Ammonia oxidation is the first and rate-limiting step in nitrification and is dominated by two distinct groups of microorganisms in soil: ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). AOA are often more abundant than AOB and dominate activity in acid soils. The mechanism of ammonia oxidation under acidic conditions has been a long-standing paradox. While high rates of ammonia oxidation are frequently measured in acid soils, cultivated ammonia oxidizers grew only at near-neutral pH when grown in standard laboratory culture. Although a number of mechanisms have been demonstrated to enable neutrophilic AOB growth at low pH in the laboratory, these have not been demonstrated in soil, and the recent cultivation of the obligately acidophilic ammonia oxidizer “Candidatus Nitrosotalea devanaterra” provides a more parsimonious explanation for the observed high rates of activity. Analysis of the sequenced genome, transcriptional activity, and lipid content of “Ca. Nitrosotalea devanaterra” reveals that previously proposed mechanisms used by AOB for growth at low pH are not essential for archaeal ammonia oxidation in acidic environments. Instead, the genome indicates that “Ca. Nitrosotalea devanaterra” contains genes encoding both a predicted high-affinity substrate acquisition system and potential pH homeostasis mechanisms absent in neutrophilic AOA. Analysis of mRNA revealed that candidate genes encoding the proposed homeostasis mechanisms were all expressed during acidophilic growth, and lipid profiling by high-performance liquid chromatography–mass spectrometry (HPLC-MS) demonstrated that the membrane lipids of “Ca. Nitrosotalea devanaterra” were not dominated by crenarchaeol, as found in neutrophilic AOA. This study for the first time describes a genome of an obligately acidophilic ammonia oxidizer and identifies potential mechanisms enabling this unique phenotype for future biochemical characterization.
presents a further challenge to cellular pH homeostasis. Almost all known prokaryotic acidophiles maintain a cytoplasmic pH higher than that in the extracellular environment (14). Extreme acidophiles usually have a reverse membrane potential (inside positive), and the proton motive force is constituted by the transmembrane pH gradient. There are several mechanisms of pH adaptation in acidophiles. In some, reverse membrane potential is achieved by uptake of cations (typically potassium), and most acidophile genomes encode a high number of cation transporters (15). The entry of protons is prevented by altering the membrane composition or modifying the cell surface structures (16, 17). Some acidophiles have proton pumps coupled to the electron transfer chain that actively remove protons from the cytoplasm (18). In addition, protons can be scavenged by buffering, e.g., by carbonic anhydrase in *Helicobacter pylori* (19), or metabolism, e.g., by arginine- and glutamate decarboxylase-based acid resistance mechanisms (20). Since small organic acids can function as uncouplers by passing through the membrane before releasing protons and acidifying the neutral pH cytoplasm, most extreme acidophiles are heterotrophs (15, 21). Acidophiles typically contain a large proportion of secondary transporters coupled to ion gradients (22). In addition, DNA and protein repair genes are prevalent in extreme acidophile genomes and are responsible for the rapid repair required after damage by low pH (15).

“Ca. Nitrosotalea devanaterra” is able to grow and oxidize ammonia within the pH range 4 to 5.5 and is unable to grow at neutral pH (6). This physiology makes “Ca. Nitrosotalea devanaterra” unique among all bacterial and archaeal ammonia oxidizers and indicates adaptations distinct from those of other cultured ammonia oxidizers. There are several major unresolved questions regarding acidophilic ammonia oxidation, as follows. (i) How does “Ca. Nitrosotalea devanaterra” overcome low NH$_3$ concentrations? Specifically, can this organism utilize NH$_4^+$, is the active site of AMO facing the cytoplasm or the periplasm and are there other N metabolism genes that can explain the distinct physiology of “Ca. Nitrosotalea devanaterra”? (ii) How does “Ca. Nitrosotalea devanaterra” fix carbon under acidic conditions where the HCO$_3^-$ concentration is low? (iii) Is cytoplasmic pH homeostasis of “Ca. Nitrosotalea devanaterra” achieved by mechanisms similar to those in other acidophiles? The aim of this study was therefore to examine the “Ca. Nitrosotalea devanaterra” genome for evidence of specific adaptations in N and C metabolism and to determine whether the genome contained genes involved in pH homeostasis mechanisms found in other model acidophiles.

