Production and Excretion of Polyamines To Tolerate High Ammonia, a Case Study on Soil Ammonia-Oxidizing Archaeon “Candidatus Nitrosocosmicus agrestis”

Liangting Liu,a Mengfan Liu,a Yiming Jiang,a Weitie Lin,a,b,c Jianfei Luo,a,b,c

School of Biology and Biological Engineering, South China University of Technology, Guangzhou, People’s Republic of China

Guangdong Key Laboratory of Fermentation and Enzyme Engineering, South China University of Technology, Guangzhou, People’s Republic of China

MOE Joint International Research Laboratory of Synthetic Biology and Medicine, South China University of Technology, Guangzhou, People’s Republic of China

ABSTRACT Ammonia tolerance is a universal characteristic among the ammonia-oxidizing bacteria (AOB); in contrast, the known species of ammonia-oxidizing archaea (AOA) have been regarded as ammonia sensitive, until the identification of the genus “Candidatus Nitrosocosmicus.” However, the mechanism of its ammonia tolerance has not been reported. In this study, the AOA species “Candidatus Nitrosocosmicus agrestis,” obtained from agricultural soil, was determined to be able to tolerate high concentrations of NH₃ (>1,500 μM). In the genome of this strain, which was recovered from metagenomic data, a full set of genes for the pathways of polysaccharide metabolism, urea hydrolysis, arginine synthesis, and polyamine synthesis was identified. Among them, the genes encoding cytoplasmic carbonic anhydrase (CA) and a potential polyamine transporter (drug/metabolite exporter [DME]) were found to be unique to the genus “Ca. Nitrosocosmicus.” When “Ca. Nitrosocosmicus agrestis” was grown with high levels of ammonia, the genes that participate in CO₂/HCO₃⁻ conversion, glutamate/glutamine syntheses, arginine synthesis, polyamine synthesis, and polyamine excretion were significantly upregulated, and the polyamines, including putrescine and spermidine, had significant levels of production. Based on genome analysis, gene expression quantification, and polyamine determination, we propose that the production and excretion of polyamines is probably one of the reasons for the ammonia tolerance of “Ca. Nitrosocosmicus agrestis,” and even of the genus “Ca. Nitrosocosmicus.”

IMPORTANCE Ammonia tolerance of AOA is usually much lower than that of the AOB, which makes the AOB rather than AOA a predominant ammonia oxidizer in agricultural soils, contributing to global N₂O emission. Recently, some AOA species from the genus “Ca. Nitrosocosmicus” were also found to have high ammonia tolerance. However, the reported mechanism for the ammonia tolerance is very rare and indeterminate for AOB and for AOA species. In this study, an ammonia-tolerant AOA strain of the species “Ca. Nitrosocosmicus agrestis” was identified and its potential mechanisms for ammonia tolerance were explored. This study will be of benefit for determining more of the ecological role of AOA in agricultural soils or other environments.

KEYWORDS ammonia tolerance, ammonia-oxidizing archaea, Nitrosocosmicus, polyamines

The ammonia-oxidizing archaea (AOA), composed of Nitrosopumilales, “Candidatus Nitrosotaleales,” “Candidatus Nitrosocaldales,” and Nitrososphaerales within the phylum Thaumarchaeota, play a major role in the global nitrogen cycle by mediating the conversion of ammonia to nitrite (1–3). AOA are ubiquitous and estimated to represent 1 to 5% of all prokaryotes in soils (1, 4). The organisms Nitrososphaera and...
TABLE 1 Ammonia and pH tolerances of AOA strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Source</th>
<th>Inhibitory NH4(^+) (mM)/NH3 (μM)(^b)</th>
<th>Temp (°C)</th>
<th>pH(^c)</th>
<th>Reference</th>
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<tr>
<td><em>Ca. Nitrospumilus maritimus</em></td>
<td>SCM1</td>
<td>Tropical marine aquarium</td>
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<tr>
<td>“Ca. Nitrospumilus koreensis” SAT1</td>
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<td>Wastewater treatment plant</td>
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<td>5.0–7.0</td>
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<td><em>Nitrospumilus replebiulus</em> HCA1</td>
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<td>Marine surface sediment</td>
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<td>*Nitrospumilus oxycline HCE1</td>
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<td>17 m depth marine water</td>
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<td>Acid soil</td>
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<td>4.0–5.5</td>
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<td><em>Nitrospumilus maritimus</em> Ga9.2</td>
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<td>Hot spring</td>
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<td>Garden soil</td>
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<td>“Ca. Nitrospumilus agrestis” SS</td>
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<td>Vegetable soil</td>
<td>200 (7.0)/1592.07</td>
<td>30</td>
<td>5.5–8.0</td>
<td>This study</td>
</tr>
<tr>
<td>AOB</td>
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<td>400 (8.0)/29712.72</td>
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<td><em>Nitrosomonas ureae</em></td>
<td></td>
<td>Oligotrophic freshwater and natural soils</td>
<td>200 (8.0)/14856.36</td>
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<tr>
<td><em>Nitrosomonas marina</em></td>
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<td>Marine environments</td>
<td>200 (8.0)/14856.36</td>
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<td>24</td>
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<tr>
<td><em>Nitrosolobus multiformis</em></td>
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<td>Soils (not acid)</td>
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<td><em>Nitrosobivrio tenuis</em></td>
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<td>Soils, rocks and freshwater</td>
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<tr>
<td><em>Nitrosospira brientis</em></td>
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<tr>
<td><em>Nitroscoccus oceanii</em></td>
<td></td>
<td>Marine environments</td>
<td>1,000 (8.0)/74281.72</td>
<td>30</td>
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</table>

\(^a\)Inhibitory NH4\(^+\) \(^b\)Free ammonia concentration, calculated according to total ammonia, pH, and temperature, estimating by formulas published by Emerson et al. (64).  
\(^c\)The range of pH at which the organism can grow.

