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Salt tolerance-based niche differentiation of soil ammonia oxidizers

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Ammonia oxidizers are key players in the global nitrogen cycle, yet little is known about their ecological performances and adaptation strategies for growth in saline terrestrial ecosystems. This study combined ^{13}C-DNA stable-isotope probing (SIP) microcosms with amplicon and shotgun sequencing to reveal the composition and genomic adaptations of active ammonia oxidizers in a saline-sodic (solonetz) soil with high salinity and pH (20.9 $cmol_c$ exchangeable Na^+ kg^{-1} soil and pH 9.64). Both ammonia-oxidizing archaea (AOA) and bacteria (AOB) exhibited strong nitrification activities, although AOB performed most of the ammonia oxidation observed in the solonetz soil and in the farmland soil converted from solonetz soil. Members of the *Nitrosococcus*, which are more often associated with aquatic habitats, were identified as the dominant ammonia oxidizers in the solonetz soil with the first direct labeling evidence, while members of the *Nitrosospira* were the dominant ammonia oxidizers in the farmland soil, which had much lower salinity and pH. Metagenomic analysis of "*Candidatus* Nitrosococcus sp. Sol14", a new species within the *Nitrosococcus* lineage, revealed multiple genomic adaptations predicted to facilitate osmotic and pH homeostasis in this extreme habitat, including direct Na^+ extrusion/H^+ import and the ability to increase intracellular osmotic pressure by accumulating compatible solutes. Comparative genomic analysis revealed that variation in salt-tolerance mechanisms was the primary driver for the niche differentiation of ammonia oxidizers in saline-sodic soils. These results demonstrate how ammonia oxidizers can adapt to saline-sodic soil with excessive Na^+ content and provide new insights on the nitrogen cycle in extreme terrestrial ecosystems.

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INTRODUCTION

Chemolithoautotrophic ammonia oxidizers are key players in ammonia oxidation, the first and often rate-limiting step of nitrification, which is a central process in the global nitrogen cycle [1, 2]. Three major groups of ammonia oxidizers, ammonia-oxidizing archaea (AOA), canonical ammonia-oxidizing bacteria (AOB), and complete ammonia-oxidizing *Nitrospira* (comammox), have been characterized as active players in autotrophic ammonia oxidation, with the activities of AOA and/or AOB dominating ammonia oxidation in soils depending on environmental conditions. Generally, one of the most important factors determining the distribution and activity of AOA and AOB in soil is pH [3, 4], and several terrestrial ammonia oxidizer clades have adapted to extreme conditions. For example, strains of the AOA lineage *Nitrosotalea* and a gammaproteobacterial AOB have been isolated from acidic soils and possess adaptations for growth at low pH [5–7], while ecological investigations have confirmed the important contributions of related clades to nitrification in acidic soils [8]. Ecological investigations have also revealed the presence of diversified alkaliphilic ammonia oxidizers, within both the AOA and AOB, in soils with pH values of up to 9 [9, 10], but their activity and ecological importance in such environments remain unclear.

Saline ecosystems represent globally distributed habitats and are often highly productive in terms of microbial diversity and related biogeochemical processes [11–14]. Notably, the area of soils affected by high salinity and sodicity is close to 1 billion hectares, accounting for nearly 7% of the Earth's land surface [15]. However, there are limited reports on the ecological performance and niche specialization of functional microbial guilds, including ammonia oxidizers, in such ecosystems [14]. Previous information on ammonia-oxidizing microbes in saline ecosystems is mainly from aquatic environments, while knowledge of ammonia-oxidizing microbes in terrestrial saline systems remains lacking [16]. For instance, AOA from marine ecosystems, including the genera *Nitrosopumilus* [17, 18] and *Ca*. Nitrosopelagicus [19], appear to be salt-tolerant based on cultivation studies, but no strains of soil AOA have been reported to grow under high saline conditions [20]. Betaproteobacterial *Nitrosomonas* strains enriched from desert soil have also demonstrated salt tolerance [21]. In addition, *Nitrosococcus* species within the Gammaproteobacteria are predominantly found in marine systems and adapted to high

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Fig. 1 Schematic diagram showing the sampling sites of agricultural soil that was reclaimed from typical saline-sodic soils in Northeastern China. The area in brown yellow represents the Songnen Plain, one of the three major regions with saline-sodic soil in the world. The solid circle in red within the Songnen Plain refers to the long-term reclamation experiment field of the Da'an Sodic Land Ecological Experiment Station of Northeast, Chinese Academy of Sciences. Soil samples were collected from the Solonetz saline-sodic field (upright panel) that contains no vegetation cover for >40 years as a control, and farmland (downright panel) that has been maintained for maize-watermelon rotation system more than 40 years.

salt concentrations [22, 23]. *Nitrosococcus*-related AOB have also been found to have a high salt tolerance in saline wastewater at low pH [24] and detected in a paddock soil with carbonate accumulations [25] and saline alkaline soils of a former lake [26]. However, several recent studies have also shown that some *Nitrosococcus*-related and other gammaproteobacterial AOB are present in non-saline systems, such as a farm biofilter [27], tea field soil [7], and grassland soil [28]. Furthermore, how AOA and AOB respond to salt stress remains unclear. Although some studies have shown that AOA are more adaptive to hypersaline conditions than AOB [20, 29], other researchers have observed the opposite [30]. These results indicate that ammonia oxidizers may differ in salt tolerance, although the mechanisms leading to the difference remain unresolved.

The saline-sodic area in the western Songnen Plain in China is one of the three major regions with saline-sodic soils worldwide, with more than three million hectares of salt-affected soils [31]. Soils in this area typically have excessive Na_2CO_3 and $NaHCO_3$ contents, leading to an extreme environment of both Na^+ toxicity and high pH stress [32]. In the present study, we investigated the ammonia oxidizers present in a natural hypersaline, alkaline field from this area, and compared them to those in a converted agricultural land in the same region with significantly reduced pH and salt content. Using ^{13}C-tracing microcosms and sequence analysis, we identified the composition of the active ammonia-oxidizing communities in both soils and obtained insights into the genetic capacity of salt-tolerant soil ammonia oxidizers.

MATERIALS AND METHODS

Site description and soil characteristics

The sampling site is located at the Da'an Sodic Land Experiment Station of the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Da'an County, Jilin Province, China (45°35′ N, 123°51′ E, Fig. 1). Samples were collected in December 2017 from two systems: (i) solonetz soil (natural saline-sodic soil) with sparse native vegetation and (ii) farmland soil that was converted from solonetz soil and has been cultivated with a maize-watermelon rotation system for >40 years. Triplicate plots (1 m × 1 m) were selected from the solonetz and farmland fields, and composite samples of five soil cores (0–20 cm) were taken at random from each plot before homogenizing through a 2.0 mm sieve and stored at 4 °C until further use. Methods for determining the soil properties are described in the Supplementary Information. All soil properties are listed in Table 1.

Table 1. Physiochemical and biological properties of soils.