**MATERIALS AND METHODS**

**DNA isolation.** “*Candidatus Nitrosotalea devanaterra*” Nd1 was grown in pure culture as previously described (7). Biomass from 10 liters of the culture was concentrated using a Pellicon XL tangential-flow filter cassette (pore size, 0.22 μm) (Merck Millipore, Billerica, MA, USA), followed by pelleting of cells by centrifugation (15 min at 18,000 × g). High-molecular-weight genomic DNA was extracted from a cell pellet by chemical lysis (23). Briefly, cells were lysed in the presence of proteinase K (final concentration, 100 μg ml$^{-1}$) and SDS (0.5% [wt/vol]), followed by addition of cetyltrimethylammonium bromide (CTAB) in NaCl solution (1% CTAB [wt/vol] and 0.7 M NaCl [final concentrations]) in a final volume of 780 μl. Proteins were removed using an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1) and DNA precipitated in the presence of 7.5 μl linear acrylamide with a 0.7 volume of 100% isopropanol. After centrifugation, the pellet was washed with 70% ethanol and resuspended in Tris-EDTA (TE) buffer. PacBio sequencing was necessary for genome closure, and as larger quantities of DNA (10 μg) were required, approximately 40 liters of culture was harvested by tangential-flow filtration and the extraction was performed using a DNeasy blood and tissue kit (Qiagen, Venlo, Netherlands). A minor modification to the manufacturer’s protocol was made: DNA was eluted five times with 50 μl prewarmed distilled water (dH$_2$O) and concentrated using a vacuum concentrator (Eppendorf, Hamburg, Germany). The molecular weight of DNA was assessed by agarose gel electrophoresis, and the concentration was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and a PicoGreen Quant-IT kit (Invitrogen, Carlsbad, CA, USA).

**Genome sequencing, assembly, and deposition.** DNA was sequenced using an Illumina MiSeq sequencer (flow cell v3 with 300-bp paired-end and 2-kb mate pair libraries) and with a PacBio RSII (GTAC, Constance, Germany) that provided 50X and 10X genome coverage, respectively. Assembly of Illumina reads was performed with CLC (CLC bio) and that of PacBio reads with Celera 8.1 (24) and SPAdes 3.1.1 (25). Illumina reads were first quality trimmed, several assembly parameters were tested, and finally the assembly was performed with a word size of 45, a bubble size of 98, and a minimum contig length of 1,000 bp. PacBio reads were error corrected using the PBcR pipeline in Celera and subsequently assembled. Neither approach alone was sufficient to close the genome, and a hybrid assembly of PacBio and Illumina reads was performed with SPAdes. Different assemblies were compared and validated using MAUVE software (26). The entire genome was annotated in MaGe (27) based on a combination of bioinformatics tools, including homology-, structure-, and synteny-based approaches. Other AOA genomes are included in MaGe, which relies on the PfGBDA relational database with data from both public data banks and manually curated genomes. Manual curation of the “Ca. Nitrosotalea devanaterra” genome was performed in MaGe, with emphasis on genes absent in other AOA, genes located at synteny breaks, genes characterized in other AOA, and functions involved in major metabolic pathways. Functional categorization into clusters of orthologous genes (COGs) and protein families ( Pfams) was performed with COGnitor and HMMMPfam software implemented in MaGe, respectively. tRNA predictions were made using tRNAscan-SE (28) and ARAGORN (29). Transporters were annotated using TransAAP implemented in TransportDB (30). Average nucleotide identity (ANI) and tetraneucleotide frequency analyses were carried out in JSpecies (31). z scores of the nucleotide word frequency analysis were used to build the matrix for the principal-component analysis in PAST software (32) and SigmaPlot v12 (Systat Software Inc., London, United Kingdom). To construct Venn diagrams, the presence of open reading frames (ORFs) in archaeal genomes was identified using reciprocal BLASTP searches (selected cutoffs were an E value of 10$^{-7}$ and 30% amino acid identity) implemented in the stand-alone BLAST-2.2.25+ suite (33). The cutoff for absence and presence of coding sequences (CDS) in comparison with other archaeal and bacterial genomes was selected as 30% DNA identity with 80% CDS coverage, as recommended in the MaGe guidelines. Synteny analysis was performed in MaGe (27).

**Identification of genes potentially linked to acidophily.** To identify candidate genes enabling acidophily, the “Ca. Nitrosotalea devanaterra” genome was compared to those of previously characterized archaeal and bacterial acidophiles to detect gene homologues conserved between “Ca. Nitrosotalea devanaterra” and acidophiles and absent in other AOA (cut-off, 30% DNA identity with 80% CDS coverage). However, as “Ca. Nitrosotalea devanaterra” represents an uncharacterized genus, many ORFs had no database hits in RefSeq, Swiss-Prot, and TrEMBL. Many ORFs also had homology only to uncharacterized proteins in databases. In addition, some (novel) acid tolerance mechanisms may be a result of convergent evolution and not share genetic homology. Therefore, a literature search was performed on pH adaptation strategies of acidophilic microorganisms, as well as on biochemical limitations of ammonia oxidizers with regard to pH, and potential functional analogues were manually identified from the curated “Ca. Nitrosotalea devanaterra” genome.
Lipid analysis. The intact polar and core lipids of “Ca. Nitrosotalea devanaterra” were analyzed as described previously (82). For analysis of intact polar lipids, an aliquot of Bligh-Dyer extracts (BDEs) was analyzed with high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS). For analysis of core glycerol dibiphytanyl glycerol tetraether lipids (GDGTs), acid hydrolysis was performed on aliquots of BDEs to cleave off polar head groups and release core GDGTs, which were analyzed by HPLC-atmospheric pressure chemical ionization–MS (HPLC-APCI-MS) using a modified procedure (34).