“*Candidatus Nitrospumilus*” in the order *Nitrososphaerales* are regarded as the main AOA groups distributed in terrestrial soil environments. Until now, four strains belonging to *Nitrososphaera* and five strains belonging to “*Ca. Nitrospumilus*” have been reported (5, 6). Although it is a sister cluster of *Nitrososphaera*, “*Ca. Nitrospumilus*” has many properties different from those of *Nitrososphaera* and other AOA clusters, among them the ability to tolerate high concentration of ammonia (7–9).

Because the ammonia affinity of AOA is dozens or hundreds of times higher than that of the ammonia-oxidizing bacteria (AOB) (10–12), members of the AOA have usually been observed to have higher abundance than AOB in habitats with low ammonia, such as the oligotrophic oceans (13, 14). Generally, AOA species have been reported to have low tolerance to un-ionized ammonia (1 to 800 μM) in contrast to the AOB species (up to 74 mM) (Table 1). In agricultural soils, overfertilization results in high concentration of ammonia, which contributes to the activation of AOB and the limitation of AOA, even though the AOA groups sometimes dominate in numbers (15, 16). Due to the high ammonia, the nitrifier denitrification activity mediated by AOB is promoted while the activity of AOA is suppressed, which makes the AOB groups the main producers of nitrous oxide (N₂O), contributing to global N₂O emission from agricultural soils (17–19).

It seems that the AOB predominate as the active nitrifiers that are responsible for the ammonia oxidation in agricultural soils. However, the AOA groups have been observed to have ammonia oxidation activity and to make a small contribution (10 to 20%) to N₂O production (17–19); they are not always inhibited by the application of large amount of nitrogen fertilizers (20–23). In particular, some strains from the genus “*Ca. Nitrospumilus*” obtained from soil, sediment, and wastewater treatment plants were observed to tolerate more than 1,000 μM un-ionized ammonia (7, 8) (Table 1).
Ammonia Tolerance of *Ca. Nitrosocosmicus agrestis* 

These findings suggested that some species of *Ca. Nitrosocosmicus* and even some unknown AOA species were probably able to tolerate high ammonia levels in agricultural soils.

Ammonia tolerance is well-known among AOB, especial for the species of *Nitrosococcus* (24). However, the mechanism for high-ammonia tolerance has been rarely reported for the AOB species, not to mention for the AOA species. In this study, an AOA strain named *Candidatus Nitrosocosmicus agrestis* SS that previously obtained from agricultural soil was observed to be able to tolerate 1,592 μM un-ionized ammonia (Table 1). Based on comparative genomic and transcriptional expression analyses, as well as metabolite determination, a potential mechanism for *Ca. Nitrosocosmicus agrestis* SS tolerance of high ammonia concentrations was proposed. This study identified some new features to more fully characterize the *Ca. Nitrosocosmicus* clade and uncovered some novel biochemical, physiological, and ecological roles of AOA in the global environment.

**RESULTS**

**New strain of *Ca. Nitrosocosmicus* from agricultural soil.** Phylogenomic analysis based on 43 concatenated universal marker protein sequences indicated that the AOA strain SS was closely related to the genus *Ca. Nitrosocosmicus* and forms a deep branch in the clade *Ca. Nitrosocosmicus* (Fig. 1A). The average nucleotide identity (ANI) of strain SS to the closely related strains was 72.03% to 72.81%, and the average amino acid identity (AAI) was 71.16% to 72.68% (see Fig. S1 in the supplemental material); the ANI and AAI values are above the proposed genus and below the proposed species boundary thresholds (25, 26). In accordance with the phylogenomic and genomic ANI/AAI analyses, strain SS was assigned to the genus *Ca. Nitrosocosmicus* and referred to as *Ca. Nitrosocosmicus agrestis.* The cells of *Ca. Nitrosocosmicus agrestis* are irregular spheres, 0.8 to 1.2 μm in diameter, and appeared in pairs or aggregates embedded in the extracellular matrix (Fig. 1B to D).

The calculation of nitrite production over time (Fig. 2A) suggested that the generation time of *Ca. Nitrosocosmicus agrestis* was 30.2 h (under 30°C), which is shorter than that of *Candidatus Nitrosocosmicus exaquare* G61 (51.7 h), *Candidatus Nitrosocosmicus franklandus* C13 (40 h), and *Candidatus Nitrosocosmicus oleophilus* MY3 (77.4 h) (7–9). After the oxidation of 1 mM NH₄⁺, the cell density in culture was determined to be approximately 7.06 × 10⁶ cells ml⁻¹, which is similar to that of *Ca. Nitrosocosmicus franklandus* C13 (7.6 × 10⁶ cells ml⁻¹) and *Ca. Nitrosocosmicus oleophilus* MY3 (3.2 × 10⁶ cells ml⁻¹) but lower than that of *Ca. Nitrosocosmicus exaquare* G61 (5.6 × 10⁷ cells ml⁻¹) (7–9).

