1132)	*Sneathiella glossodoripedis*	Marine invertebrate symbiont	Yes	Yes

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(1) Orthologues identified in database were clustered at 95% amino-acid identity prior to phylogenetic analysis.

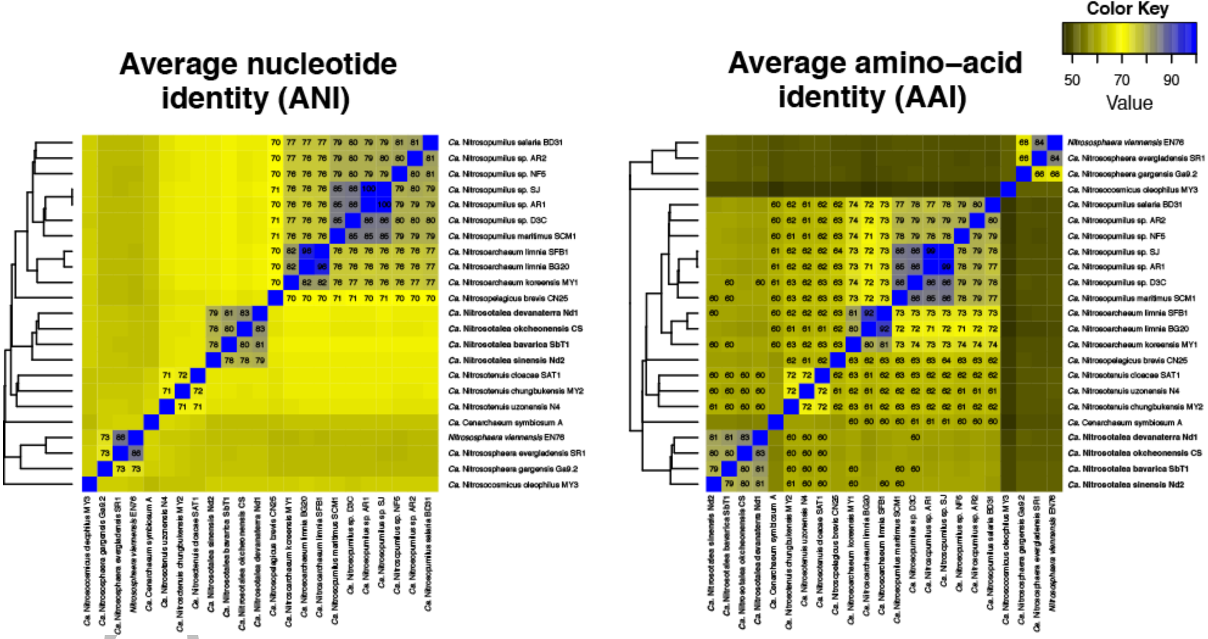
Table 3. Re-evaluation of *Ca.* N. devanaterra-specific genes proposed to be involved in acidophily in Lehtovirta-Morley *et al.* 2016. In that paper, *Ca.* N. devanaterra-specific genes that possessed homologues to other acidophilic microbes were considered as candidate genes involved in acidophily. In total, 51 genes were identified by that procedure. This table shows that only 10 of these genes are present in all four *Ca.* Nitrosotalea genomes and have no homologues in other non-acidophilic thaumarchaeotes. In addition, four of the previously identified genes are present in all four *Ca.* Nitrosotalea genomes and in the AOA *Ca.* Nitrosocosmicus oleophilus MY3.