Soil	Solonetz	Farmland
Exchangeable sodium percentage (ESP, %)	26.9***	0.511
Exchangeable Na^+ (E_{Na}, $cmol_c \cdot kg^{-1}$)	20.8***	0.376
Soil pH	9.64***	7.76
Soil bulk density (SBD, $g \cdot cm^{-3}$)	1.38**	1.24
HCO^{3-} ($mmol \cdot L^{-1}$)	3.94**	1.36
CO_3^{2-} ($mmol \cdot L^{-1}$)	6.79***	0.00
Microbial biomass carbon (MBC, $mg \cdot kg^{-1}$)	62.8	194***
Microbial biomass nitrogen (MBN, $mg \cdot kg^{-1}$)	19.4	29.1**
MBC/MBN	3.26	6.70***
Cation exchange capacity (CEC, $cmol_c \cdot kg^{-1}$)	77.2**	73.4
Total carbon (TC, $mg \cdot g^{-1}$)	13.6	13.5
Total nitrogen (TN, $mg \cdot g^{-1}$)	0.393	0.872***
TC/TN	34.7***	15.5
Available phosphorus (AP, $g \cdot kg^{-1}$)	6.57	25.5***
Available potassium (AK, $mg \cdot kg^{-1}$)	171**	155
Archaeal *amoA* gene abundance (10^6 copies g^{-1})	3.35	495***
Bacterial *amoA* gene abundance (10^7 copies g^{-1})	17.0	330***
Nitrification potential (NP, μg N g^{-1} day^{-1})	23.5*	17.1

Significance levels are *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Soil microcosms for stable-isotope probing of active ammonia oxidizers

Triplicate microcosms were constructed for both the solonetz and farmland soils as previously described [33]. Briefly, fresh soil equivalent to 5.0 g dry weight soil was incubated at a 60% maximum water-holding capacity in a 120-ml serum bottle tightly capped with a butyl stopper at 28 °C in the dark. The soil microcosms were incubated with a 5% (v/v) isotopically-

enriched labeled $^{13}C–CO_2$ (Sigma-Aldrich, St. Louis, MO, USA) or $^{12}C–CO_2$ headspace in the absence or presence of 100 pa acetylene (C_2H_2), an inhibitor of microorganisms that use ammonia monooxygenase. The bottles were flushed with pressurized synthetic air (20% O_2, 80% N_2) for 1 min to maintain oxic conditions and resupplied with $^{13}C–$ or $^{12}C–CO_2$ at 7-day intervals. In addition, the soil microcosms were amended with 100 μg urea-N per gram of dry soil on a weekly basis, starting from the first day of incubation. For the $^{13}CO_2$ amended microcosms, the supplemented CO_2 and urea (Shanghai Research Institute of Chemical Industry, Shanghai, China) were >99-atom% ^{13}C-labeled. Isopycnic density gradient centrifugation with 2.0 μg of extracted DNA was performed as previously described [33]. Additional details are provided in the Supplementary Information.

Quantification and sequencing of 16S rRNA and *amoA* genes

The abundance of prokaryote 16S rRNA, archaeal *amoA,* and bacterial *amoA* genes were determined using quantitative PCR with the primer sets 515F/907R [33], Arch-amoAF/Arch-amoAR [34], and A189F/A682R [35], respectively (Table S1). The amplification efficiencies for all genes were 91.5–100%, with R^2 values of 0.991–1.000. The 16S rRNA gene was amplified for sequencing using the primer pair 515F/907R [33] with a barcoded (12 bp) forward primer. Archaeal and bacterial *amoA* genes were amplified for clone library construction with the same primer sets used in quantitative PCR (Table S1). Detailed descriptions of the reaction conditions, sequencing protocols, and sequence analyses are provided in the Supplementary Information.

Metagenome sequencing and analysis

The ^{13}C-DNA of the heavy fractions (5–7 for both AOA and AOB in the solonetz soil, and 5–7 and 8–10 for AOB and AOA in the farmland soil, respectively) from the DNA-SIP soil microcosms were used to construct libraries for metagenomic sequencing using a VAHTS Universal Plus DNA Library Prep Kit for Illumina (Vazyme Biotech, Nanjing, Jiangsu, China) following the manufacturer's instructions. The size of each metagenome was 32.05±1.59 Gb, resulting in a total data output of 288.45 Gb. The metagenome sequencing and analysis are described in detail in the Supplementary Information.

Statistical analysis

One-way analysis of variance (ANOVA) with Duncan's post hoc test was performed for multiple comparisons using the statistical package SPSS version 23.0, and $p < 0.05$ was considered to indicate a significant difference. Distance-based linear modeling (DISTLM) was performed to identify the potential abiotic driver(s) for different community compositions of ammonia oxidizers.

RESULTS

Changes in saline-sodic soil properties under agricultural reclamation

Agricultural reclamation resulted in significant alleviation of soil salinity and sodicity (Table 1). The mean concentration of exchangeable sodium (E_{Na}) decreased significantly by 55.3-fold from 20.8 in the solonetz soil to 0.376 in the farmland soil. Moreover, the exchangeable sodium percentage (ESP), carbonate and bicarbonate contents also showed consistently significant declines. In addition, significant decreases in soil bulk density (SBD) and pH were observed (Table 1). Both microbial biomass carbon (MBC) and nitrogen (MBN) of the solonetz soils were much lower than those of the farmland soils. Furthermore, the mean abundance of AOB *amoA* genes was 19.4-fold lower in the solonetz soil than in the farmland soil, while AOA *amoA* gene abundance was 147.8-fold lower (Table 1). Intriguingly, nitrification potential was significantly higher (1.4-fold) in the solonetz soil than in the farmland soil (Table 1).

^{13}C-labeling of active ammonia oxidizers

Soil nitrification was assessed as the net change in NO_x^--N content (Fig. 2a). Urea fertilization led to a remarkable accumulation of NO_x^--N at approximately 800 μg g^{-1} dry soil in both solonetz and farmland soils, whereas no NO_x^--N accumulation was detected in the water-amended (control) soils (Fig. 2a). The presence of C_2H_2 completely inhibited NO_x^--N production, and the stoichiometric relationship between NH_4^+-N accumulation in C_2H_2-inhibited soils (Fig. S1) and NO_x^--N production (Fig. 2a) indicated that autotrophic nitrification predominated in both soils. Similar results were also observed for N_2O emissions (Fig. 2b), and the weekly emission flux was generally higher in solonetz soils than in farmland soils (Fig. S2). The abundance of *amoA* genes of both AOA (Fig. 2c) and AOB (Fig. 2d) showed an increasing trend only in the urea-amended soils, particularly for AOB during the 56-day incubation period.

Following ultracentrifugation of the total DNA extracted from ^{13}C-labeled and ^{12}C-control microcosms, quantification of *amoA* gene distribution as a function of the buoyant density of the DNA demonstrated labeling of growing ammonia oxidizers in both urea-amended soils (Fig. 2e). For the solonetz soil, high peaks of AOA and AOB *amoA* genes occurred in the ^{13}C-labeled 'heavy' DNA (fractions 5–7) from the ^{13}C-microcosms when compared to those from ^{12}C-microcosms. The distribution of *amoA* genes in farmland soil DNA also showed distinct labeling patterns, as the abundance of ^{13}C-AOA *amoA* genes appeared in DNA fractions 8–10, whereas ^{13}C-AOB *amoA* genes remained in DNA fractions 5–7 (Fig. 2e). Notably, no labeling of comammox *amoA* genes was detected despite the use of different primers and PCR conditions [36, 37] (Fig. S3).

Population dynamics of active ammonia oxidizers

Phylogenetic analysis of *amoA* genes in total DNA (Day 0 and Day 56) and ^{13}C-DNA at Day 56 revealed distinct changes in the community structure of active ammonia oxidizers in solonetz soil upon agricultural reclamation (Fig. 3). The population size of distinct phylotypes was further determined based on their relative proportion (Fig. 3a, b) and the total abundance of ammonia oxidizers as inferred from *amoA* gene abundance (Table S2). In the solonetz soil, the populations of AOB were exclusively within the *Nitrosococcus*-related Gammaproteobacteria, while betaproteobacterial AOB were not detected under any conditions by sequence analysis of either 16S rRNA (Fig. S4a) or *amoA* (Fig. 3a) genes. Urea amendment stimulated significantly the autotrophic growth of *Nitrosococcus*-related AOB in this solonetz soil (Fig. 3c), whereas *Nitrosospira*-related Betaproteobacteria predominated the AOB populations in the farmland soil (Fig. 3c).