The ammonia-oxidizing activity of *Ca. Nitrosocosmicus agrestis* was observed to be optimal at 37°C and nearly inhibited at 42°C (Fig. 2B); the activity was optimal at 0 to 0.2% NaCl and completely suppressed by 2% NaCl (Fig. 2C). *Ca. Nitrosocosmicus agrestis* was able to tolerate NaCl (i.e., it retained ~35% of activity under 1% NaCl), which is much higher than the levels of many other terrestrial AOA strains, such as *Candidatus Nitrototenuis aquarius* AQ6f (0.1%) (27), *Candidatus Nitrosotenuis cloacae* SAT1 (0.03%) (28), *Candidatus Nitrosotenuis uzonensis* N4 (0.1%) (29), and *Candidatus Nitrosoarchaeum koreensis* MY1 (0.4%) (30). Ammonia-oxidizing activity was observed at pH values ranging from 5.5 to 8.0 and determined to be optimal at pH 6.5 to 7.0 (Fig. 2D).

*Ca. Nitrosocosmicus agrestis* was also able to use urea as the sole energy and nitrogen source; the ammonia oxidation rate when urea was the sole energy source was only 1/10 of that when ammonia was the energy source, while the relative expression levels of genes relating to urea transport and hydrolysis were conversely 11 to 13 times higher (Fig. S2). The half-maximal inhibitory concentrations (IC₅₀) of nitrification inhibitors, including allylthiourea (ATU), dicyanodiamide (DCD), and 3,4-dimethylpyrazole phosphate (DMP), for *Ca. Nitrosocosmicus agrestis* were determined to be 445.1, 947.1 and 488.0 μM, respectively (Fig. S3), which are similar to observations for other AOA species (31–33). In contrast, the IC₅₀ of nitrpyrin (NP) for *Ca.
Nitrosocosmicus agrestis’ ammonia tolerance was only 0.599 mM (Fig. S3), which is about 1/100 to 6/100 of that for Nitrososphaera viennensis EN76 (32), Candidatus Nitrososphaera sp. strain JG1 (34), Ca. Nitrosoarchaeum koreensis MY1 (30), and Candidatus Nitrosoarchaeum devanaterra (31).

Ammonia tolerance of “Ca. Nitrosocosmicus agrestis” and the other AOA strains. The ammonia oxidation activity of “Ca. Nitrosocosmicus agrestis” decreased with increases in the initial ammonia concentration; about 75%, 35%, and 5% of the activity were maintained when the concentration of un-ionized ammonia (NH₃) reached 400, 800, and 1,592 mM, respectively (Fig. 3A). Ammonia-oxidizing strains of “Ca. Nitrosocosmicus” were usually reported to have higher tolerance either to NH₃ or to ionized ammonia (NH₄⁺) than the other AOA strains. The inhibitory concentration of NH₃ for strains of the genus “Ca. Nitrosocosmicus” was determined to range from 796 to 3,894 mM; except for strains of “Ca. Nitrosocosmicus” and the terrestrial strains Nitrososphaera viennensis EN76 and “Ca. Nitrostotenuis aquarius” AQ6f, the inhibitory concentrations of NH₃ for other strains are less than 500 μM (Fig. 3B). In fact, the inhibition by NH₃ of known AOA varies between strains as well as between genera; only
strains of “Ca. Nitrosocosmicus” are able to tolerate high ammonia levels, which suggests that ammonia tolerance is probably prevalent among strains of this genus.

**Genomic features of “Ca. Nitrosocosmicus agrestis.”** A genome of “Ca. Nitrosocosmicus agrestis” was recovered from a metagenomic assembly and contains 43 contigs with a total length of 3.22 Mbp (Fig. S4). The completeness, contamination, and strain heterogeneity of the genome were 96.1%, 2.91% and 0%, respectively. The genome has an average G+C content of 33.42%, includes 3,513 protein-coding sequences (CDS), and contains 45 tRNA genes, one 5S rRNA gene, and two 16S/23S rRNA operons (Table 2). The genes involved in central carbon metabolism, including autotrophic CO2 fixation (3HP/4HB pathway), tricarboxylic acid cycle, gluconeogenesis, and nonoxidative pentose phosphate pathways, are presented (Table S1; Fig. 4). Genes encoding pathways of ammonia oxidation (single copies of amoA, amoB, and amoX and three copies of amoC) and urea hydrolysis (urease subunits, urea accessory proteins, and urea transporter), which participate in energy metabolism, are also found in the genome (Table S1; Fig. 4). Urease utilization genes were always found in the terrestrial genera “Ca. Nitrosocosmicus” and *Nitrososphaera* and sometimes in the marine genus *Nitrosopumilus* (Table 2).

During carbon metabolism in AOA, the first step is the fixation of inorganic carbon into cells via the 3HP/4HB pathway, with the help of carbonic anhydrase (CA), which is responsible for the reversible hydration of CO2 to HCO3− (35, 36). Three genes encoding CA were identified in the genome of “Ca. Nitrosocosmicus agrestis” (Table 2; Table S1); they were assigned to the β class (d clade) and γ class (Cam and CamH) according to the phylogenetic analysis (Fig. S5). Until now, the CA genes had been identified only in the genomes of terrestrial AOA strains from the genera “Ca. Nitrosocosmicus,” *Nitrososphaera*, and “Ca.
Nitrosotalea” (Table 2). In contrast to the periplasmic location of Cam, CamH and β-CA are located in the cytosol in the absence of signal peptide. Meanwhile, a full set of genes encoding the key enzymes and transporters that participate in polysaccharide metabolism were also found in the genome, including members of the glycosyltransferase (GT) family, members of the glycoside hydrolase (GH) family, members of the carbohydrate esterase (CE) family, multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase, members of the lipopolysaccharide exporter (LPSE) family, etc. (Table S1) (37). These proteins are important for cell surface modification and exopolysaccharide (EPS) production of biofilm-forming bacteria and archaea. An extensive set of genes encoding these proteins was identified among the AOA groups from the Nitrososphaerales, such as Nitrososphaera viennensis, “Ca. Nitrososphaera gargensis,” and “Candidatus Nitrososphaera evergladensis” (37). Interestingly, the genes encoding LPSE are unique among the genomes of genus “Ca. Nitrosocosmicus,” suggesting the potential of polysaccharide secretion for this AOA clade.

