1 **Physiological and genomic analysis of “*Candidatus Nitrosocosmicus agrestis*”, an**
2 **ammonia tolerant ammonia-oxidizing archaeon from vegetable soil**

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23 ABSTRACT

24 The presences of ammonia tolerant ammonia-oxidizing archaea (AOA) in environments are always
 25 underestimated and their adaption to complex habitats has also rarely been reported. Here we present
 26 the physiological and genomic characteristics of an ammonia tolerant soil AOA strain *Candidatus*
 27 *Nitrosocosmicus agrestis*. This strain was able to form aggregates and adhere on the surface of
 28 hydrophobic matrix. Ammonia-oxidizing activities were still observed at 200 mM NH_4^+ (> 1500 μM of
 29 free ammonia) and 50 mM NO_2^- . Urea could be used as sole energy source but exogenous organics had
 30 no significant effect on the ammonia oxidation. Besides the genes involving in ammonia oxidation,
 31 carbon fixation and urea hydrolysis, the genome also encodes a full set of genes (GTs, GHs, CEs, MOP,
 32 LPSE, etc) that responsible for polysaccharide metabolism and secretion, suggesting the potential
 33 production of extracellular polymeric substances (EPS). Moreover, a pathway connecting urea cycle,
 34 polyamines synthesis and excretion was identified in the genome, which indicates the NH_4^+ in
 35 cytoplasm could potentially be converted into polyamines and excreted out of cell, and then contributes
 36 to the high ammonia tolerance. Genes encoding the cytoplasmic carbonic anhydrase and putative
 37 polyamine exporter are unique in *Ca. Nitrosocosmicus agrestis* or the genus *Ca. Nitrosocosmicus*,
 38 suggesting the prevalence of ammonia tolerance in this clade. The proposed mechanism of ammonia
 39 tolerance via polyamines synthesis and export was verified by using transcriptional gene regulation and
 40 polyamines determination.

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42 IMPORTANCE

43 AOA are ubiquitous in different environments and play a major role in nitrification. Though AOA have
 44 higher affinities for ammonia, their maximum specific cell activity and ammonia tolerance are usually

45 much lower than AOB, resulting in low contribution to the global ammonia oxidation and N₂O
 46 production. However, in some agricultural soils, the AOA activity would not be suppressed by the
 47 fertilization with high concentration of ammonium nitrogen, suggesting the presence of some ammonia
 48 tolerant species. This study provides some physiological and genomic characteristics for an ammonia
 49 tolerant soil AOA strain *Ca. Nitrosocosmicus agrestis* and proposes some mechanisms of this AOA
 50 adapting to a variety of environments and tolerating to high ammonia. Ammonia tolerance of AOA was
 51 always underestimated in many previous studies, physiological and genomic analyses of this AOA
 52 clade are benefit to uncover the role of AOA playing in global environmental patterns.

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67 INTRODUCTION

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69 Ammonia-oxidizing archaea (AOA) of the phylum *Thaumarchaeota* play a major role in nitrification
70 that mediating the conversion of ammonia to nitrate via nitrite (Stahl and de la Torre, 2012; Stein and
71 Klotz, 2016; Kuypers *et al.*, 2018; Stein, 2019). AOA are ubiquitous and able to thrive in different
72 environments. It was estimated that the AOA represents up to 1-5% of all prokaryotes in soils and
73 20–40% of all marine bacterioplankton in marine system (Stahl and de la Torre, 2012). AOA within the
74 phylum *Thaumarchaeota* is composed of four orders (Kerou *et al.*, 2016): (i) *Ca. Nitrosopumilales*:
75 mainly (*Ca. Nitrosopumilus*, *Ca. Nitrosopelagicus*) derived from the oligotrophic ocean (Könneke *et*
76 *al.*, 2005; Hatzenpichler, 2012; Qin *et al.*, 2014; Santoro *et al.*, 2015; Bayer *et al.*, 2016) and some of
77 them (*Ca. Nitrosoarchaeum*, *Ca. Nitrosotenuis*) come from terrestrial habitats (Jung *et al.*, 2011, 2014;
78 Li *et al.*, 2016; Sauder *et al.*, 2018). (ii) *Ca. Nitrosotaleales*: represented by the genus *Ca. Nitrosotalea*
79 that mainly distributed in acidic soil (Lehtovirta-Morley *et al.*, 2011). (iii) *Ca. Nitrosocaldales*:
80 members of thermophilic AOA belonging to genus *Ca. Nitrosocaldus* (Hatzenpichler *et al.*, 2008; Abby
81 *et al.*, 2018; Daebeler *et al.*, 2018). (iv) *Nitrososphaerales*: *Nitrososphaera*, *Ca. Nitrosocosmicus* and
82 many uncultivated AOA that inhabit in soils (Tourn *et al.*, 2011; Zhelnina *et al.*, 2014;
83 Lehtovirta-Morley *et al.*, 2016), sediments (Jung *et al.*, 2016), terrestrial hot springs (Hatzenpichler *et*
84 *al.*, 2008) and wastewater treatment plants (Sauder *et al.*, 2017). Up to now, four strains belonging to
85 *Nitrososphaera* and five strains belonging to *Ca. Nitrosocosmicus* have been obtained (Alves *et al.*,
86 2019; Liu *et al.*, 2019). As a sister cluster of *Nitrososphaera*, the *Ca. Nitrosocosmicus* owns many
87 different properties from *Nitrososphaera* and the other AOA clusters, especially the ability to tolerate
88 high concentration of ammonia (Jung *et al.*, 2016; Lehtovirta-Morley *et al.*, 2016; Sauder *et al.*, 2017).