Analysis of *amoA* (Fig. 3b) and 16S rRNA genes (Fig. S4b) indicated that all AOA fell within the order *Nitrososphaerales* [38]. The AOA in the solonetz soil consisted of clades NS-α (*Nitrososphaera* spp.), NS-β, and NS-γ, but only clades NS-α and NS-γ exhibited growth during microcosm incubation (Fig. 3d). The AOA in the farmland soils were in clades NS-α, NS-γ, and NS-δ (Fosmid clone 54d9 cluster), and showed autotrophic growth after urea fertilization (Fig. 3d). In both soils, the activity of AOA was dominated by clade NS-α, as suggested by the ^{13}C-DNA-SIP results (Fig. 3d). The changes in these active populations were further supported by analysis of AOB and AOA in the 16S rRNA gene amplicon analysis of total DNA and ^{13}C-DNA (Fig. S5, Table S3, Supplementary results).

Moreover, the DISTLM analysis indicated that the concentration of exchangeable Na^+ (E_{Na}) alone could explain 99.89% of the variation in active communities (Fig. 3e), suggesting that salt-tolerance was a major factor in shaping the niche separation of ammonia-oxidizing prokaryotes.

Metagenome assembly of active ammonia oxidizers

Metagenomic analysis of ^{13}C-DNA further demonstrated a significant shift in active ammonia oxidizers in the two soils. Taxonomic classification of the scaffold sequences (1340–1752 nt on average, Table S4) showed that the dominant clades of AOB were affiliated with *Nitrosococcus* and *Nitrosospira* in the solonetz

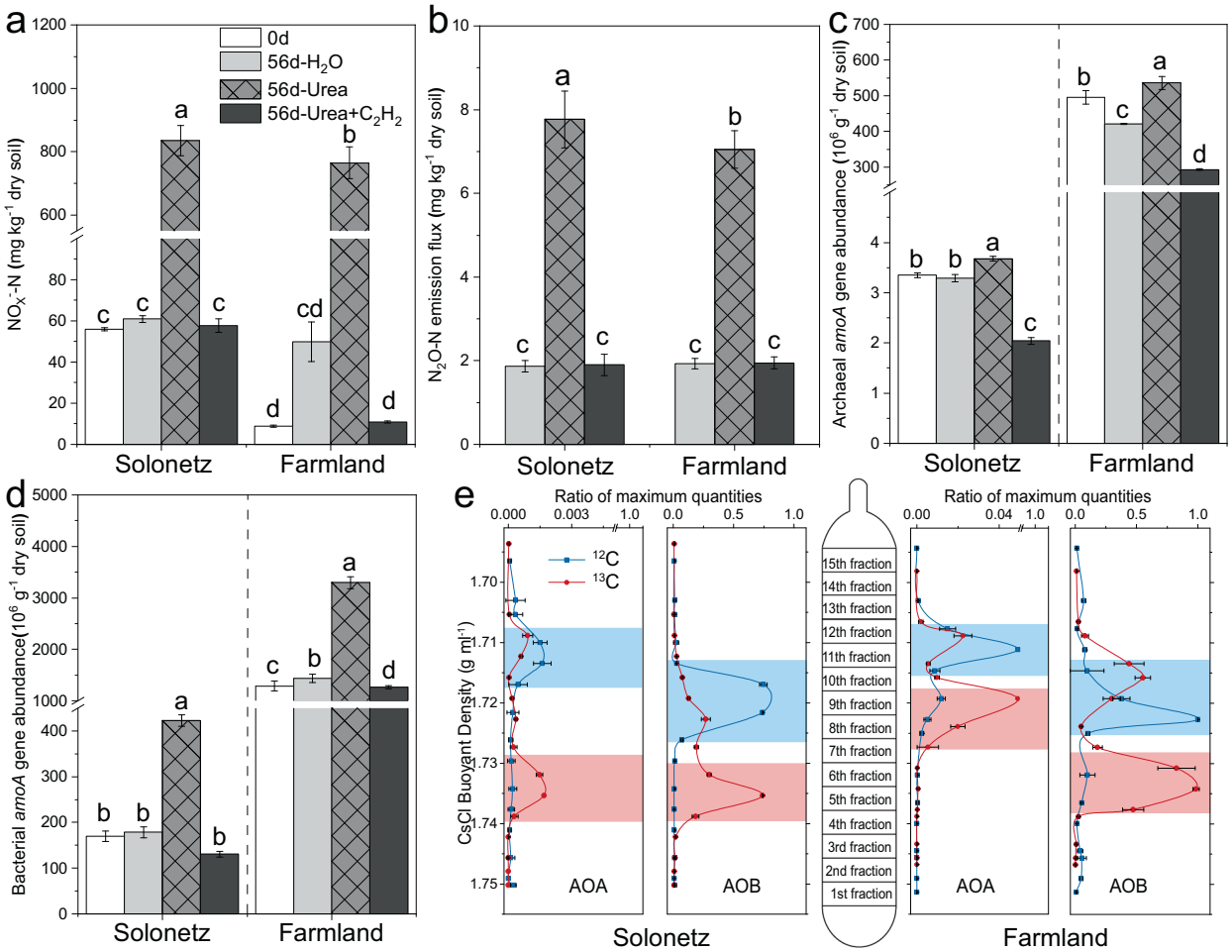

Fig. 2 Stable-isotope probing (SIP) of active ammonia oxidizers in solonetz and farmland soil. Changes in the concentration of soil nitrite-plus nitrate-N (NO_x^--N) (**a**), N_2O emissions (**b**), and *amoA* gene abundances (**c**, **d**) of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in SIP microcosms over an incubation period of 56 days. The ^{13}C-DNA of AOA and AOB was revealed by quantitative analysis of archaeal and bacterial *amoA* gene abundances across the entire buoyant density gradient of the fractionated DNA from SIP microcosms at day 56 (**e**). SIP microcosms were incubated with either ^{13}C (CO_2 and urea) or ^{12}C (CO_2 and urea), and an equal volume of H_2O instead of urea solution was amended as a control to monitor nitrification activity due to ammonia released from soil mineralization. The designations "0 d" and "56 d" denote days 0 and 56, respectively. "56 d-H_2O" and "56 d-urea" indicate samples from microcosms that received water or urea every seven days, respectively. The designation "56d-Urea+C_2H_2" represents the sample at day 56 from SIP microcosms incubated with both urea and 100 Pa C_2H_2. Different letters above the columns indicate significant differences ($p < 0.05$) (**a**, **b**). Different letters above the columns in each soil indicate significant differences ($p < 0.05$) (**c**, **d**). The data are normalized units (**e**) using the ratio of the *amoA* gene copy number in each DNA gradient fraction to the maximum quantity of two soils, and the "^{13}C-DNA" ("heavy DNA") and "^{12}C-DNA" ("light DNA") fractions are indicated by the shaded rectangles in red and blue, respectively.

and farmland soils, respectively, while the dominant clades of AOA were closely associated with NS-α in both soils (Table S5), and largely consistent with the amplicon sequencing results (Fig. 3, Fig. S5).