During nitrogen metabolism, the ammonia nitrogen that diffuses through cytoplasmic membrane (in the form of NH3) or is transferred from the extracellular environment by ammonium transporters (Amt) (in the form of NH4+) is integrated into glutamine/glutamate syntheses. A single copy of the gene encoding Amt was identified in the genome of “Ca. Nitrosocosmicus agrestis” (Table 2; Fig. S6A; Table S1). Based on the phylogenetic analysis, it was assigned to Amt-2, a cluster of Amt showing low affinity to NH4+ (Fig. S6A); the ammonium-binding site of Amt-2 is as conserved as that of Amt-1 (Fig. S6B), suggesting their specific binding to ammonium. The known strains of “Ca. Nitrosocosmicus” were found to have only one copy of the Amt-2 gene in their genomes; however, almost all of the other AOA strains have genes for two types of Amt in their genomes, the high-affinity Amt-1 and the low-affinity Amt-2 (Table 2; Fig. S6B). Besides the genes encoding glutamine synthetase (GS) and glutamate dehydrogenase (GDH), the genes encoding type II carbamoyl phosphate synthetase (CPS) were also found in the genome (Table S1), suggesting the organisms’ ability to hydrolyze glutamate to glutamine and produce carbamoyl phosphate as a by-product (Fig. 4) (38). Along with CPS, the key enzymes, involved in arginine synthesis, including ornithine transcarbamylase (OTC), argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), were also found in “Ca. Nitrosocosmicus agrestis” (Table S1). When arginine was used as the substrate, it could be further catalyzed to polyamines via the
<table>
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<tr>
<th>AOA strain</th>
<th>Genome size (Mb)</th>
<th>GC (%)</th>
<th>No. of protein coding genes</th>
<th>Coding region (%)</th>
<th>tRNA operons</th>
<th>tRNA genes</th>
<th>Motility</th>
<th>Urease</th>
<th>β-CA</th>
<th>γ-CA-Cam</th>
<th>γ-CA-CamH</th>
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<th>Amt-2</th>
<th>DME</th>
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<td>33.41</td>
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<td>–</td>
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<td>–</td>
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<td>1</td>
<td>–</td>
<td>–</td>
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*The number in parentheses indicates the number of copies of the putative polyamine exporter gene.

bThe complete gene sequence cannot be obtained by gene prediction.
activities of arginine decarboxylase (ADC), agmatinase (AUH), spermidine synthase (SDS), and deoxyhypusine synthase (DHS). Except for the SDS gene, these genes were identified in the genome of “Ca. Nitrosocosmicus agrestis,” which suggests that arginine could be converted into putrescine and some other polyamines after a series of enzymatic catalysis steps (Fig. 4).

The genes encoding the pathways of arginine synthesis as well as polyamine synthesis are found in most of the AOA genomes, suggesting their importance for amino acid synthesis or stress responses among AOA species. Although significant functions of polyamine have been reported for bacteria and archaea (39), excessive accumulation of polyamines and the related metabolites in intracellular spaces likely causes toxic effects (40). Therefore, an effective means of secreting the synthesized polyamine from the cells is required, or the polyamine synthesis would be repressed. Unfortunately, no gene has been annotated as the transporter protein or efflux pump used for the excretion of polyamines in AOA. In some bacteria and yeasts, members of the drug/metabolite exporter (DME) family have been reported to be able to mediate the

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**FIG 4** Schematic reconstruction of the predicted metabolic modules and other genome features of “Ca. Nitrosocosmicus agrestis.” Dashed lines indicate reactions for which the enzymes have not been identified. CE, carbohydrate esterase; GH, glycoside hydrolase; CA, carboxyl anhydrase; NirK, nitrite reductase; Ure, urease; GDH, glutamate dehydrogenase; GS, glutamine synthetase; ADC, arginine decarboxylase; AUH, agmatinase; DHS, deoxyhypusine synthase. The unique enzymes or transporters of *Nitrososocmicus* clade are in red. Candidate enzymes, gene accession numbers, and transporter classes are listed in the supplemental tables.
excretion of amino acids and polyamines (41, 42). Based on sequence analysis, four copies of the gene encoding DME were identified in the genome of “Ca. Nitrosocosmicus agrestis” (Fig. S7A). In fact, the genes encoding DME were found to be prevalent among the AOA genomes (Table 2). According to phylogenetic analysis, these DMEs were clustered into three separate groups, with the sequences from “Ca. Nitrosocosmicus” in two groups and the sequences from other genera, including “Ca. Nitrosopumilus,” “Ca. Nitrosotalea,” Nitrosophphaera, “Ca. Nitrosotenuis,” “Ca. Nitrosoarchaeum,” and “Ca. Nitrosococcus,” in one group (Fig. S7A). Interestingly, one DME group belonging to the genus “Ca. Nitrosocosmicus” are annotated as the probable exporters of amino acids or other metabolites, while the DMEs from other AOA genera are annotated as the Co²⁺/Ni²⁺ efflux porter or uncharacterized; moreover, the phylogenetic analysis indicated that the DMEs of “Ca. Nitrosocosmicus” are closely related to the DMEs of Escherichia and Saccharomyces (Fig. S7B), which have been identified to act as the efflux pump of polyamines (43, 44).