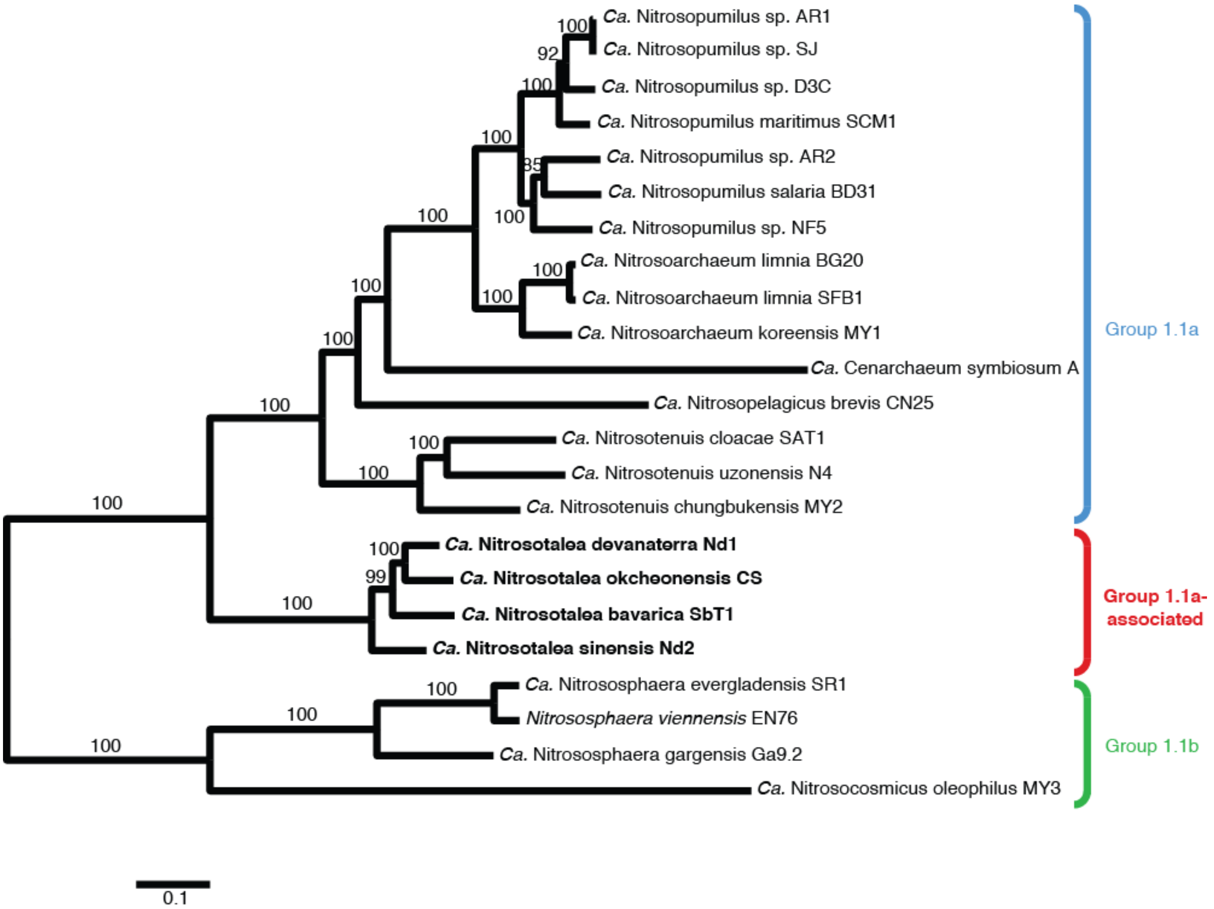
Locus ID	Product	*Ca. N. sinensis Nd2*	*Ca. N. bavarica SbT1*	*Ca. N. okcheonensis NCS*	Acquired by HGT	Detected in soil metatranscriptome	Detected in proteome of NCS	Detected in proteome of Nd2
Genes present in the *Ca.* Nitrosotalea–specific core that were previously postulated as *Ca.* N. devanaterra-specific genes involved in acidophily								
NDEV_0529	FKBP-type peptidyl-prolyl cis-trans isomerase	▨	▨	▨	■	■	■	
NDEV_0651	Coiled-coil motif protein	▨	▨	▨		■		
NDEV_0721	Protein of unknown function	▨	▨	▨		■		■
NDEV_0771	Exported protein of unknown function	▨	▨	▨		■		
NDEV_1085	NRAMP family Mn^{2+}/Fe^{2+} transporter	▨	▨	▨	■	■		
NDEV_1297	Na+/solute symporter	▨	▨	▨	■	■		
NDEV_1333	Exported protein of unknown function	▨	▨	▨		■		
NDEV_1368	Chromosome segregation ATPase-like protein	▨	▨	▨		■		■
NDEV_1562	Protein of unknown function	▨	▨	▨				■
NDEV_1577	Membrane protein of unknown function	▨	▨	▨				
Genes present in the *Ca.* Nitrosotalea–specific core that were previously postulated as *Ca.* N. devanaterra-specific genes involved in acidophily and that have homologues in *Ca.* Nitrosocosmicus oleophilus MY3								
NDEV_1587	Na^{+}/H^{+} exchanger	▨	▨	▨		■		
NDEV_1999	Archaeal/V-type ATP synthase subunit I	▨	▨	▨		■		■
NDEV_2005	Archaeal/V-type ATP synthase subunit F	▨	▨	▨		■		■
NDEV_2006	Archaeal/V-type ATP synthase subunit C	▨	▨	▨		■	■	■

Figure legends

Figure 1: Heat maps showing pairwise ANI and AAI values inferred from the four *Ca.* Nitrosotalea genomes (bold) and other available Thaumarchaeota genomes. Dendrograms were calculated by hierarchical clustering (hclust, method=complete linkage) in R with distances calculated as (100% - %identity, i.e. 100% ANI = 0 distance). For ANI and AAI, values above 70% and 60%, respectively, are indicated on the heat map.

Figure 2: Phylogenetic relationship of *Ca.* Nitrosotalea genomes (bold) with other sequenced AOA based on a RaxML phylogenetic analysis of 198 concatenated single-copy universal arCOG markers. Bootstrap values for internal branches are shown.




Ca. Nitrosopumilus sp. AR1
Ca. Nitrosopumilus sp. SJ
Ca. Nitrosopumilus sp. D3C
Ca. Nitrosopumilus maritimus SCM1
Ca. Nitrosopumilus sp. AR2
Ca. Nitrosopumilus salaria BD31
Ca. Nitrosopumilus sp. NF5
Ca. Nitrosoarchaeum limnia BG20
Ca. Nitrosoarchaeum limnia SFB1
Ca. Nitrosoarchaeum koreensis MY1
Ca. Cenarchaeum symbiosum A
Ca. Nitrosopelagicus brevis CN25
Ca. Nitrosotenuis cloacae SAT1
Ca. Nitrosotenuis uzonensis N4
Ca. Nitrosotenuis chungbukensis MY2
Ca. Nitrosotalea devanaterra Nd1
Ca. Nitrosotalea okcheonensis CS
Ca. Nitrosotalea bavarica SbT1
Ca. Nitrosotalea sinensis Nd2
Ca. Nitrososphaera evergladensis SR1
Nitrososphaera viennensis EN76
Ca. Nitrososphaera gargensis Ga9.2
Ca. Nitrosocosmicus oleophilus MY3
Group 1.1a
Group 1.1a-associated
Group 1.1b
100
92
100
100
85
100
100
100
100
100
100
100
100
100
100
100
99
100
100
100
100
100
0.1