Sequence alignments, protein structure predictions, and phylogenetic analysis. Sequence alignments of AmoB, AmoC, and Amo/Rh genes were generated using ClustalW implemented in BioEdit (35) and used to examine active-site conservation and for phylogenetic analysis. For AMO, all AOA amoB and amoC sequences deposited in GenBank (July 2014) were used in alignments, and the active site of AMO was modeled against PmoB and PmoC of Methylococcus capsulatus Bath and other previously characterized model organisms (36–39). This resulted in an alignment of 115 and 147 sequences for amoB and amoC, respectively. Transmembrane helices and protein topology were predicted initially using TMHMM (40). However, TMHMM failed to detect some of the helices that experimentally have been indicated to be present in AOA AmoB sequences (39), and TMPred (41) and TopPred (42) were subsequently used. Signal peptides were initially predicted by SignalP (43), which also failed to detect signal peptides of AmoB of AOA (39), and prediction was implemented in Phobius (44). Phobius was used additionally for validation of the transmembrane helix predictions. Phobius uses both signal peptides and transmembrane helices in its topology prediction, which requires that amino acid residues immediately downstream from the signal peptide cleavage site must be extracellular.

For Amo/Rh, the database was compiled from a selected set of sequences (total, 89) from GenBank, with the emphasis on sequences that had been previously characterized electrophysiologically and structurally. Phylogenetic analysis of derived Amo/Rh protein sequences used 228 unambiguously aligned positions. Maximum-likelihood analysis was performed with PhyML (45) with invariable sites and eight variable gamma rate modes modeled. Bootstrap support was also calculated using parsimony and distance analyses (MEGA6 [46]) with 1,000 replicates.

Transcriptional and physiological response to pH change. Exponentially growing “Ca. Nitrosotalea devanaterra” cultures (pH 5) were transferred into prerinsed and autoclaved dialysis bags (Spectra/Por 4, molecular mass cutoff, 12 to 14 kDa [Spectrum Labs, Rancho Dominguez, CA, USA]) and transferred twice to fresh medium (pH 5) to remove nitrite to avoid inhibition before transfer to media at pH 4.0, 5.0, and 6.0. The pH was recorded at the start and end of the experiments, and mean pH values were 4.2, 5.5, and 6.2 and 4.2, 5.6, and 6.5, respectively. At each time point, three replicate dialysis bags were destructively sampled. Nitrite was measured inside and outside the bag, and 1 ml of cells was used for transcriptional analysis. Cell and amoA abundances were estimated at all time points for all replicates. Phenol–ethanol (1:19) stop solution was added immediately after sampling to preserve transcripts before storing at −80°C until RNA extraction. Nucleic acid extraction was performed by phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were precipitated in the presence of 7.5 µg linear acrylamide, 0.1 volume 3 M sodium acetate, and an equal volume of 100% ethanol, followed by DNase treatment and reverse transcription (47). Primers were designed for selected transporters and putative pH homeostasis genes using Primer3 (see Table S1 in the supplemental material) (48), and quantitative PCR (qPCR) was performed with Quantitative PCR (Qiagen) for all the assays using a two-step cycle: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 10 s, combined annealing/extension at 60°C for 30 s, and fluorescence reading at 72°C, followed by a final extension at 72°C for 10 min, followed by the melt curve from 55°C to 95°C. The quality of qPCR products was verified by melt curve analysis and agarose gel electrophoresis. amoA qPCR was performed as previously described (6). Statistical analysis was performed in SigmaPlot v12 (Systat Software Inc., London, United Kingdom). To determine whether there was a significant increase or decrease in transcript abundance over time, linear regression was performed using individual (rather than averaged) values of transcript/gene data as the dependent variable and time as the independent variable. This analysis was additionally performed for nitrite accumulation, assessed with Griess reagent as previously described (7).

Nucleotide sequence accession number. The closed genome sequence with full annotations has been deposited in ENA with accession number LN902860.