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90 Nitrification is a central process in the global nitrogen cycle. Owing to the ammonia affinities of AOA

91 are dozens or hundreds of times higher than AOB (Könneke *et al.*, 2005; Martens-Habbena *et al.*, 2009;

92 Kits *et al.*, 2017), members of AOA have usually been observed to have higher abundance than AOB in

93 the habitats containing low ammonium, such as the oligotrophic oceans (Berg *et al.*, 2015; Trimmer *et*

94 *al.*, 2016). However, in agricultural soils, the overdose fertilization always results in high concentration

95 of ammonia. Though AOA have higher affinities for ammonia, their maximum specific cell activity

96 (V_{max}) and ammonia tolerance are much lower than AOB (Prosser and Nicol, 2012; Kits *et al.*, 2017;

97 Liu *et al.*, 2019). High ammonium in agricultural soils usually contributed to the activation of AOB and

98 the limitation of AOA, even though the AOA groups dominated in numbers (Sterngren *et al.*, 2015;

99 Egan *et al.*, 2018). Aerobic ammonia oxidation that performed by AOA and AOB is usually regarded as

100 the main source of nitrous oxide (N_2O) and contributes to the global N_2O emissions from agriculture

101 (Reay *et al.*, 2012; Oertel *et al.*, 2016; Lam *et al.*, 2017). In general, the AOA species were reported to

102 have low ammonia tolerance (only up to 1-20 mM) (Table 1) while AOB species were able to tolerate

103 7-50 mM (Prosser and Nicol, 2012) even up to 400 mM (Hunik *et al.*, 1992). Then, AOB groups were

104 usually regarded as the main contributor who was responsible for the N_2O emission in agricultural

105 fields after fertilizing with ammonium nitrogen (Wang *et al.*, 2016; Hink *et al.*, 2017; Meinhardt *et al.*,

106 2018). However, the AOA groups were still observed to have a small contribution (10-20%) to the N_2O

107 emission (Wang *et al.*, 2016; Hink *et al.*, 2017; Meinhardt *et al.*, 2018). Moreover, in some agricultural

108 soils, the ammonia oxidation and N_2O production of AOA would not be suppressed by the fertilization

109 with high ammonium (Schauss *et al.*, 2009; Stopnišek *et al.*, 2010; Lu *et al.*, 2015; Duan *et al.*, 2019).