**Gene expression and protein and polyamine production of “Ca. Nitrosocosmicus agrestis” at high ammonia concentrations.** In comparison with the gene expression levels at a low ammonia concentration (7.96 μM NH₃), the expression patterns of genes involved in ammonia oxidation, arginine synthesis, polyamine synthesis, glutamine/glutamate syntheses, carbonic anhydrase, and polyamines transport when “Ca. Nitrosocosmicus agrestis” responded to high ammonia had significant changes. For example, amoA and theAmt gene were significantly downregulated at 796 μM NH₃; however, all of the genes participating in arginine synthesis, polyamine synthesis, and polyamine excretion had significant upregulation, with values ranging from 1.28 to 5.63 times (Fig. 5A). The genes encoding GDH, GS and intracellular CA (β-CA and CamH of γ-CA) were also significantly upregulated; for the genes encoding GDH2 and CA-γ2, expression levels were about 8.3 and 7.5 times higher, respectively (Fig. 5A).

During chemoautotrophic growth of “Ca. Nitrosocosmicus agrestis” using ammonia as the sole energy source, the energy generated from ammonia oxidation is mainly used for inorganic carbon fixation, biomass production, and life maintenance. During growth of “Ca. Nitrosocosmicus agrestis” with 7.96, 79.6, and 796 μM ammonia, the protein production rates were determined to be 13.16, 9.68, and 1.11 μg ml⁻¹, respectively (Fig. 5B). The average ammonia oxidation rate of “Ca. Nitrosocosmicus agrestis” exposed to high ammonia (796 μM NH₃) decreased by 17% in contrast to that seen with low levels of ammonia (7.96 μM NH₃) (Fig. 5B); however, the protein synthesis efficiency decreased by 89.8% (Fig. 5B). It is suggested that a large amount of energy was used for syntheses of other compounds, such as polyamines, in order to tolerant high ammonia levels.

The concentrations of putrescine, cadaverine, spermidine, and spermine in the supernatant of the “Ca. Nitrosocosmicus agrestis” cultures grown with different concentrations of ammonia were determined by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Polyamines including putrescine, cadaverine, and spermidine were identified, with concentrations ranging from 1.37 to 10.25 μg liter⁻¹ (Fig. 5C). Relative to the rates of production in the presence of a low ammonia concentration (7.96 μM NH₃), cadaverine production was not significantly different but spermidine production was about three times higher; notably, putrescine was detected only with a high level of ammonia (796 μM NH₃) and was identified as the predominant polyamine in the culture (Fig. 5C). Putrescine is usually regarded as the precursor for the syntheses of other kinds of polyamines (45). The accumulation of putrescine in the presence of high ammonia concentrations suggested that polyamine synthesis could be a strategy of the “Ca. Nitrosocosmicus agrestis” response to ammonia stress.

**DISCUSSION**

The terrestrial environments of agricultural soils are usually more variable and complex than marine environments. This requires the terrestrial AOA to evolve different strategies to survive in these environments. Generally, the genomes of terrestrial AOA
are much larger than those of the marine AOA (Table 2); they have evolved and obtained more genes, probably in order to encode more proteins in response to different kinds of environmental stresses. For example, genes encoding the pathways of polysaccharide metabolism, such as the synthesis enzymes (GTs, GHs, and CEs) and transporter proteins (MOP and LPSE), are prevalent among the genomes of terrestrial AOA; they probably synthesize polysaccharide to modify the cell surface and then form biofilms, which provides protection and promotes survival in rapidly changing environmental conditions, such as fixation on solid matrices, cooperation with bacteria, and tolerance to high levels of ammonia.

The newly nominated genus “Ca. Nitrosocosmicus” is an important part of the AOA community in agricultural soils (8, 46, 47); though low in numbers, they contribute more to ammonia oxidation than bacterial oxidizers (47, 48). The known species of “Ca. Nitrosocosmicus” are able to grow at NH₃ concentrations that inhibit other cultivated AOA (Table 1), reflecting their identity as ammonia-tolerant AOA groups. Due to the presence of this kind of AOA group, AOA-mediated ammonia oxidation and greenhouse gas N₂O production in soils were not completely suppressed by high ammonia (18, 49). The finding of “Ca. Nitrosocosmicus” indicates that ammonia toxicity does not clearly differentiate AOA from AOB. It is well known that AOB species have high tolerance to ammonia; however, the related mechanism has rarely been reported. The

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**FIG 5** Metabolic characteristics of “Ca. Nitrosocosmicus agrestis” at different un-ionized-ammonia concentrations. (A) Relative gene expression levels in response to high ammonia. RNA was extracted from triplicate pooled cultures. Error bars indicate standard errors of the means for technical triplicates of qPCR. (B) Nitrite production and protein synthesis by “Ca. Nitrosocosmicus agrestis” in 2 days with 7.96 to 796 μM ammonia. (C) Effect of ammonia on the accumulation of polyamine by “Ca. Nitrosocosmicus agrestis.” Put, putrescine; Cad, cadaverine; Spd, spermidine. Error bars indicate standard errors of the means for biological triplicates. RNA extraction and protein and polyamine quantification were carried out after the addition of 7.96 to 796 μM un-ionized ammonia for 2 days.
rhesus (Rh) proteins, belonging to the Amt/MEP/Rh (ammonium transporter/methylammonium-ammonium permease/rhesus protein) family and acting as bidirectional transporters of NH$_4^+$/$\text{NH}_3$, have been reported in *Nitrosomonas* (50, 51). They could mediate ammonia efflux, which then reduces the NH$_3$ toxicity, making it possible for them to detoxify ammonia for fish (52). Unfortunately, the gene encoding Rh protein or related protein is absent in the AOA genome; the AOA seem to prefer to use Amt as the transporter of NH$_4^+$. Some different mechanisms for the ammonia tolerance are probably used by the AOA species of the genus "Ca. Nitrosocosmicus."