RESULTS AND DISCUSSION

Genome summary. The closed and complete genome of “Ca. Nitrosotalea devanaterra” is a single circular 1,805,304-bp chromosome with 2,205 predicted genes (Table 1). The genome was automatically annotated prior to manual examination and curation, and a putative function was successfully assigned to 58.8% of ORFs and seven small RNA (smRNA) genes, 40 tRNA genes, and 1 copy of each rRNA gene (5S, 16S, and 23S), with putative introns and a putative function assigned to 58.8% of ORFs (Table 1). Approximately 15% (364) of ORFs of “Ca. Nitrosotalea devanaterra” are absent from other soil AOA genomes (see Fig. S1 in the supplemental material). Approximately 15% (364) of ORFs of “Ca. Nitrosotalea devanaterra” are unique when all aquatic AOA are included in this comparison. The distinction of genus Nitrosotalea from other AOA is supported by the low average nucleotide identity (ANI) scores between “Ca. Nitrosotalea devanaterra” and group 1.1a and 1.1b representatives (66.5 to 68.0% and 62.5 to 62.9%, respectively) (see Fig. S1 in the supplemental material).

“Ca. Nitrosotalea devanaterra” has major metabolic pathways that are conserved in all AOA and discussed elsewhere (49, 50), including biosynthesis of amino acids, lipids, and sugars and central C metabolism (see Table S2 in the supplemental material). In addition, “Ca. Nitrosotalea devanaterra” contains an Ni-Fe hydrogenase and genes for flagellar motility, gas vacuoles, and phosphorus utilization, which are present in some but not all AOA (see Tables S2 and S3 in the supplemental material). It is, however, beyond the scope of this article to discuss all of these genes individually, and this article highlights those features that could be associated with the adaptation of “Ca. Nitrosotalea devanaterra” to low-pH environments.

Similarities between “Ca. Nitrosotalea devanaterra” and other acidophiles. The fully annotated “Ca. Nitrosotalea devanaterra” genome contains 364 ORFs without homologues in neutrophilic AOA genomes, of which 45 were shared with characterized bacterial and archaeal acidophiles (see Table S4 in the supplemental material). No homologues were found exclusively in all acidophilic and archaeal acidophiles (see Table S4 in the supplemental material). It is, however, beyond the scope of this article to discuss all of these genes individually, and this article highlights those features that could be associated with the adaptation of “Ca. Nitrosotalea devanaterra” to low-pH environments.

Primers were designed for selected transporters and putative pH homeostasis genes using Primer3 (see Table S1 in the supplemental material) (48), and quantitative PCR (qPCR) was performed with Quantitative PCR (Qiagen) for all the assays using a two-step cycle: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 10 s, combined annealing/extension at 60°C for 30 s, and fluorescence reading at
NDEV_1462), and several subunits of archaeal A-type ATP synthase (complex V) which correspond to the membrane-bound A0 domain and the central stalk (NDEV_1999 [atpF], NDEV_2002 [atpD], NDEV_2005 [atpF], and NDEV_2006 [atpC]) (51) (see Table S5 in the supplemental material). Of these, cation transporters may function in cation uptake, ATP synthase may function in proton translocation, and FKBP-type peptidyl-prolyl cis-trans isomerase may facilitate protein folding. This modest conservation probably reflects the fact that bacterial and archaeal acidophiles have several strategies for pH adaptation (15). In addition, “Ca. Nitrosotalea devanaterra” may possess mechanisms enabling acidophily that are distinct from those of other acidophiles. The genome, with the emphasis on genes absent in other AOA, was therefore further investigated to identify potential mechanisms contributing to pH homeostasis and facilitating growth of AOA with low concentrations of NH₃ and HCO₃⁻.

**Ion transport.** Extreme acidophiles contain a large number of secondary transporters (22), but “Ca. Nitrosotalea devanaterra,” surprisingly, possesses a similar number of secondary transporters to the number possessed by other AOA (Table 1; see Table S5 in the supplemental material). Of these, cation transporters may function in cation uptake, ATP synthase may function in proton translocation, and FKBP-type peptidyl-prolyl cis-trans isomerase may facilitate protein folding. This modest conservation probably reflects the fact that bacterial and archaeal acidophiles have several strategies for pH adaptation (15). In addition, “Ca. Nitrosotalea devanaterra” may possess mechanisms enabling acidophily that are distinct from those of other acidophiles. The genome, with the emphasis on genes absent in other AOA, was therefore further investigated to identify potential mechanisms contributing to pH homeostasis and facilitating growth of AOA with low concentrations of NH₃ and HCO₃⁻.