110 It seems that some unknown AOA species was able to tolerate high ammonia in these soils. No

111 evidence indicated that the presence of ammonia tolerant AOA until obtaining the enrichments of
112 *Nitrosocosmicus* from soil, sediment and WWTP (Jung *et al.*, 2016; Lehtovirta-Morley *et al.*, 2016;
113 Sauder *et al.*, 2017).
114
115 Generally, ammonia tolerance of AOA groups that derived from soils are always higher than the groups
116 from marine and fresh water, and the AOA strains from genus *Ca. Nitrosocosmicus* have the highest
117 currently known tolerance (Table 1). For example, the ammonia tolerance of *Ca. Nitrosocosmicus*
118 franklandus C13 that enriched from arable soil is about 50 times higher than the *Nitrosopumilus*
119 maritimus SCM1 that isolated from tropical marine aquarium, and 2-10 times higher than many soil
120 AOB (Lehtovirta-Morley *et al.*, 2016). Because the ammonia tolerant AOA were always
121 undistinguished in many previous studies, archaeal ammonia oxidation and N₂O production might be
122 underestimated in agricultural soils. Obtaining much more AOA strains from environments and
123 figuring out their biochemical, physiological and ecological features are benefit to uncover the role of
124 AOA playing in global environmental patterns. In our previous work, three AOA strains that affiliating
125 with the genus *Ca. Nitrosocosmicus* were obtained from agricultural soils by using a two-step strategy
126 (Liu *et al.*, 2019). In this study, one of these AOA strains that named as *Ca. Nitrosocosmicus* agrestis
127 SS was observed to be able to tolerate 200 mM ammonium (1592.07 µM free ammonia) (Table 1).
128 Based on the physiological and genomic studies, an ammonia tolerant mechanism of the strain *Ca.*
129 *Nitrosocosmicus* agrestis SS was proposed and verified by using transcriptional gene regulation and
130 metabolites determination, supplying some new features to full the *Nitrosocosmicus* clade or uncover
131 novel roles of AOA in the global environment.

132

133 **RESULTS AND DISCUSSION**

134

135 *Phylogeny and Morphology of “Ca. Nitrosocosmicus agrestis”*

136 Based on the phylogenomic analysis of 43 concatenated universal marker protein sequences, AOA
137 strain SS was found to be closely related to the genus *Ca. Nitrosocosmicus* and form a deep branch in
138 this clade (Fig. 1). The average nucleotide identity (ANI) that calculated between the genomes of strain
139 SS and the other three *Nitrosocosmicus* strains are 72.05%–72.81% (Fig. S1), which are above the
140 proposed genus and below the proposed species boundary thresholds (Varghese *et al.*, 2015; Daebeler
141 *et al.*, 2018). In accordance with the phylogenomic and genomic ANI analyses, strain SS was assigned
142 to the genus *Ca. Nitrosocosmicus* and referred to as “*Ca. Nitrosocosmicus agrestis*”.

143

144 The TEM and SEM micrographs indicated that these archaeal cells are irregular sphere and 0.8–1.2 μm
145 in diameter (Fig. 2); they often appeared in pairs or aggregation (Fig. 2, Fig. S2). Based on the
146 micrographs, cells of *Ca. Nitrosocosmicus agrestis* were observed to be embedded in a thick
147 extracellular matrix (up to 500 nm), forming an aggregate or biofilm (Fig. 2, Fig. S2). This
148 extracellular matrix could be the putative extracellular polymeric substances (EPS) that secreted during
149 the chemoautotrophic growth of AOA cells. The same feature has also been observed in the cells of *Ca.*
150 *Nitrosocosmicus oleophilus* MY3 (Jung *et al.*, 2016) and *Ca. Nitrosocosmicus franklandus*
151 (Lehtovirta-Morley *et al.*, 2016), suggesting the secretion of EPS is probably prevalent in the
152 *Nitrosocosmicus* clade. Even though the flagella that was reported to be essential to the biofilm
153 formation for bacterial cells (Pratt and Kolter, 1998; van Wolferen *et al.*, 2018) was not observed on the
154 micrographs (no related gene was identified in the following genomic analysis), the hydrophobic

155 feature of EPS that secreted by cells of *Nitrosocosmicus* clade would facilitate the cells aggregation and
156 adhesion on hydrophobic matrix.

157

158 *Physiology of “Ca. Nitrosocosmicus agrestis”*

159 The ammonia oxidation resulted in the nitrite generation at near-stoichiometric levels (Fig. 3A). A
160 minimum generation time that calculated to be 30.2 h was obtained at 30 °C. This value is shorter than
161 the generation times of strains *Ca. Nitrosocosmicus* exaquare G61 (51.7 h), *Ca. Nitrosocosmicus*
162 franklandus C13 (40 h), and *Ca. Nitrosocosmicus* oleophilus MY3 (77.4 h). After the oxidation of 1
163 mM substrate (NH_4^+), the cell density was determined to be approximately 7.06×10^6 cells mL^{-1} , which
164 is similar to the levels of *Ca. Nitrosocosmicus* franklandus C13 (7.6×10^6 cells mL^{-1}) and *Ca.*
165 *Nitrosocosmicus* oleophilus MY3 (3.2×10^6 cells mL^{-1}), but lower than the “*Ca. Nitrosocosmicus*
166 exaquare G61” (5.6×10^7 cells mL^{-1}).