A total of eight genome bins of ammonia oxidizers were obtained (Table 2). Three *Nitrososcoccus* and two NS-α MAGs were recovered from the solonetz soil, but only one *Nitrososcoccus* genome (bin14) was of high quality (91.7% completeness and 0.5% contamination) (Table 2). The genome-wide average nucleotide identities (gANI) between pairwise sets of three *Nitrososcoccus* MAGs (Fig. S6) were higher than the species threshold of 95% [39], indicating that the three MAGs should represent closely related strains within the same species. Similarly, gANI and genome-wide average amino acid identities (gAAI) between bin14 and all known cultured *Nitrososcoccus* representatives (Table S6) were 74–75% [39] and 70–73% (higher than the genus cutoff of approximately 65%) [40, 41], respectively (Fig. 4a), suggesting the *Nitrososcoccus* MAGs represented a new species. This finding was further supported by the phylogenetic analysis of a concatenation of 120 conserved bacterial marker proteins (Fig. S7a) [42]. This *Nitrososcoccus* MAG was thus designated "*Candidatus* Nitrososcoccus sp. Sol14".

Three high-quality NS-α-related MAGs were obtained from the farmland soil (Table 2) and shared a gANI of 99–100%, indicating that they represented closely related strains of the same species. These MAGs likely represented a novel species according to the pairwise comparison of gANI (75–78%) and gAAI (69–77%) with known NS-α representatives (Fig. 4b, Table S6), which was verified by phylogenetic analysis (Fig. S7b) of a concatenation of 122 conserved archaeal marker proteins [42]. We propose "*Candidatus* Nitrososphaera sp. Far49", "*Candidatus* Nitrososphaera sp. Far3" and "*Candidatus* Nitrososphaera sp. Far68" as names for these three AOA MAGs, which we collectively refer to as "*Candidatus* Nitrososphaera sp. FarX" hereafter. In addition, the two NS-α bins

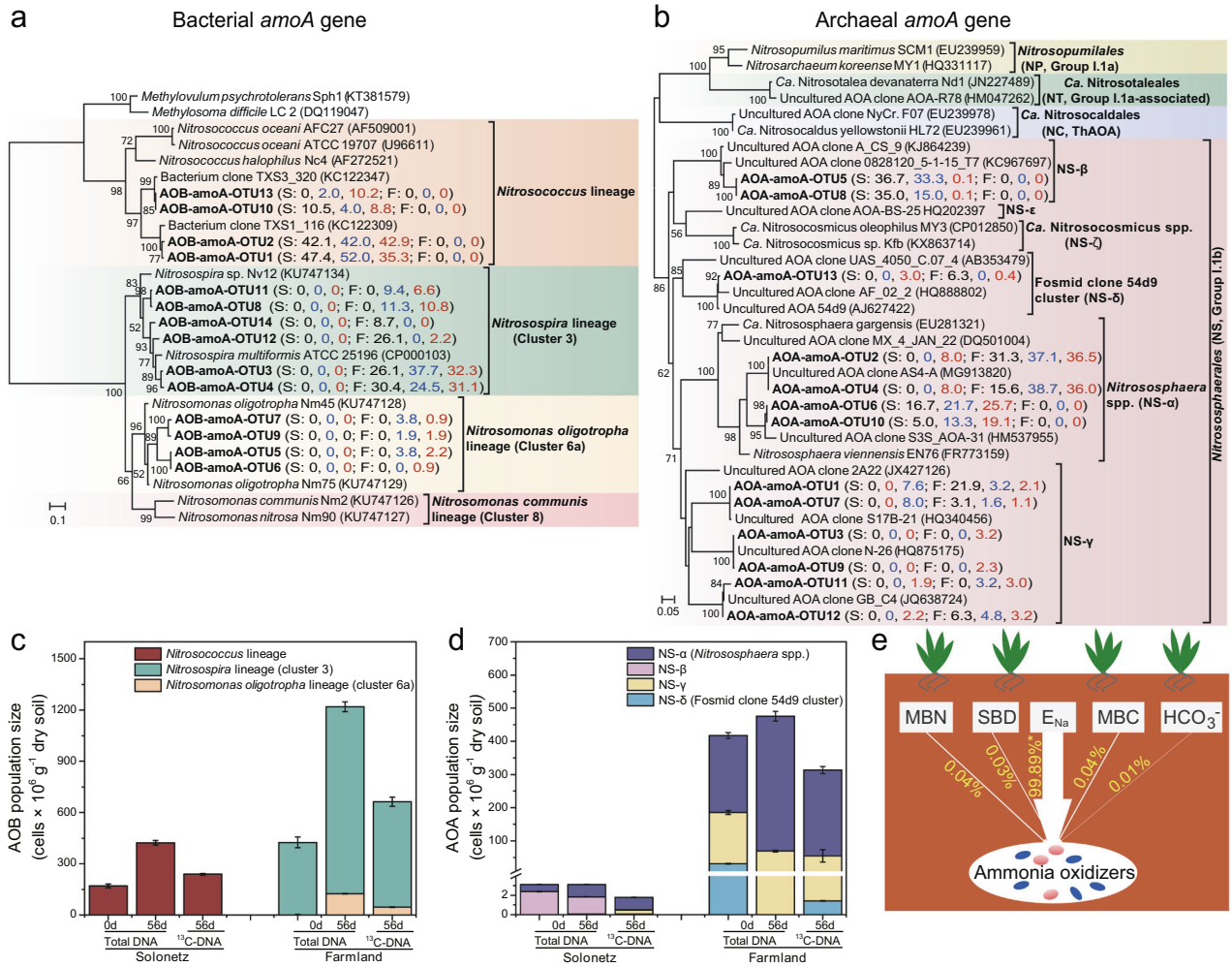

Fig. 3 Population dynamics of active ammonia oxidizers in solonetz and farmland soil. Total DNA (day 0 and 56) and ^{13}C-DNA (day 56) were sequenced for phylogenetic identification of ammonia oxidizers in soils based on *amoA* genes of AOB (**a**) and AOA (**b**). Numbers in black, blue, and red represent the relative abundance of each operational taxonomic unit (OTU) sequences to the total *amoA* gene sequences in the total DNA at day 0, total DNA at day 56, and ^{13}C-DNA, respectively. "S" and "F" in parentheses refer to solonetz and farmland soil, respectively. For instance, the designation of AOB-amoA-OTU-1 (S: 47.4, 52.0, 35.3; F:0, 0, 0) indicates that OTU-1 of AOB accounts for 47.4, 52.0, and 35.3% of the total AOB *amoA* gene sequences in the total DNA at day 0, total DNA at day 56 and ^{13}C-DNA from solonetz soils, respectively. The population size of AOB (**c**) and AOB (**d**) was determined by multiplying the relative abundance of different lineages/clusters by the total AOA or AOB abundance (Table S2). OTUs were clustered at 93% identity. The phylogeny of AOA and AOB was generated using IQtree 1.6.12 with the best fit SYM+I+G4 and TPM2u+F+G4 model selected using the BIC. Bootstraps are based on 1000 replicated trees. *Methylovulum psychrotolerans* Sph1 and *Methylosoma difficile* LC 2 were included as an outgroup within the class Gammaproteobacteria. The results of distance-based linear modeling (DISTLM) analysis of the ammonia oxidizer compositions using the physicochemical properties of soils as predictor variables (**e**), where the explanatory proportion of each variable is shown beside the arrow line. The significance level is $*p < 0.05$. MBN microbial biomass nitrogen, SBD soil bulk density, E_{Na} exchangeable Na^+ content, MBC microbial carbon, HCO_3^- HCO_3^- content.

Table 2. Characteristics of ammonia oxidizer genome bins from ^{13}C-labeled metagenomes of solonetz and farmland soils.