**TABLE 1 Genomic features of selected thaumarchaea**

| Parameter                      | “Ca. Nitrosotalea devanaterra” Nd1 | “Ca. Nitrosopumilus koreensis” MY1 | “Ca. Nitrosopumilus maritimus” SCM1 | “Ca. Nitrososphaera viennensis” EN76 | “Ca. Nitrososphaera gargensis” Ga9.2 |
|--------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Length (Mb)                    | 1.81                              | 1.61                              | 1.65                              | 2.53                              | 2.83                              |
| Coding density (%)             | 92.1                              | 89.99                             | 91.7                              | 87.0                              | 83.4                              |
| No. of predicted CDS           | 2,205                             | 1,957                             | 1,967                             | 3,123                             | 3,997                             |
| % of annotated CDS            | 58.8                              | 62.1                              | 56.0                              | 46.7                              | 43.1                              |
| No. of:                        |                                   |                                   |                                   |                                   |                                   |
| 16-23S rRNA operons            | 1                                 | 1                                 | 1                                 | 1                                 | 1                                 |
| Separate 5S rRNAs              | 1                                 | 1                                 | 1                                 | 1                                 | 1                                 |
| tRNAs                         | 40                                | 42                                | 44                                | 44                                | 40                                |
| Other RNAs                     | 7                                 | 3                                 | 1                                 | 5                                 | 0                                 |
| Primary transporter genes      | 51                                | 38                                | 45                                | 66                                | 59                                |
| Secondary transporter genes    | 42                                | 41                                | 31                                | 44                                | 54                                |
| Other transporter genes        | 14                                | 14                                | 18                                | 22                                | 26                                |
| Ratio of secondary to primary transporter genes | 0.8:1 | 1.1:1 | 0.7:1 | 0.7:1 | 0.9:1 |

a Ratios of secondary to primary transporter genes in other archaeal acidophiles (41): *Thermoplasma acidophilum*, 10:1; *Picrophilus torridus*, 5.6:1; *Sulfolobus solfataricus*, 2.7:1.

Several of the “Ca. Nitrosotalea devanaterra” genes potentially involved in pH/ion homeostasis are colocalized on the chromosome (*mgta*, *mntH*, a carbonic anhydrase gene [NDEV_1082], a universal stress protein gene [NDEV_1080], and an α-acetolactate decarboxylase gene [NDEV_1077]). Interestingly, the region is flanked by a transposase gene, located at a synteny break, and the gene cluster shares a degree of synteny and homology with bacterial genomes (Fig. 2B; see Table S7 in the supplemental material). Potassium is typically a critically important solute for maintaining the reverse membrane potential in acidophiles (15), and it is therefore surprising that the “Ca. Nitrosotalea devanaterra” genome does not contain genes for potassium transporters which are absent in all neutrophilic AOA.

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Several bacterial acid resistance mechanisms are based on cytoplasmic proton consumption by various decarboxylases (20). All AOA contain an arginine decarboxylase (NDEV_1165) that is involved in arginine and polyamine metabolism but also in arginine-based acid resistance in bacteria. Due to its presence in neutrophilic AOA, it is an unlikely candidate for pH adaptation of “Ca. Nitrosotalea devanaterra.” In contrast to neutrophilic AOA, “Ca. Nitrosotalea devanaterra” harbors a homologue of α-acetolactate decarboxylase (NDEV_1077), which is upregulated in bacteria during pH stress (52) and is predicted to catalyze the conversion of α-acetolactate to acetoin (Fig. 1 and 2). This is unexpected, as acetoin production is energetically costly for an autotrophic organism and α-acetolactate decarboxylase activity is generally greater under anaerobic conditions (53). How-

May 2016 Volume 82 Number 9 Applied and Environmental Microbiology aem.asm.org 2611
ever, α-acetolactate is produced in all AOA from pyruvate as part of valine, leucine, and isoleucine biosynthesis.

Cell wall and membrane. The membranes of some acidophiles, e.g., *Picrophilus oshimae*, require a low pH for stability (16), and the lipid composition and proportion of glycolipids change with varying pH (17). "Ca. Nitrosotalea devanaterra" contains two large cell surface modification gene clusters (31 and 34 ORFs) that are absent in neutrophilic AOA, with one cluster located next to genes encoding the main S-layer protein. These genes share only modest homology (30 to 61.4% amino acid identity) with bacterial and a limited number of archaeal genes. However, COG- and Pfam-based functional classification and their synteny with bacterial genomes support their involvement in cell surface glycosylation (see Table S8 and Fig. S2 in the supplemental material). The gene clusters of "Ca. Nitrosotalea devanaterra" do not belong to the recognized archaeal S-layer glycosylation pathways of *Euryarchaeota* and *Crenarchaeota* (54, 55). In bacteria, these homologues function in synthesis of the polysialic acid capsule (*neu/kps*), pseudoaminic acid glycosylation of flagella (*pse*), and outer spore coat glycosylation of Gram-positive bacteria (*sps*) (but not in bacterial peptidoglycan synthesis or modification) (56–58). There is also no evidence for the presence of peptidoglycan or pseudopeptidoglycan in "Ca. Nitrosotalea devanaterra" compared to other AOA in agreement with culture studies of acidophilic archaea which show an increase in the number of cyclopentane moieties in GDGTs with decreasing pH (62, 63). Furthermore, the increase in rings is thought to increase the packing density of the lipid membrane (64), which may reduce membrane permeability. The head groups of the "Ca. Nitrosotalea devanaterra" GDGTs are dominated by sugar moieties (glycolipids), in agreement with previous reports on acidophilic archaea which showed an increase in glycolipids versus phospholipids with decreasing pH (16). An increase in sugar units on the outside cell wall has been suggested to provide protection against proton entry (65). Hydroxylated GDGTs were also detected as cores, as in group 1.1a AOA (60), consistent with the 16S rRNA and *amoA* gene phylogenetic placement of *Nitrosotalea* (6).