167

168 *Ca. Nitrosocosmicus* agrestis was able to grow over broad ranges of temperature and salinity. The
169 ammonia-oxidizing activity was observed to be optimal at 37 °C and nearly inhibited at 42 °C (Fig. 3B,
170 Fig. S3); the activity was optimal under the concentration of 0 - 0.2% NaCl and completely suppressed
171 by 2% NaCl (Fig. 3C, Fig. S3). *Ca. Nitrosocosmicus* agrestis was able to tolerate 1% NaCl (kept ~35%
172 of its initial activity), which is much higher than the NaCl tolerance of other terrestrial AOA strains,
173 such as *Ca. Nitrosotenuis* aquarius AQ6f (0.1%) (Sauder *et al.*, 2018), *Ca. Nitrosotenuis* cloacae SAT1
174 (0.03%) (Li *et al.*, 2016), *Ca. Nitrosotenuis* uzonensis N4 (0.1%) (Lebedeva *et al.*, 2013) and *Ca.*
175 *Nitrosoarchaeum* koreensis MY1 (0.4%) (Jung *et al.*, 2011).

176

177 Ammonia oxidation is usually inhibited by the commercial nitrification inhibitors, such as
178 dicyanodiamide (DCD), allylthiourea (ATU), 3,4-dimethylpyrazole phosphate (DMPP) and nitrapyrin
179 (NP); the inhibition varies depending on the type of ammonia oxidizer (Beeckman *et al.*, 2018). In the
180 present study, the half-maximal inhibition (IC_{50}) of ATU, DCD and DMPP to strain SS were
181 determined to be 445.1, 947.1 and 488.0 μ M, respectively (Fig. 3D, Fig. S4, Table 2). These results are
182 similar with the observations in other AOA species (Lehtovirta-Morley *et al.*, 2013; Shen *et al.*, 2013;
183 Beeckman *et al.*, 2018). Differently, the IC_{50} of NP to *Ca. Nitrosocosmicus agrestis* was only 0.599 μ M
184 (Table 2), which is far lower than the concentration to *Nitrososphaera viennensis* EN76 (Shen *et al.*,
185 2013), *Ca. Nitrososphaera* sp. JG1 (Kim *et al.*, 2012), *Ca. Nitrosoarchaeum koreensis* MY1 (Jung *et al.*,
186 2011) and *Ca. Nitrosotalea devanaterre* (Lehtovirta-Morley *et al.*, 2013). NP is a kind of
187 water-insoluble nitrification inhibitor and might have a higher affinity with the hydrophobic surface of
188 *Nitrosocosmicus* (Jung *et al.*, 2016) than the other AOA clades. However, *Ca. Nitrosocosmicus agrestis*
189 cannot be extracted by p-xylene like *Ca. Nitrosocosmicus oleophilus* (Jung *et al.*, 2016). It may be
190 related to their isolated environment (vegetable soil versus coal tar-contaminated sediment). Therefore,
191 NP has the potential to act as a specific inhibitor of this clade of ammonia oxidizer, but the mechanism
192 is still to be further studied.

193

194 Although the mechanisms for ammonia tolerance are largely unknown, AOA strains from the genus *Ca.*
195 *Nitrosocosmicus* were usually reported having higher ammonia tolerance than other AOA (Table 1). In
196 the present study, the ammonia oxidation activity of *Ca. Nitrosocosmicus agrestis* was greatest at 2 mM
197 NH_4^+ and decreased with increasing initial ammonium concentration; more than 70% of the initial
198 activity was maintained when the initial ammonium was less than 20 mM (Fig. 4A, B). *Ca.*