In this study, an AOA strain "Ca. Nitrosocosmicus agrestis" obtained from agricultural soil was determined to have high tolerance to ammonia (Fig. 3). According to studies on comparative genomic analysis, gene expression quantification, and protein and extracellular metabolite determination, the potential mechanisms of "Ca. Nitrosocosmicus agrestis" and possibly the genus "Ca. Nitrosocosmicus" that allow tolerance of high ammonia were proposed, as follows (Fig. 6).

The first notable finding in this regard is the presence of only the Amt-2 transporter in genomes (Table 2; Fig. S6); it shows low affinity to NH$_4^+$ and would be significantly downregulated in response to high ammonia (Fig. 5A), suggesting that entrance of ammonia into the cytoplasm occurs mainly by spontaneous diffusion. In general, the ammonia concentrations in terrestrial habitats are usually higher than those in the ocean, which carries the evolutionary implication that the terrestrial AOA do not need the high-affinity ammonium transporter Amt-1, because the amount of NH$_3$ diffused
across the inner membrane is sufficient for their metabolism. As a result of this evolutionary step, the ammonia tolerance of terrestrial AOA would be higher than that of the marine AOA. With respect to the mechanism, the NH₃ that diffuse through inner membrane is combined with H⁺ (derives from the chemical equilibrium: H₂CO₃ \rightleftharpoons HCO₃⁻ + H⁺) to form NH₄⁺. However, if it presents Amt-1, NH₄⁺ transport into the cytoplasm via Amt-1 would be the main way for ammonia to enter cells. At this stage, the assimilation of NH₄⁺ and bicarbonate will produce a large amount of H⁺, which in turn will decrease the cytoplasmic pH and significantly suppress cell growth.

Second, cytoplasmic CAs are unique to the genus “Ca. Nitrosocosmicus” (Table 2) and are significantly upregulated in response to high ammonia (Fig. 5A); they are capable of supplying plenty of HCO₃⁻ for the 3HP/4HB pathway and arginine synthesis, as well as supplying H⁺ for the conversion of NH₃ to NH₄⁺.

The third mechanism is the presence of an incomplete ornithine cycle pathway in the genome of “Ca. Nitrosocosmicus agrestis.” Due to the lack of arginase, the arginine that is synthesized via the pathway mediated by the enzymes CPS, OTC, ASS, and ASL could not be catalyzed to ornithine and urea and then be used as the precursor for polyamine synthesis. The genes that encode the enzymes involved in glutamine (as the substrate for CPS-II) and arginine syntheses were significantly upregulated under high-ammonia conditions, which suggested that the NH₃ that diffuses through the cell membrane was probably used for arginine synthesis and further polyamine synthesis.

Fourth, “Ca. Nitrosocosmicus agrestis” probably uses DME as an exporter of polyamine. The DME genes of “Ca. Nitrosocosmicus agrestis” that are closely related to the bacterial putative DME were determined to be upregulated 2.5- to 7.5-fold when growing with high levels of ammonia (Fig. 5A), and the production of extracellular polyamines were also much higher (Fig. 5C), suggesting that polyamines were not only synthesized in cells but also excreted from the cells. As a result, the maintenance of cytoplasmic pH and production of polyamine are probably responsible for the ammonia tolerance of “Ca. Nitrosocosmicus agrestis” and for other species of “Ca. Nitrosocosmicus.”

Polyamines are a class of small cation that contain multiple amino groups. They play important roles in nucleic acid stabilization, protein translation, stress responses, and cell division (39). However, high intracellular accumulation of polyamines is very harmful to the cells. For example, excess polyamines in the cytoplasm are likely to interfere with cellular metabolism, including protein synthesis, and the oxidative decomposition of polyamines generates reactive oxygen species (ROS) that are highly damaging to cells (40). Because the positively charged polyamines are unable to diffuse through cell membranes by themselves, certain types of transporter are needed to excrete them from the cells. The DME family, belonging to the DMT superfamily, has been reported to be able to export various compounds such as amino acids and polyamines to maintain intracellular homeostasis (53, 54). Some marine AOA can exude a suite of organic nitrogen-containing compounds, including amino acids, thymidine, and B vitamins, out of cells to fuel prokaryotic heterotrophs or release labile organic matter to recruit H₂O₂-detoxifying heterotrophs to self-rescue (55, 56). Unfortunately, no related transporter of amino acid was identified in the genus “Ca. Nitrosocosmicus.” Though the genes encoding DME protein are ubiquitous in the genomes of AOA, the function of DME in the excretion of amino acids among these marine AOA needs further research. In this study, the amino acid in the supernatant of “Ca. Nitrosocosmicus agrestis” growing with low or high concentrations of ammonia is almost undetectable. Other than the excreted amino acid being used by its bacterial consortium in cultures, there is no evidence at present to prove that “Ca. Nitrosocosmicus agrestis” could exude amino acid.