Soil	Taxonomic identity		Completeness (%)	Contamination (%)	GC (%)	Size (Mbp)	Genes
Solonetz	AOB	*Ca.* Nitrosococcus sp. Sol14	91.7	0.5	51.4	2.71	2521
		Nitrosococcus bin19	62.2	0.1	52.3	1.94	1783
		Nitrosococcus bin74	65.1	0.2	52.3	1.99	1843
	AOA	*Nitrososphaera* bin12	58.1	0	47.3	1.13	1327
		Nitrososphaera bin62	60.2	0	47.4	1.13	1323
Farmland	AOA	*Ca.* Nitrososphaera sp. Far3	97.3	1.9	51.3	1.59	2092
		Ca. Nitrososphaera sp. Far49	96.1	1.0	51.4	1.54	2048
		Ca. Nitrososphaera sp. Far68	98.5	1.9	51.1	1.65	2168

Only genome bins that were above 50% completion with less than 10% contamination are included.

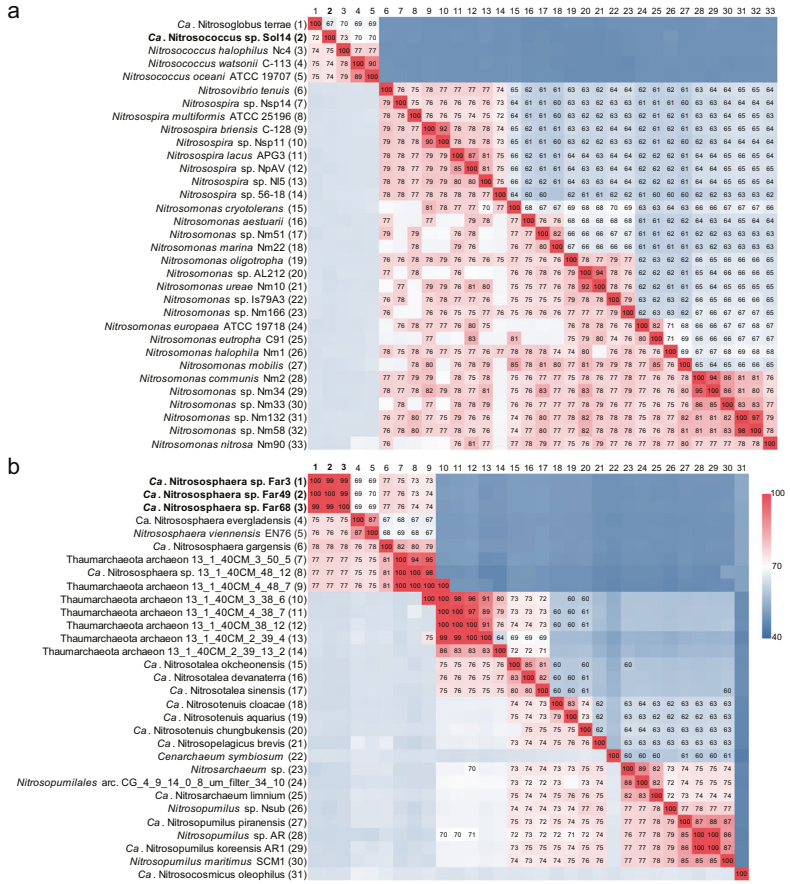

Fig. 4 Genome-wide, pairwise comparisons of the average nucleotide identity (gANI) and average amino acid identity (gAAI) values between MAGs (highlighted in bold) and known genomes of ammonia oxidizers. a Symmetrical matrix of pairwise gANI and gAAI between AOA MAGs (*Ca*. Nitrososphaera sp. Far3, *Ca*. Nitrososphaera sp. Far49 and *Ca*. Nitrososphaera sp. Far68) and known AOA genomes (Table S6). The gANI is presented in the lower left triangle and values ≥70. The gAAI is presented in the upper right triangle and values ≥60 are provided. **b** Symmetrical matrix of pairwise gANI and gAAI between AOB MAG (*Ca*. Nitrosococcus sp. Sol14) and known AOB genomes (Table S6). The gANI is presented in the lower left triangle and values ≥70. The gAAI is presented in the upper right triangle and values ≥60 are provided.

of the solonetz soil and *Ca*. Nitrososphaera sp. FarX were not the same species according to a pairwise comparison of gANI and gAAI (Fig. S6).

Salt-tolerance mechanisms in active ammonia oxidizers

Comparative metagenomic analysis of active ammonia oxidizers identified gene repertoires that could potentially enable increased salt-tolerance of *Nitrosococcus* strains compared to *Nitrosospira* and NS-α (Table S7). As there was no MAG available for AOB in the farmland soils, we parsed all the genes from the *Nitrosospira*-affiliated scaffolds in the metagenome dataset for comparison (Table S7). Metabolic reconstruction revealed the key role of three modes of saline adaption in driving niche differentiation of ammonia oxidizers in saline-sodic soil (Fig. 5).

Na^+ extrusion. The *Ca*. Nitrosococcus sp. Sol14 genome encodes proteins involved in four Na^+ extrusion mechanisms, i.e., NhaA, NhaD, NhaP, and Mrp Na^+/H^+ antiporters [43–45] (Fig. 5). However, *Nitrosospira* from the farmland soil appeared to possess only NhaA according to the annotation of the scaffold genes (Fig. 5). None of Na^+ extrusion mechanisms in *Ca*. Nitrosococcus sp. Sol14 was detected in *Ca*. Nitrososphaera sp. FarX and NS-α bins of the solonetz soil (Fig. 5).

Inorganic compatible solute uptake. The Trk transporter is a major transport system for K^+ accumulation in cells [46], and the genes *(trkAH)* encoding these proteins were identified in *Ca*. Nitrosococcus sp. Sol14 but were absent in all the MAGs of AOA and *Nitrosospira* scaffolds (Fig. 5). Genes encoding MgtE and CorA proteins for Mg^{2+} uptake [47] were identified in the *Ca*. Nitrosococcus sp. Sol14 genome, while the *Nitrosospira* and AOA MAGs contained genes encoding only CorA (Fig. 5). In addition, *Ca*. Nitrososphaera sp. FarX and *Nitrosospira* (Fig. 5) might import Ca^{2+} and export Na^+ by YrbG [47].

Organic compatible solute transport and biosynthesis. The identification of *opuCA* genes only in *Ca*. Nitrosococcus sp. Sol14 suggests the potential to import glycine betaine [47]. The genes (*treS* and *ectABCD*) encode proteins involved in the synthesis of the compatible solutes trehalose, ectoine and hydroxyectoine [44, 48, 49] and were identified only in *Ca*. Nitrosococcus sp. Sol14 (Fig. 5). Glutamate is also a compatible solute [50], which may be synthesized through the reversible reactions of glutamate dehydrogenase (GDH2) and glutamine synthetase (GlnA) [50] and the reaction catalyzed by glutamate synthase (GltDB) in *Ca*. Nitrosococcus sp. Sol14 (Fig. 5a). However, *Ca*. Nitrososphaera sp. FarX might encode another glutamate dehydrogenase (GdhA) and GlnA (Fig. 5b). GdhA and GlnA were also identified in NS-α bins from the solonetz soil and *Nitrosospira* (Fig. 5), respectively. In addition, *Ca*. Nitrosococcus sp. Sol14 might be able to convert glutamate into proline as another compatible substance, catalyzed by glutamate 5-kinase (ProB), glutamate-5-semialdehyde dehydrogenase (ProA), and pyrroline-5-carboxylate reductase (ProC) (Fig. 5a). *Nitrosospira* may also perform this conversion because the key enzyme (ProA) was detected