199 *Nitrosocosmicus agrestis* was able to tolerate high concentration of ammonium; at 100 and 200 mM
200 NH_4^+ , the activities were reduced to approximately 26% and 14% of that at 2 mM NH_4^+ (Fig. 4B).
201
202 *Ca. Nitrosocosmicus agrestis* is a neutrophilic microbial strain. Under the low-ammonium
203 concentration (1 and 10 mM), the ammonia-oxidizing activity was detectable during pH range from 6.0
204 to 7.5, with optimal values being observed in pH 6.5-7.0; the optimal activity was also observed in the
205 range of pH 6.5-7.0 when using 50 mM ammonium as substrate, only the activity was undetectable in
206 pH 7.5 (Fig.4 C, Fig. S6). However, the activities were only detectable in the range of pH 6.5-7.0 when
207 ammonium concentrations were used more than 100 mM. Under these concentrations, the optimal
208 activities were determined in pH 6.5 (Fig.4 C, Fig. S6). Because the higher the pH reaches, the more
209 the free ammonia generates through a pH-dependent equilibrium $\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+$, which could freely
210 penetrate cell membrane and limit the ammonia oxidation. Then, the more the free ammonia was
211 presented, the lower the ammonia-oxidizing activity was observed; when the ammonia concentration
212 reached 1592.0 μM , ammonia-oxidizing activity was hardly detected (Fig. 4D).
213
214 Same as the tolerance to high ammonium, the AOA strains from the genus *Ca. Nitrosocosmicus* were
215 found to have higher nitrite tolerance than the AOA from other genera (Table 1). *Ca. Nitrosocosmicus*
216 *agrestis* was able to tolerate the nitrite with initial NO_2^- concentration as high as 50 mM; under this
217 concentration, the ammonia-oxidizing activity was reduced to approximately 18% of that at optimal
218 nitrite concentration (Fig. S5). It is obvious that *Ca. Nitrosocosmicus agrestis* has higher nitrite
219 tolerance than the *Nitrosocosmicus* strains *Ca. Nitrosocosmicus oleophilus* (Jung et al., 2016), *Ca.*
220 *Nitrosocosmicus franklandus* (Lehtovirta-Morley et al., 2016), *Ca. Nitrosocosmicus exaquare* (Sauder

et al., 2017), as well as the other AOA strains.

Besides the ammonia, *Ca. Nitrosocosmicus agrestis* was able to use urea as sole energy and N source, only the ammonia oxidation rate was one tenth of their growth using ammonia as energy source (Fig. S7). Urea is an abundant form of nitrogen fertilizer in agricultural soils. Many soil AOA strains affiliating with the genera *Nitrososphaera* and *Nitrosocosmicus* have usually been reported to use urea as an energy source (Tourn et al., 2011; Jung et al., 2016; Lehtovirta-Morley et al., 2016; Sauder et al., 2017). *Ca. Nitrosocosmicus franklandus* is an ureolytic strain capable of completely converting urea to ammonia and growing on urea as the sole source of ammonia (Lehtovirta-Morley et al., 2016). However, the accumulation of ammonia from urea hydrolysis was not observed in *Ca. Nitrosocosmicus agrestis* (Fig. S7B). The ureolytic activity probably varies depending on the type of AOA strain.

Besides being used by the soil AOA, urea is suggested to be the energy source for AOA throughout the ocean (Kitzinger et al., 2019).

Exogenous supplement of organics were observed to have no significant effect on the ammonia oxidation of *Ca. Nitrosocosmicus agrestis* (Fig. S8). The effect of organic carbon on AOA growth varies between strains. For example, the ammonia oxidation and growth of both *Nitrosotalea* isolates were inhibited by many TCA cycle intermediates but their growth yield increased in the presence of oxaloacetate (Lehtovirta-Morley et al., 2014); the pure culture *Nitrososphaera viennensis* requires pyruvate for growth in the laboratory (Tourn et al., 2011); the ammonia oxidation of *Ca. Nitrosocosmicus exaquare* that obtained from a municipal WWTP was greatly stimulated by the addition of organics (Sauder et al., 2017); α -keto acids could support the growth of some marine AOA

243 via peroxide detoxification (Kim *et al.*, 2016). Due to the physiological characters of *Nitrosocosmicus*
244 are largely unknown, the effects of organics on their growth or ammonia oxidation are still needed
245 further profound studies.

246

247 *Genome of “Ca. Nitrosocosmicus agrestis”*

248 The genome of *Ca. Nitrosocosmicus agrestis* was recovered by binning from metagenomic assembly
249 (Fig. S9), in which contains 43 contigs with a total length of 3224501 bp. The genome has an average
250 G + C content of 33.42%, contains 3513 protein coding sequences (CDS), and encodes 45 tRNA genes,
251 one 5S rRNA gene, two 16S/23S rRNA operons (Fig. S10). The proportion of coding region of this
252 strain is 70.74%, which is probably the lowest over all AOA strains at present (Table 3).