Central carbon metabolism and acidophily. "Ca. Nitrosotalea devanaterra" possesses a carbonic anhydrase (NDEV_1082) (Fig. 1 and 2), which is present in many acidophiles (see Table S4 in the supplemental material) and predicted to catalyze rapid re-

![FIG 1 Predicted mechanisms of cytoplasmic pH regulation in “Ca. Nitrosotalea devanaterra” based on the presence of putative functional genes in the genome.](http://aem.asm.org/)}
versatile interconversion of \( \text{HCO}_3^- \) and \( \text{CO}_2 \). Although found also in \textit{Nitrososphaera viennensis} and \textit{Candidatus Nitrososphaera vegglandensis}, carbonic anhydrase may have a dual function for carbon accumulation and cytoplasmic buffering in \textit{Ca. Nitrosotalea devanaterra}.

Depending on the direction of the reaction, carbonic anhydrase may prevent cytoplasmic acidification by breakdown of \( \text{HCO}_3^- \) or conversion of membrane-permeative \( \text{CO}_2 \) to \( \text{HCO}_3^- \) for carbon fixation. The reverse mechanism was proposed for carbon assimilation in \textit{N. europaea} (66), with \( \text{HCO}_3^- \) transporter and carbonic anhydrase genes colocated and \( \text{HCO}_3^- \) taken up, converted to \( \text{CO}_2 \), and fixed by the Calvin cycle.

**Ammonia oxidation at low pH.** The closed genome of \textit{Ca. Nitrosotalea devanaterra} does not carry any genes participating in N metabolism that could explain its adaptation to apparently low substrate concentrations at acid pH (e.g., with urease). As AMO genes and ammonium transporters are found in all AOA (49), this suggests that the adaptations enabling \textit{Ca. Nitrosotalea devanaterra} to grow with low ammonia concentrations either are based on differences from the active sites of AMO and transporters or reflect features shared by all AOA.

Since AOB cannot oxidize ammonia at low pH (8, 83), the substrate acquisition systems of \textit{Ca. Nitrosotalea devanaterra}, AOA, and AOB were compared. The three histidine residues (His 33, His 37, and His 139) in \textit{M. capsulatus} Bath [37]) of AmoB/PmoB coordinating a periplasmic dicopper center are conserved in methanotrophs, AOB, and AOA (Fig. 4) but not in the acidophilic methane-oxidizing \textit{Verrucomicrobia} (36, 37). In contrast, the variable metal binding site of AmoC is present in \textit{Verrucomicrobia}, AOB, and AOA (38) (Fig. 4). In \textit{silico} protein topology prediction for AmoB and AmoC favors an extracellular (outward-facing) location of the active site in \textit{Ca. Nitrosotalea devanaterra} (see Fig. S3 and S4 in the supplemental material). The active site of AMO is conserved between \textit{Ca. Nitrosotalea devanaterra}, AOA, and AOB and thus cannot explain the differences between these organisms.

Ammonium transporters of AOA and AOB are distantly related and belong to two functionally and phylogenetically distinct families: Amt and Rh, respectively (67). The substrate preferences (\( \text{NH}_4^+ \) versus \( \text{NH}_3 \)) often differ between Amt and Rh types (68, 69). Rh transporters are thought to perform bidirectional diffusion equilibrating \( \text{NH}_3 \) (70), and Amt transporters are energy dependent (71) (Fig. 5C). Furthermore, Amt transporters function better or equally well at acidic pH, whereas Rh transporters favor neutral/high pH (68, 72). Although archaean ammonium transporters were extensively reviewed recently (67), the transported species (\( \text{NH}_3 \) versus \( \text{NH}_4^+ \)) of Amt/Rh transporters remains uncertain, and no hypothesis has been put forward for the preferred substrate.