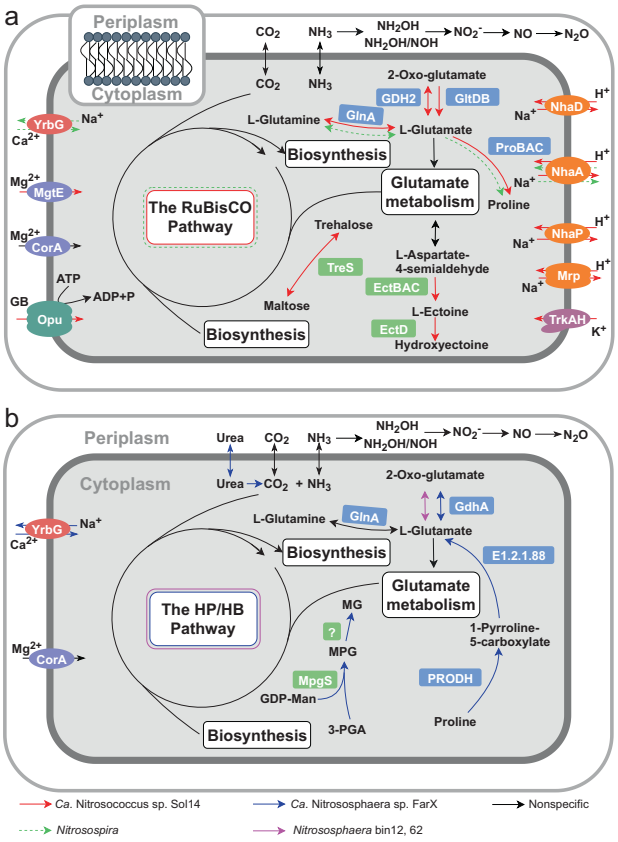

Fig. 5 Metabolic reconstruction of active ammonia-oxidizing bacteria and archaea in response to agricultural reclamation of Solonetz saline-sodic soil. Cell metabolism diagrams of AOB (**a**) and AOA (**b**) were constructed from the genome annotation of *Ca.* Nitrosococcus sp. Sol14, *Ca.* Nitrososphaera sp. FarX and Nitrososphaera bin12, 62 and the scaffold annotation of the genus Nitrosospira. Putative adaptations to high salinity and selected core metabolic pathways of ammonia oxidizers are shown. NhaA NhaA Na^+/H^+ antiporter, NhaD NhaD Na^+/H^+ antiporter, NhaP NhaP Na^+/H^+ antiporter, Mrp Mrp Na^+/H^+ antiporter, Trk Trk K^+ uptake system, MgtE/CorA magnesium uptake mediated by facilitated diffusion, Opu glycine betaine uptake transporter, TreS trehalose synthase, EctA diaminobutyrate acetyl transferase, EctB diaminobutyrate transaminase, EctC ectoine synthase, EctD ectoine hydroxylase, GDH2 and GdhA glutamate dehydrogenase, GlnA glutamine synthetase, GltDB glutamate synthase, ProA glutamate-5-semialdehyde dehydrogenase, ProB glutamate-5-kinase, ProC pyrroline-5-carboxylate reductase, YrbG Ca^{2+}/Na^+ antiporters, PRODH proline dehydrogenase, E1.2.1.88 1-pyrroline-5-carboxylate dehydrogenase, MpgS mannosyl-3-phosphoglycerate synthase, question mark (?) uncharacterized phosphatase, GB glycine betaine. See Table S7 for detailed gene presence/absence.

(Table S7). In contrast, only *Ca.* Nitrososphaera sp. FarX could convert proline into glutamate through 1-pyrroline-5-carboxylate dehydrogenase (E1.2.1.88, PCD) and proline dehydrogenase (PRODH) (Fig. 5b). Moreover, *Ca.* Nitrososphaera sp. FarX may synthesize the compatible solute mannosylglycerate (MG), as the gene encoding the key enzyme mannosyl-3-phosphoglycerate synthase (MpgS) was detected (Fig. 5b) [51].

Genomes of previously characterized ammonia oxidizers in the NCBI database were also examined for the presence of genes encoding the above-described proteins (Fig. 6). These salt-tolerant mechanisms were unevenly distributed among ammonia-oxidizing microorganisms, especially Na^+ extrusion mechanisms, which are relatively scarce (Fig. 6).

DISCUSSION

Salinity-based niche differentiation of ammonia oxidizers

Our results showed a significant shift in the active ammonia-oxidizing communities in solonetz soil under reclamation. Stable-isotope probing revealed the growth and activity of both AOB and AOA in saline-sodic soil. In particular, *Nitrosococcus*- and *Nitrosospira*-affiliated clades dominated the active AOB in the solonetz and farmland soils, respectively, and were most likely the primary contributors to ammonia oxidation, considering the higher population size of AOB than AOA in these two soils (Fig. 3c, d) and the higher specific cell activities of AOB than AOA usually observed in pure cultures [8, 52, 53]. Furthermore, the increased ammonia availability caused by alkaline pH in both soils is likely to favor AOB over both AOA and comammox [54, 55]. *Nitrosospira* is frequently identified as the major contributor to ammonia oxidation in soils, especially those under agricultural management [33, 56, 57]. The estimated cell apparent activity of AOA far exceeded the activities of pure cultures, further suggesting the dominant activity of *Nitrosococcus* in solonetz soils (Table S2). Gammaproteobacterial AOB are predominantly found in marine environments from which all previously cultured *Nitrosococcus* species were isolated [22, 23]. Intriguingly, *Nitrosococcus*-related and other gammaproteobacterial AOB have been demonstrated using molecular surveys and physiological studies to be present in a diverse range of habitats, including wastewaters [24], saline alkaline soils [26], a biofilter [27], a tea field [7] and rangelands [25, 28], also indicating that gammaproteobacterial AOB may have a higher diversity and more widespread distribution than previously appreciated (Fig. S8). Nonetheless, the ecophysiologies of these gammaproteobacterial AOB remain poorly understood in complex natural environments. Our labeling results provide the first direct evidence of *Nitrosococcus* dominating autotrophic ammonia-oxidizing activity in upland soil under salt stress. The NS-α clade was the dominant group of AOA in both soils investigated in our study, which is consistent with previous findings in alkaline farmland soil [33].

E_{Na} is potentially the most critical factor driving the niche specialization of different ammonia-oxidizer clades in these two soils (Fig. 3e). The solonetz soil exhibited a 40-fold higher E_{Na} than the converted farmland soil, which may have selected microbes with different tolerances to elevated salinity. Similarly, high salinity has been observed in wastewater reactors [24], marine ecosystems [22, 58], and salt lakes [23], which also contain dominant or highly abundant *Nitrosococcus* organisms similar to those in the solonetz soil. The adaptation of AOA to high salinity has also been previously demonstrated in aquatic systems based on the isolation of several strains of marine AOA [17, 50, 59–61], and ecological studies have shown high abundances of *Nitrosopumilales*-related AOA in marine systems, saline wastewater and floodplains [62–64], NS-α dominated groups in mangrove sediment [65] and the dominant activity of a *Nitrosocosmicus*-related clade (NS-ζ) following salinization of activated sludge [20]. Interestingly, the NS-α and NS-γ clades were present consistently in both soils, suggesting that these clades of AOA could adapt to a wide range of environmental conditions and may be important in maintaining the diversity and activity of AOA in soils subjected to severe environmental disturbances, e.g., during land conversion. In addition, growth of NS-δ was detected in the farmland soil, while NS-β was present but did not grow during incubations of the solonetz soil (Fig. 3c, d). Therefore, NS-β is likely specialized for highly saline environments and replaced by NS-γ in soils with much less salinity, leading to the reassembly of communities of AOA following agricultural conversion. The average cell apparent activity of total active ammonia oxidizers in the solonetz soil was 4.5-fold higher than that in the farmland soil (Table S2), which suggested that active ammonia oxidizer communities dominated by *Nitrosococcus*-related AOB in the solonetz soil possessed a much higher average specific-cell-energy-yield efficiency and