253

254 *Ca. Nitrosocosmicus agrestis* encodes several genes related to ammonia oxidation, including single
255 copy of the *amoA*, *amoB* and *amoX*, and three copies of *amoC*. (Table S3). Multiple copies of *amoC*
256 have been reported in the genomes of group I.1b AOA (Tourna *et al.*, 2011; Jung *et al.*, 2016; Sauder *et*
257 *al.*, 2017); they probably involve in the regulation of stress response to complicated soil environment,
258 as same as they performances in the soil AOB (Berube *et al.*, 2007; Berube and Stahl, 2012). A single
259 copy of the gene encoding ammonia transporters (*Amt*) was also identified in the genome. In general,
260 two types of *Amt* referring to the high-affinity *Amt*-1 and the low-affinity *Amt*-2 were usually found to
261 occur simultaneously in AOA genomes (Offre *et al.*, 2014). However, *Ca. Nitrosocosmicus agrestis* and
262 other strains from genus *Ca. Nitrosocosmicus* were found only in the presence of *Amt*-2 (Table S6, Fig.
263 S11). This is probably closely related to the feature of agricultural soils that the presence of high
264 ammonium; the soil AOA strains, such as the *Nitrosocosmicus*-like are able to rely entirely on *Amt*-2 to

265 meet the ammonia demand at high level of ammonium.

266

267 In addition, the genome contains a cluster of genes for the urea degradation, including the urease

268 subunits, urea accessory proteins and urea transporter (Table S3, S6). These genes were highly

269 expressed under the stimulation of urea (Fig. S7C). The urea degradation supplies ammonia to the

270 ammonia oxidation and could be used as an alternative pathway to produce energy. However, the

271 ammonia oxidation rate of *Ca. Nitrosocosmicus agrestis* on urea was much slower than on ammonia

272 (Fig. S7A). Owing to urease is located in the cytoplasm (predicted by SignalP (Almagro Armenteros

273 *et al.*, 2019) and Pred-Signal (Bagos *et al.*, 2009)), the ammonia that generated from intracellular

274 urea hydrolysis could be used as N source for the growth but not enough for the energy production via

275 ammonia oxidation. Because of the ammonia in the form of NH_4^+ is unable to transport from cytoplasm

276 into periplasm, only through the diffusion in the form of NH_3 , which results in the low substrate

277 concentration for ammonia oxidation that taking place in the periplasmic location (Walker *et al.*, 2010;

278 Kozłowski *et al.*, 2016). The ureolytic strain *Ca. Nitrosocosmicus franklandus* is able to completely

279 hydrolyze urea to ammonia and grow on urea as the sole source of ammonia (Lehtovirta-Morley *et al.*,

280 2016). However, the mechanism of how urea hydrolysis or ammonia transport in this strain has not yet

281 been given. In addition, the urease gene-containing AOA were observed to dominate the autotrophic

282 ammonia oxidation in acid soils (Lu and Jia, 2013). Expectedly, the mechanism of how urea activates

283 AOA cells remains undefined. It is thus clear that the pathway of urea metabolism fueling ammonia

284 oxidation of AOA would vary between strains and is still far from understood.

285

286 Pathways of the central carbon metabolism including CO_2 fixation, tricarboxylic acid cycle,

287 gluconeogenesis and nonoxidative pentose phosphate pathways were found in the genome of *Ca*.

288 *Nitrosocosmicus agrestis* and generally similar to those of other AOA (Table S4, Fig. 5). Intriguingly,

289 the genes encoding β (D-clades) and γ -classes (Cam and CamH) carbonic anhydrase (CA) were

290 identified in the genome (Fig. S12, Table 3, Table S4,). CA is responsible for the reversible hydration

291 of CO_2 to HCO_3^- and plays an important role in facilitating carbon transfer into the cell by extracellular

292 converting HCO_3^- to CO_2 , which is subsequently diffuse through the cell membrane (Kerou, Offre, *et*

293 *al.*, 2016). The genes encoding γ -class Cam have also been identified in the genomes of *Nitrososphaera*

294 *viennensis*, *Ca. Nitrososphaera evergladensis* and *Ca. Nitrosotalea devanaterre*, and genes encoding α

295 and β -classes CA were usually found in AOB species (Chain *et al.*, 2003). In contrast to the location of

296 Cam in periplasmic, the CamH and β -CA of *Ca. Nitrosocosmicus* with no signal peptide are located in

297 cytosolic. Periplasmic CA might constitute an adaption to the discontinuous soils with low CO_2

298 availability (Kerou, Offre, *et al.*, 2016). The presence of cytosolic CA in the genome suggested that *Ca.*

299 *Nitrosocosmicus agrestis* has an advantage in advantage in maintaining substrate concentration as well

300 as stabilizing pH in the cytoplasm.