It is also suggested that the excretion of amino acids (especially the glutamate group of amino acids) from cells would not be a mechanism of ammonia tolerance. However, polyamines, including putrescine, cadaverine, and spermidine, were identified in the supernatant of “Ca. Nitrosocosmicus agrestis,” and their concentrations increased along with the upregulation of DME genes under high-ammonia conditions (Fig. 5C),
suggesting the possibility of DME acting as the transporter of polyamines. According to the phylogenetic analysis, a certain DME of “Ca. Nitrosocosmicus” is distinct from that of other AOA and closely related to the polyamine exporter of bacteria and yeasts (Fig. S7). It is proposed that the DME could potentially act as an exporter of polyamines in “Ca. Nitrosocosmicus agrestis.”

Inexplicably, the polyamines detected in the cultures of “Ca. Nitrosocosmicus agrestis” grown with either a high or low concentration of ammonia were far from corresponding to the amount of ammonia oxidized. Several explanations for this observation are possible. First, the role of polyamines is probably as an active molecule, like their functions in bacteria and archaea (39); a low concentration of polyamine was enough to help AOA cells to tolerate high ammonia, which implies that there is therefore no need to synthesize large amounts of polyamines to decrease the concentration of ammonia and reduce ammonia toxicity. Second, the culture of “Ca. Nitrosocosmicus agrestis” was not pure, as it comprised about 5% (much more in the presence of high ammonia) bacteria belonging to the Rhizobiales (Fig. S3); therefore, the polyamines synthesized and secreted by AOA cells were immediately used to fuel the growth of heterotrophic bacteria. Based on the metagenome data of “Ca. Nitrosocosmicus agrestis” culture, one genome assigned to Ensifer sp. strain SSB1 was recovered, which represented the predominant bacterial partner in the culture (Fig. S4B; Table S1). The genome of Ensifer sp. strain SSB1 contains 14 copies of potABCD and one of ydcSTUV (Table S1), which have been reported to encode the polyamine transporters used for polyamine uptake (57). The gene copy number of potABCD is much higher than in the related strains Ensifer adhaerens OV14 (4 copies) and Ensifer adhaerens Casida A (6 copies) (58, 59). In addition, the genome of Ensifer sp. strain SSB1 also contains several different coding genes responsible for polyamine degradation (Table S1) (60, 61). The presence of multiple copies of polyamine transporter and metabolism genes indicates that Ensifer sp. strain SSB1, as well as some other coexisting heterotrophic bacteria, could probably use the polyamines as an organic carbon source. Third, at high concentrations of ammonia, ammonia nitrogen entered the cytoplasm mainly by spontaneous diffusion and not by membrane-crossing transport via Amt, which makes the concentration of intracellular ammonia much lower than that of extracellular ammonia. Therefore, the synthesis of a small amount of polyamines from intracellular ammonia is enough to reduce the ammonia toxicity.

The polyamines were almost certainly present in the cells of the last universal common ancestor (LUCA) of all life and widely distributed among the hyperthermophilic, acidophilic, and thermoacidophilic archaea, suggesting their potential role in the response to adverse environmental conditions (39). The polyamine structures in different microbial species are diverse, as are their lifestyles, which makes many of them are still unknown. Moreover, microbial interactions are widespread in nature. The relationship between the genus “Ca. Nitrosocosmicus” and heterotrophs has barely been reported, and so some unknown or complex interactions between them may occur, with the result that many terrestrial AOA species are uncultivated or even unclassified (62). The polyamine-mediated interaction between AOA and bacteria would be most likely to expand the ecological role of AOA in nature. This case study was performed on only one strain from the genus “Ca. Nitrosocosmicus.” In order understand why “Ca. Nitrosocosmicus agrestis” or the genus “Ca. Nitrosocosmicus” is able to tolerate high ammonia levels, additional work on more AOA strains is needed to elucidate whether the polyamine is responsible for ammonia tolerance and what happens between “Ca. Nitrosocosmicus” and its bacterial consortia.

MATERIALS AND METHODS
AOA strain and culture maintenance. “Ca. Nitrosocosmicus agrestis” strain SS was obtained from agricultural soil by using a two-step strategy and maintained in a mineral salts medium (MSM) (5). The culture was incubated at 30°C in the dark without shaking; an antibiotic mixture containing ciprofloxacin (50 mg/liter), azithromycin (50 mg/liter), and natamycin (10 mg/liter) was added to the culture to maintain a high abundance of archaeal cells. Ten percent (wt/vol) quartz (1 mm in diameter) was supplied for the attachment of archaeal cells during the seed cultivation.
Physiological characterization. AOA cells attached to quartz were washed out by vortex-shaking in fresh liquid MSM; the cell suspension with a rate of 10% (vol/vol) was inoculated into the fresh MSM without supplying quartz and subjected to the determination of physiological characteristics. To avoid pH deviation due to hydrolysis or dissociation of bicarbonate, the MSM used for pH characterization was not supplied with NaHCO₃ (the diffused CO₂ is sufficient for SS growth) but was buffered with 10 mM MES [2-(N-morpholino)ethanesulfonic acid], HEPES, or Tris to maintain the pH in the ranges of 6 to 7, 7 to 8, and 8 to 9, respectively. Concentrations of nitrite and ammonium were determined by the standard Griess-llosvay method and the indophenol blue method, respectively (63). The concentration of un-ionized ammonia was calculated using the formula of Emerson et al. (64). The mean ammonia oxidation rate was calculated basing on the determination of nitrite concentration. The abundance of AOA and bacteria in the cultures was analyzed using absolute quantitative PCR based on 16S rRNA gene primers (SS16S-1F/SS16S-1R for “Ca. Nitrosocosmicus agrestis” and 1369F/1492R for bacteria), following the method described in our previous study (5).

Microscopy assays. Fluorescence in situ hybridization (FISH) was observed on a scanning confocal microscope (LSM 710; Carl Zeiss, Germany); transmission electron microscopy (TEM) was performed on an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV; scanning electron microscopy (SEM) was performed using a Merlin field emission scanning electron microscope (Carl Zeiss, Jena, Germany).