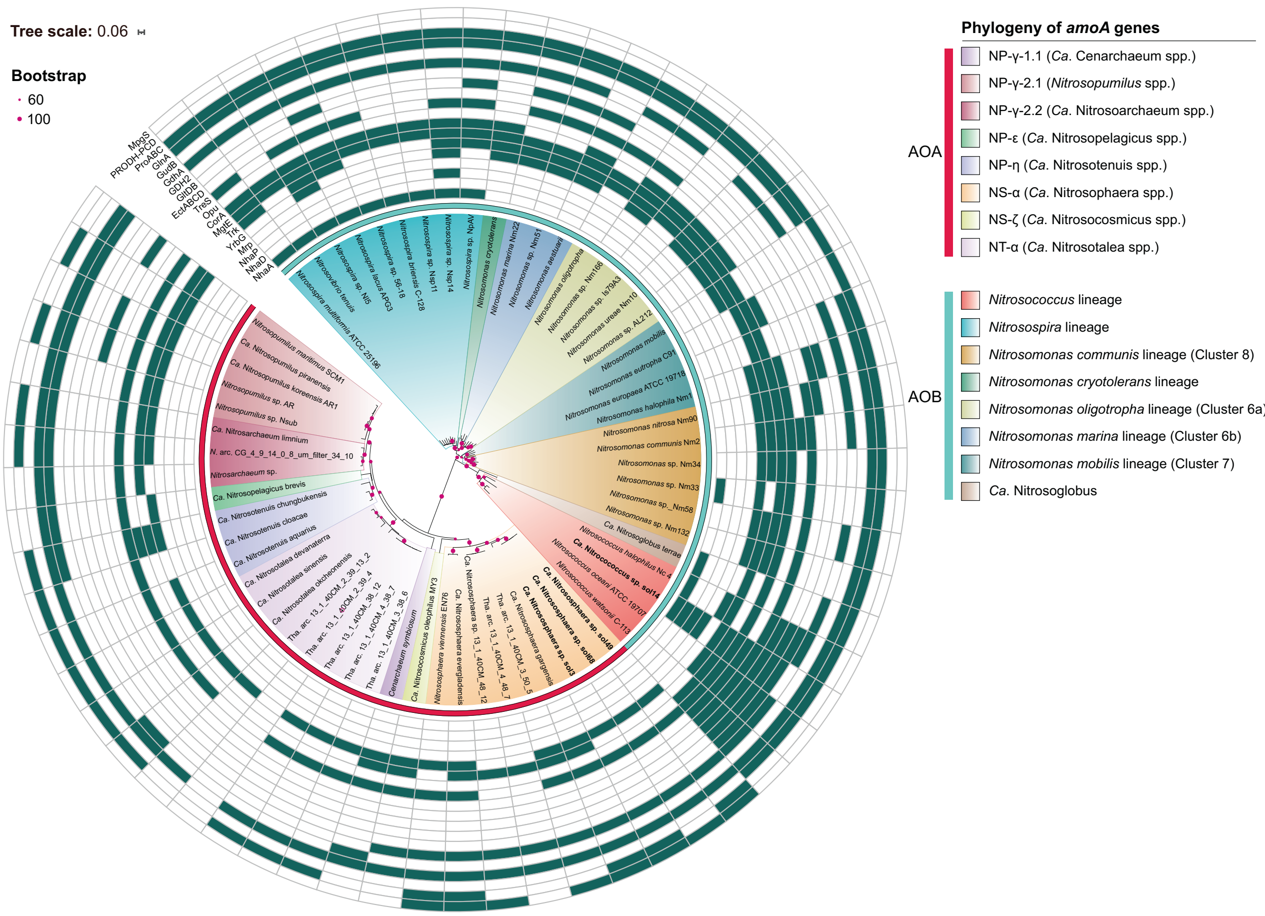

Fig. 6 Meta-analysis of salt resistance genes in phylogenetically distinct ammonia-oxidizing bacteria and archaea. Phylogenetic tree of *amoA* genes from typical AOA and AOB species, with known genomes (see Table S6), including those from the MAGs in this study, highlighted in bold. Representative *amoA* sequences were phylogenetically analyzed with MEGA version 7.0 using the neighbor-joining method and the maximum composite likelihood model with 1000 replicates to generate bootstrap values. Outside the tree, the phylogenetic grouping of AOA and AOB is shown in the first internal ring with the colored strip in red and blue, respectively. The salt tolerance proteins identified in active ammonia oxidizers in this study (Fig. 5) are shown from the internal 2nd ring to the 20th ring including those encoding proteins of NhaA; NhaD; NhaP; Mrp; YrbG; Trk; MgtE; CorA; Opu; TreS; EctABCD; GltDB; GDH2; GdhA; GudB; GlnA; ProABC; PRODH-PCD; and MpgS. The presence and absence of these proteins in ammonia oxidizers are indicated in green and white, respectively. GudB, glutamate dehydrogenase (K00260); PCD, E1.2.1.88/1-pyrroline-5-carboxylate dehydrogenase.

appeared to have more energy to overcome the limitation of the low energy-yield of nitrification and tolerate salt stress [66].

Genomic adaptations to salt stress

High salinity leads to high osmotic pressure that can severely constrain the survival of ammonia oxidizers in such environments [50, 66]. In our study, comparative genomics analysis indicated that a range of different mechanisms are adopted by ammonia oxidizers to cope with excessive Na^+ and high osmotic stress. NhaA, NhaD, NhaP and Mrp, identified in *Ca.* Nitrosococcus sp. Sol14, represents a group of membrane transporter proteins, with secondary Na^+/H^+ antiporter activity energized by the proton motive force for exporting Na^+ and importing H^+ (Fig. 5) [67]. NhaA, NhaD, and NhaP are encoded by single genes and are widely distributed in a wide variety of microorganisms involved in the maintenance of ion homeostasis under high sodium stress [43, 45], while Mrp is typically encoded by six or seven genes and has been shown to play an important role in Na^+ resistance [67]. As these antiporters are involved in H^+ uptake in exchange for cytoplasmic Na^+ (Fig. 5, Fig. S9, Supplementary results), they could also contribute to maintaining a lower intracellular pH compared with the exceedingly high alkaline saline-sodic soils [44, 68, 69]. The variety of Na^+ transport mechanisms identified in the *Nitrosococcus* MAG may allow it to survive under severe salt stress even in oligotrophic conditions because the energetic cost of establishing ionic gradients for salt tolerance is less than that of biosynthesizing compatible solutes [70]. The multiple Na^+/H^+ antiporters, which were very scarce or absent in *Nitrosospira* and NS-α in our soils and other known ammonia oxidizers (Fig. 6), may contribute to protecting *Nitrosococcus* against Na^+ toxicity and high pH stress. In addition, adjusting the pH and osmotic homeostasis with the same transporter is obviously an effective strategy for efficient energy utilization, particularly considering the low energy yield of ammonia oxidation [50, 70].

Inorganic compatible solutes can be absorbed by cells to maintain osmotic equilibrium when the external salt concentration is high, whereas inorganic ions are maintained mostly outside the cells [46, 71, 72]. For instance, cytoplasmic K^+ is less toxic to enzyme activity and metabolic function than Na^+ [73], and many bacteria actively accumulate K^+ to achieve osmotic equilibrium at elevated saline concentrations [74, 75]. Similarly, the Trk system harbored by *Ca.* Nitrosococcus sp. Sol14 is widely distributed in haloarchaea [47] and has been demonstrated to play a vital role in Na^+ resistance in the halophilic bacterium *Halomonas elongate* of the family *Halomonadaceae* [46] that was also detected in the solonetz soil (Table S8), suggesting that K^+ uptake likely alleviated the salt stress of *Ca.* Nitrosococcus sp. Sol14. Moreover, Mg^{2+} uptake transporters possessed by the ammonia oxidizers in our

soils may also alleviate Na^+ stress and appear to be important in stabilizing halophilic enzymes in many microbes [47]. However, Mg^{2+} transport does not seem to be a specific factor in the salt tolerance of ammonia oxidizers in the solonetz soil, because almost all ammonia-oxidizing microorganisms contain Mg^{2+} uptake transporters (Fig. 6). In addition, the *Ca*. Nitrososphaera sp. FarX and *Nitrosospira* (Fig. 5) in our soil possess YrbG antiporters to extrude Na^+ by Ca^{2+} uptake, but this process might function only under high Ca^{2+} content conditions [47], which are not available in saline-sodic soil. YrbG proteins were consistently detected in 41 ammonia oxidizers (Fig. 6) but not in the genome of *Ca*. Nitrosococcus sp. Sol14, possibly indicating that YrbG was not an effective antiporter for salt tolerance in our soil.