301

302 A full set of genes encoding for key enzymes involving in the degradation and transport of

303 polysaccharide were found in the genome, including Glycosyl transferases (GTs) family, glycoside

304 hydrolases (GHs) family, carbohydrate esterases (CEs) family,

305 multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase, lipopolysaccharide exporter (LPSE)

306 family, etc (Table S6, S7). These protein families are important to the cell surface modification and

307 EPS production of biofilm-forming bacteria and archaea; an extensive gene encoding these proteins

308 was identified among the AOA groups (such as *N. viennensis*, *N. gargensis* and *Ca. Nitrososphaera*

309 evergladensis) from Nitrososphaerales (Kerou, Offre, *et al.*, 2016). Interestingly, the genes encoding
 310 LPSE are found to be unique in the genomes of *Nitrosocosmicus*, indicating the ability to secrete
 311 polysaccharide potentially for this AOA clade (Fig. 5). In addition, genes encoding divalent anion/Na⁺
 312 symporter (DASS) and carbohydrate uptake transporter-1 (CUT1) were also found in the genome
 313 (Table S6). They catalyze the uptakes of C4-dicarboxylate and complex sugars in bacterial cells
 314 (Hugouvieux-Cotte-Pattat *et al.*, 2001; Strickler *et al.*, 2009). In *Ca. Nitrosocosmicus agrestis*, the
 315 sugars derived from the EPS degradation by CEs and GHs are potentially transported into the cells (Fig.
 316 5). However, addition of organic carbons to the cultivation were proved have no effect on the growth or
 317 ammonia oxidation of *Ca. Nitrosocosmicus agrestis* (Fig. S8). The uptake of organic carbons (such as
 318 the oligosaccharides) by CUT1 is probably not used as part of the energy metabolism but recycled for
 319 the EPS production.

320

321 *Potential mechanism of ammonia tolerance in “Ca. Nitrosocosmicus agrestis”*

322 All genes encoding the pathway of arginine synthesis were identified in the genome of *Ca.*
 323 *Nitrosocosmicus agrestis*, including carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase
 324 (OTC), argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Fig. 5, Table S5). Some
 325 genes encoding arginine decarboxylase (ADC), agmatinase (AUH) and deoxyhypusine synthase (DHS)
 326 were also found in the genome, which catalyze the conversion of arginine to polyamines, such as
 327 putrescine and spermidine (Fig. 5). Meanwhile, four copies of the gene encoding drug/metabolite
 328 exporter (DME) were identified in the genome of *Ca. Nitrosocosmicus agrestis* (Fig. 6A). In fact, the
 329 DME encoding genes were found in the genomes of almost all of the isolated AOA (Table 3); the genes
 330 from genera *Ca. Nitrosopumilus*, *Ca. Nitrosotalea*, *Nitrososphaera*, *Ca. Nitrosotenuis*, *Ca.*

331 Nitrosoarchaeum and *Ca. Nitrosocosmicus* were clustered into one branch, which is widely distinct
332 from the other branch consisting of *Nitrosocosmicus* and some bacteria and yeast (Fig. 6A). The DME
333 family that existed in these bacteria or yeast were reported be able to mediate the excretion of amino
334 acids and polyamines (Igarashi and Kashiwagi, 2010; Nguyen *et al.*, 2015; Lubitz *et al.*, 2016),
335 suggesting the active function of DME in AOA strains from genus *Ca. Nitrosocosmicus*. Based on
336 these enzymes and transporters, the NH_4^+ in the cytoplasm that derived either from urea hydrolysis or
337 transmembrane diffused NH_3 could potentially be converted into polyamines and then excreted out by
338 specific transporters, contributing to the high ammonia tolerance of *Ca. Nitrosocosmicus agrestis*.
339
340 The relative expressions of genes involved in ammonia oxidation, arginine synthesis, polyamine
341 synthesis, glutamine/glutamate syntheses, carbonic anhydrase, and polyamines transport when
342 responded to high concentration of ammonia were analyzed (Fig. 6B). In comparison with the gene
343 expressions under low ammonia (1 mM NH_4^+), the *amoA* had no significant up-regulation and the
344 AMT gene was observed to be down-regulated; however, the arginine synthesis, polyamine synthesis
345 and putative DME polyamine exporter related genes were observed to have up-regulation with the
346 increase of ammonia concentration. In addition, the gene encoding putrescine aminotransferase (PA)
347 that catalyzes the first step of putrescine degradation was also up-regulated in response to high
348 ammonia, suggesting the more production of putrescine under high ammonia. The glutamate
349 dehydrogenase (GDH) and glutamine synthetase (GS) genes were also found to be significantly
350 up-regulated, which depleted cytoplasmic ammonium and resulted in the production of glutamine,
351 providing more precursors for ornithine synthesis. Up-regulation of carbonic anhydrase (CA) genes
352 that locating in the intracellular (β -CA and CamH of γ -CA) can provide H^+ to avoid cytoplasmic pH

rise caused by ammonia uptake and provide more bicarbonate for carbamoylphosphate synthesis. In summary, the up-regulations of genes involving in polyamines synthesis and secretion when in response to high ammonia were probably responsible for the high ammonia tolerance of *Ca. Nitrosocosmicus agrestis*. (Fig. 6C).