Genome sequencing, assembly, and annotation. The genomic DNA from 3,000 ml batch cultures was extracted according to a previously described protocol (5). Purified DNA was fragmented to ~400 bp with the aid of an M220 focused ultrasonicator (Covaris, Woburn, MA, USA) and subsequently used for library preparation using a TruSeq DNA sample prep kit (Illumina, San Diego, CA, USA). Metagenomic sequencing was performed on a HiSeq X Ten sequencing system (Illumina, San Diego, CA, USA). The raw data were length and quality filtered using Trimmomatic v0.38 (65) and de novo assembled using metaSPAdes v3.13.0 (66) with 127-mers. The GapCloser module of SOAPdenovo2 v1.12 (67) was used with default parameters to fill a proportion of gaps of the assembled scaffolds. Genome binning of the metagenomic assemblies was conducted using MaxBin2 v2.26 (68) with the universal marker gene sets (69). Scaffolds in the assembly with lengths of <1,000 bp were removed. The completeness, contamination, and strain heterogeneity of genome were evaluated using CheckM v1.013 (70). Gene prediction and annotation were done using JGI-IMG/MER (71) and MicroScope platform (72). KEGG pathways were generated using KASS (73). Putative transport proteins and carbohydrate-active enzymes were identified using TCDB (74) and dbcCAN2 (75), respectively. The average nucleotide identity (ANI) and average amino acid identity (AAI) of five Nitrosocosmicus genomes were calculated using JSpeciesWS (76) and compareM (https://github.com/dparks1134/CompareM), respectively.

A phylogenetic tree was obtained using the automatically generated alignment of 43 concatenated universal marker protein sequences, which were identified by CheckM v1.013 (70). The best-fit model of evolution was selected with ModelFinder (77), and phylogenetic trees were inferred by maximum likelihood with IQ-TREE v1.6.1 (78).

Relative transcriptional expression quantitative analysis. During the total RNA extraction, AOA cells in cultures were collected by centrifugation, normalized to the endogenous control 16S rRNA. Primer pairs (Table S2) were designed on the gene sequences. Quantitative Real-Time PCR (qRT-PCR) was performed on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using TransStart Tip Green qPCR SuperMix (Transgen, Beijing, China). The reaction conditions were as follows: 1 min at 94°C and 40 cycles of 10 s at 94°C and 34 s at 60°C. The 2^ΔΔCT method was used to estimate fold change in gene expression, normalized to the endogenous control 16S rRNA. Primer pairs (Table S2) were designed on the function genes from the “Ca. Nitrosocosmicus agrestis” genome. All experiments were prepared in triplicate.

Protein determination. To measure the total protein in AOA cells, about 20 ml of culture was collected and filtered through a 0.22-μm cellulose filter; the filter was cut into pieces and ground in a tube containing 0.5 g quartz sand. The supernatant was collected and filtered through a 0.22-μm cellulose filter. The filtrate was mixed with 200 μl of sulfosalicylic acid (10%) and incubated at 4°C for 1 h, following centrifugation at 14,000 × g for 15 min. The supernatant was filtered through a 0.22-μm cellulose filter again and used for the quantitative analysis of amino acids or polypeptides.

Quantification of polypeptides and amino acids in supernatant. An 800-μl portion of the supernatant was collected and filtered through a 0.22-μm cellulose filter. The filtrate was mixed with 200 μl of sulfosalicylic acid (10%) and incubated at 4°C for 1 h, following centrifugation at 14,000 × g for 15 min. The supernatant was filtered through a 0.22-μm cellulose filter again and used for the quantitative analysis of amino acids or polypeptides.

Concentrations of putrescine, cadaverine, spermidine, and spermine in the supernatant were determined using the LC-20A HPLC system (Shimadzu, Kyoto, Japan) coupled with an API 4000 Quantiva triple-quadrupole tandem mass spectrometer (SCIEX, Framingham, MA, USA) according to a previous study (79). HPLC separation was performed at 0.30 ml/min with the column compartment at 40°C. The stationary phase was an Inertsil ODS-3 column (2.1 by 100 mm; 3 μm), and the mobile phase was an isocratic mixture (20:80, A to B) of 5 mmol/liter CH₃COONH₄ in H₂O (A) and 0.2% HCOOH in H₂O (vol/vol) (B). Amino acids in the supernatant were determined by using the amino acid analyzer A300-advanced (membrapure, Berlin, Germany).
Ammonia Tolerance of "Ca. Nitrosocosmicus agrestis"

Statistical information. Results are expressed as means and standard errors (n = 3) as noted. Student’s t-test was used to calculate the P value as noted. P values of <0.05 and <0.01 were considered to indicate significant differences and highly significant differences, respectively.

Data availability. The genomes described in this study have been deposited in NCBI under GenBank accession number VUY500000000 for "Ca. Nitrosocosmicus agrestis" and JAEPRR0000000000 for heterotroph Ensifer sp. SS81.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.4 MB.
FIG S2, TIF file, 0.4 MB.
FIG S3, TIF file, 0.4 MB.
FIG S4, TIF file, 0.3 MB.
FIG S5, TIF file, 1.7 MB.
FIG S6, TIF file, 2.8 MB.
FIG S7, TIF file, 1.1 MB.
TABLE S1, XLSX file, 0.04 MB.
TABLE S2, XLSX file, 0.01 MB.

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REFERENCES


Ammonia Tolerance of Ca. Nitrosocosmus agristes