Glycine betaine is another compatible solute that many prokaryotes and eukaryotes are known to accumulate under salt stress to regulate their osmotic pressure. For example, in the presence of a high concentration of sodium chloride, the addition of glycine betaine could improve the salt-tolerance of *Rhizobium meliloti* Be 151 [76]. Opu was identified in *Ca*. Nitrosococcus sp. Sol14 and two other *Nitrosococcus* strains from marine ecosystems [22, 77] as a transport system for glycine betaine (Fig. 6), and may be a commonly adopted salt tolerance mechanism for *Nitrosococcus*. Among all active ammonia oxidizers in our study, only *Ca*. Nitrosococcus sp. Sol14 appeared to possess the enzyme for the biosynthesis of trehalose, which is an organic molecule produced in many microorganisms and is typically associated with halotolerance [48, 78]. Both ectoine and hydroxyectoine are osmolytes [49] that are synthesized by *Nitrosopumilus maritimus* in response to increased osmotic stress [50, 79]. The gene cluster *ectABCD* encodes proteins for the biosynthesis of ectoine and hydroxyectoine from the substrate aspartate semialdehyde [80, 81], and was detected in *Ca*. Nitrosococcus sp. Sol14 and all three known *Nitrosococcus* genomes (Fig. 6) but absent in the MAGs of AOA and *Nitrosospira* scaffolds in our soils (Fig. 5). TreS and EctABCD proteins might confer an advantage to *Nitrosococcus* activity and competition over other ammonia oxidizers in our solonetz soil. Moreover, the cytoplasmic glutamate concentrations in some bacteria increase after exposure to highly osmotic media [82]. Similarly, glutamate may be synthesized in *Nitrosopumilus* by reversible reactions of glutamate dehydrogenase and glutamine synthetase [50], which were also identified in *Ca*. Nitrosococcus sp. Sol14 and *Ca*. Nitrososphaera sp. FarX (Fig. 5). Analysis of the *Nitrosospira* scaffolds and AOA MAGs of the solonetz soil indicated that these organisms may be able to synthesize glutamate via glutamine synthetase and glutamate dehydrogenase, respectively (Fig. 5). However, *Nitrosopumilus* may also take up and synthesize glutamate through a glutamate/aspartate symporter and the ornithine-glutamate reaction, respectively [50], which were not found in *Ca*. Nitrosococcus sp. Sol14 or AOA MAGs or *Nitrosospira* (Fig. 5). It is noteworthy that only some AOB, including *Ca*. Nitrosococcus sp. Sol14, have the potential to synthesize glutamate through the reaction of glutamate synthase [83] (Fig. 6). These multiple reactions may indicate that glutamate plays an important role in the adaptation of *Ca*. Nitrosococcus sp. Sol14 to its highly saline habitat.

Proline is a vital osmoprotectant for many Gram-positive bacteria, such as the moderately halophilic *Salinicoccus roseus* and *Salinicoccus hispanicus* [84]. Many bacteria increase their proline concentrations under osmotic stress by synthesizing or taking up proline [82]. Proline synthesis proteins [85] were identified in *Ca*. Nitrosococcus sp. Sol14. *Nitrosospira* in our soils may also synthesize proline based on the identified key enzyme (ProA) (Table S7) and the wide distribution of ProABC in known *Nitrosospira* genomes (Fig. 6). These results potentially illustrate that proline synthesis is not the key mechanism underlying the salt resistance of *Ca*. Nitrosococcus sp. Sol14. Interestingly, as in *Nitrosopumilus* [50], proteins that convert proline to glutamate were detected in *Ca*. Nitrososphaera sp. FarX, suggesting the potential for osmolyte switching [50]. However, the significance of this reaction with regard to salt tolerance is unclear. *Ca*. Nitrososphaera sp. FarX may be capable of forming MG, which is absent in *Ca*. Nitrosococcus sp. Sol14 and *Nitrosospira* (Fig. 5), as a compatible solute for cellular osmotic adjustment and thermal protection; this inference is based on the identification of a key enzyme similar to that found in *Nitrososphaera* and *Nitrosocaldus* lineages [51, 86]. MG is distributed widely among thermophilic and hyperthermophilic organisms [87]. However, in a few *Rhodothermus marinus* strains, MG accumulates only at supraoptimal growth temperatures during salt stress, suggesting that the synthesis of MG may require a higher temperature [87]. As the average annual temperature of the sites used in this study is only 4.7 °C [31], it seems unlikely that AOA use MG for effective defense against salt stress.

In summary, diverse salt tolerance mechanisms are key to the competitive adaptation of *Ca*. Nitrosococcus sp. Sol14 to its highly saline environment. *Ca*. Nitrosococcus sp. Sol14 contains more Na^+/H^+ antiporters than other *Nitrosococcus* representatives, suggesting greater salt-tolerance. These results also indicate that the ecological significance of active *Ca*. Nitrosococcus sp. Sol14 could be largely represented by pure culture studies, wherein the maximum salt tolerance of *Nitrosococcus* representatives (80–180 cmol per liter) [77, 88] was shown to be much higher than that of *Nitrosospira briensis* (only 25 cmol per liter) [88], which has far fewer salt-resistance mechanisms.

CONCLUSIONS

The present study showed salt tolerance-based niche differentiation of soil ammonia oxidizers. The *Nitrosococcus* species, which are predominantly found in marine environments and salt lakes, was demonstrated in soil ecosystems with markedly high sodium salt content and high pH. The targeted reconstruction of metagenome-assembled ^{13}C-labeled genomes revealed that *Nitrosococcus* in saline-sodic soil possesses a more sophisticated assembly of salt tolerance mechanisms including Na^+/H^+ antiporters, a K^+ uptake system and the transport and biosynthesis of organic compatible solutes (glycine betaine, trehalose, ectoine, hydroxyectoine, and glutamate) than other ammonia oxidizers (Thaumarchaeota and *Nitrosospira*) detected in the soil. These findings extend our understanding of important salt-tolerant microbes contributing to the nitrogen cycles, and suggest that the ecological importance of gammaproteobacterial ammonia oxidizers need to be re-assessed in salt-affected environments.

DATA AVAILABILITY

Raw 16S rRNA and *amoA* gene sequences were deposited in the NCBI Sequence Read Archive (SRA) database under the BioProject ID PRJNA641227. Metagenomics and metagenome-assembled genomes (MAGs) data are available at MG-RAST under the study names DAAN_WGS and DAAN_MAGs, respectively.

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AUTHOR CONTRIBUTIONS

XS and ZJ designed the study. XS performed the experiments and analyzed the data. JZ, XZ, and WX helped data mining. QB constructed the metagenome-assembled genomes. BZ helped acquire soil data. ZJ and J-BZ supervised the project and approved the final version. XS wrote the manuscript with input from all authors.

COMPETING INTERESTS

The authors declare no competing interest.

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