Homology and conservation of coordinating residues with other transporters indicate that \( \text{NH}_4^+ \) is bound to the Amt of \textit{Ca. Nitrosotalea devanaterra} (Fig. 5; see Table S9 in the supplemental material). The cation binding site (required for \( \text{NH}_4^+ \) transport) remains unoccupied in every Amt of all AOA and is absent in betaproteobacterial AOB (Fig. 5B) (73), as confirmed by the crystal structure of the N. europaea Rh protein (74). In addition, ammonium transporters may play a more critical role in AOA than in AOB, as all AOA genomes contain \( \approx 2 \) Amt transporters, while five of 10 publically available AOB genomes \textit{(N. eutropha C71, Nitrososomas cryotolerans ATCC 49181, Nitrosococcus watsonii C113, Nitrosococcus oceani AFC27, and N. oceani ATC19707)} lack ammonia transporters. Unlike neutrophilic AOA, \textit{Ca. Nitrosota-
“Ca. Nitrosotalea devanaterra” has two putative low-affinity Amts (Amt1a and Amt1b) and one high-affinity Amt (Amt2) with a conserved signal peptide (75). The conservation of the cation binding site suggests that the mechanism enabling “Ca. Nitrosotalea devanaterra” to acquire ammonium is shared with all AOA but not AOB.

Pii homologues are one of the most widespread signal transduction proteins in prokaryotes and have a vital and exclusive role in regulating N metabolism (76). Pii homologues are found in the vicinity of low-affinity Amt genes in neutrophilic group 1.1a AOA (Fig. 4C) (67). In addition, a Pii homologue is also located near the high-affinity Amt in “Ca. Nitrosotalea devanaterra” (Fig. 4C). Some AOB, e.g., *N. europaea*, lack Pii genes despite the presence of the Rh transporter (66).

**Transcriptional activity of genes potentially involved in pH homeostasis, ammonia acquisition, and ammonia oxidation.**

Several genes potentially associated with the acidophilic lifestyle of “Ca. Nitrosotalea devanaterra” were chosen for further transcriptional analysis: the genes for potassium-transporting ATPase B chain (*kdpB* [NDEV_1460]), carbonic anhydrase (*cam* NDEV_1082), α-acetolactate decarboxylase (*alsD* [NDEV_1077]), or ammonium acquisition and oxidation (*amt1a* [NDEV_1347], *amt1b* [NDEV_1108], *amt2* [NDEV_1784], and *amoA* [NDEV_1777]). All ammonium transporter genes, *amoA*, and putative pH homeostasis genes, including those absent in neutral-pH AOA, were transcriptionally active. The transcript/gene ratios were highest at the optimal growth pH of 5 (see Fig. S5 and Table S10 in the supplemental material), and transcription rates were generally associated with nitrification activity rather than being in response to pH change. Nevertheless, transcription of ammonium transporter genes, *amoA*, and homeostasis genes at pH 5 highlights their necessity for growth and metabolism in “Ca. Nitrosotalea devanaterra.” Transcription of the putative high-affinity Amt2 gene was >10-fold higher than that of the low-affinity Amt1a and Amt1b genes in “Ca. Nitrosotalea devanaterra,” as reported for nonstarved neutrophilic AOA in culture and metatranscriptomic studies (75, 77).

**Obligate acidophily.**

Representatives of the *Nitrosotalea* lineage are found overwhelmingly in acidic, rather than neutral, soils (4), and characterized *Nitrosotalea* isolates cannot grow at a pH of >6.1 (7), but the genome analysis provided no explanation for this inability. Ammonia may be toxic at neutral pH due to its higher concentration and its ability to permeate membranes. Neutral pH could also disable secondary transporters coupled to membrane pH gradients and change the bioavailability of many metals compared to that at acid pH (78).

**How does acidophilic ammonia oxidation occur in “Ca. Nitrosotalea devanaterra”?** Several lines of evidence favor an extracellular (outward-facing) orientation of the AMO active site in

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**FIG 3** Membrane lipid composition of “Ca. Nitrosotalea devanaterra.” Base peak chromatograms of core glycerol dibiphytanyl glycerol tetraether lipids (GDGTs) (A) and GDGT-based intact polar lipids (B) are shown. Cren’ refers to a regioisomer of crenarchaeol. Percentages below “4+Crenarchaeol” indicate the relative contributions of GDGT-4 and crenarchaeol to the peak, respectively. Peaks belong to various core GDGTs from 0 to 4, including GDGTs with hydroxy cores.
“Ca. Nitrosotalea devanaterra.” First, the active site of the pMMO of *M. capsulatus* Bath is periplasmic (36), and there is a high degree of conservation across methanotrophs, AOB, and AOA (38). Second, *in silico* topology prediction for AmoB and AmoC of “Ca. Nitrosotalea devanaterra” supports the outward orientation of the AMO active site. Third, AMO activity generates reactive intermediates, and it is advantageous to exclude them from the cytoplasm by performing the ammonia oxidation in the pseudoperiplasmic space (79).

Acidophilic growth could be facilitated by high substrate affinity: the whole cells of the marine AOA “*Candidatus Nitrosoarchaeum limnia*” *SFB1* and “*Candidatus Nitrotenuis uzonensis*” genomes cannot grow at acidic pH. This may be explained by the absence of the cytoplasmic pH regulation mechanisms found in “*Ca. Nitrosotalea devanaterra*,” e.g., ion transport and cell envelope modification, from other AOA. Conversely, even if AOB possessed genes for acid pH homeostasis, starvation would result from a lack of ammonia (without ureolytic activity). If AOA are able to use ammonium for transport as our results suggest, then AOA rather than AOB were in a better predisposition to evolve an acidophilic phenotype.

**Conclusions.** Despite its ecological significance, the mechanism of acid-tolerant ammonia oxidation is a long-standing paradox. This investigation has examined, for the first time, how an ammonia-oxidizing organism may perform this process at low pH by examining an archaela genome with specific emphasis on (i) N metabolism, (ii) C metabolism, and (iii) pH homeostasis mechanisms. We predict the following. (i) Previously proposed mechanisms of acid tolerance in AOB (e.g., urease, biofilm, or aggregate formation) are not required for the growth of the obligate acidophile “*Ca. Nitrosotalea devanaterra*.” Further, predictions suggest that the AMO of “*Ca. Nitrosotalea devanaterra*” oxidizes NH₃ and has an extracellular (outward-facing) active site and that Amt transporters of “*Ca. Nitrosotalea devanaterra*” bind NH₄⁺ in contrast to AOB, which transport NH₃. (ii) HCO₃⁻, required for carbon fixation, may be supplied by carbonic anhydrase, which is actively transcribed in “*Ca. Nitrosotalea devanaterra*.” (iii) “*Ca. Nitrosotalea devanaterra*” shares aspects of pH homeostasis mechanisms with other moderate acidophiles, including having a high number of cation transporters, cytoplasmic proton-scavenging mechanisms, and altered cell membrane and surface compositions compared to neutrophilic AOA. In contrast to the case for

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**FIG 4** Ammonia oxidation machinery of “*Ca. Nitrosotalea devanaterra.*” (A and B) Conservation of the periplasmic active site in PmoB/AmoB (A) and PmoC/AmoC (B) sequences. (C) AMO gene cluster, Amt transporter, and PII gene arrangement. Longer alignments and protein topology predictions for AmoB and AmoC are in Fig. S3 and S4 in the supplemental material, respectively. Illustrated ORFs are drawn to scale, except for genes that are not involved in ammonia metabolism (truncated, white). The asterisks indicate that the “*Candidatus Nitrosoarchaeum limnia*” SFB1 and “*Candidatus Nitrotenuis uzonensis*” genomes are not closed and distance cannot be estimated.

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extreme acidophiles, secondary transporters are not overrepresented in "Ca. Nitrosotalea devanaterra." We postulate that unlike AOB, all AOA, including "Ca. Nitrosotalea devanaterra," have the genetic potential to oxidize ammonia under acidic conditions and that acidophilic adaptation of "Ca. Nitrosotalea devanaterra" is achieved by cytoplasmic pH regulation mechanisms not shared by neutrophilic AOA.

The current study (as with all genome studies) is limited by
existing sequence databases but proposes hypothetical mechanisms for growth of acidophilic archaea that can provide the basis for future experimental testing. Although the presence and activity of previously uncharacterized genes were associated with the acidophilic growth of "Ca. Nitrosotalea devanaterra," one caveat of this approach is that "Ca. Nitrosotalea devanaterra" cannot grow at neutral pH. It is therefore impossible to unequivocally confirm that these genes are a prerequisite for acidophily. This question could be further explored by heterologous expression of candidate acidophily genes in a neutrophilic organism (preferably a neutrophilic AOA) to examine whether they confer an acidophilic phenotype on the host. An alternative approach would be site-directed mutagenesis, although unfortunately neither of these techniques has yet been developed for AOA. Further, acetoin production could be determined, transcription of further genes (e.g., Na+/solute symporter or Na+/H+ exchanger genes) studied, and membrane lipid composition determined over a range of pH. Other important future research directions include genome sequencing, lipid profiling, and S-layer glycome profiling of other strains of Nitrosotalea (and potentially acid-tolerant urease-positive AOB).

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