To prove the above proposed mechanism, the energy utilization and the polyamines production were studied. In contrast to the ammonia oxidation in low substrate (1 mM), the average ammonia oxidation rate of *Ca. Nitrosocosmicus agrestis* exposing to high substrate (100 mM) was decreased to 83% (Fig. 7A); if these energy that derived from ammonia oxidation were completely converted into biomass (determined by protein concentration), the energy utilization efficiency was only 10% (Fig. 7B). It is suggested that a large amount of energy was used for some other syntheses in order to tolerant high ammonia, such as the polyamines synthesis. Based on the detection by HPLC-MS/MS, the polyamines putrescine, cadaverine and spermidine were identified from the supernatant, with concentrations ranging from 1.37 to 10.25 $\mu\text{g L}^{-1}$ (Fig. 7C). In contrast to the productions under low ammonium, the cadaverine concentration had no difference but the spermidine concentration was about three times higher and the putrescine (10.25 $\mu\text{g L}^{-1}$) was only detected in high ammonium. In the actual situation, the polyamines would have higher concentrations due to (i) the presence of other type of polyamines, (ii) the absorption by EPS for they have positive charge, and (iii) the uptake by bacterial partners. The polyamines are kinds of dissolved organic carbon (DOC), which could be served as carbon source for the growth of heterotrophic bacteria when being released into ambient environment. Recently, some marine AOA *Nitrosopumilus* spp. were reported to be able to release hydrophobic amino acid through passive diffusion and then fuel the prokaryotic heterotrophy in the ocean (Bayer *et al.*, 2019). Though

375 very few amino acid was detected in the supernatants neither from low ammonium culture nor from
376 high ammonium culture (Fig. S13), the released polyamines were still able to fuel the heterotrophic
377 partners. In fact, during the cultivation of *Ca. Nitrosocosmicus agrestis*, the utilization of high
378 ammonium as substrate has undoubtedly resulted in lower abundance of AOA in comparison with the
379 low ammonium culture (data not shown), suggesting the promotion of bacterial growth by high
380 ammonium stress. Further studies are needed to figure out if the polyamines that secreted under high
381 ammonium are responsible for the interaction between AOA and heterotrophy, which subsequently
382 hinder the AOA enrichment and even isolation.

383

384

385 **CONCLUSION**

386

387 Physiological characterization of *Ca. Nitrosocosmicus agrestis* has revealed its extensive
388 environmental adaptability, especial for the high ammonia tolerance. Genomic analysis indicated that
389 *Ca. Nitrosocosmicus agrestis* harbors a variety of carbohydrate-active enzymes and transporters
390 relating to the EPS synthesis, which is potentially responsible for they adaption to the complex
391 environment. Surprisely, *Ca. Nitrosocosmicus agrestis* has a complete pathway for the polyamines
392 synthesis and secretion in genome, suggesting a mechanism to tolerate high ammonia. High ammonium
393 resulted in the higher productions of putrescine and spermidine, proving the polyamine synthesis is the
394 adaption strategy in response to high ammonia.

395

396 Strain SS is an ammonia oxidizing archaeon enriched from soil, we propose the following

397 provisional taxonomic assignment:

398 *Nitrososphaerales* order

399 *Nitrososphaeraceae* fam. and

400 *Candidatus Nitrosocosmicus agrestis* sp. nov.

401 Etymology: nitrosus (Latin masculine adjective), nitrous; cosmicus (Latin masculine adjective):

402 cosmopolitan; agrestis (Latin masculine adjective), belonging to the field.

403 Locality: vegetable field in Suishi village, Guangzhou, Guangdong, China

404 Diagnosis: an ammonia oxidizer growing chemolithoautotrophic, appearing as 0.8-1.2 μm

405 irregular cocci covered by putative exopolysaccharides, optimum pH 6.5-7.0, optimum temperature

406 37 °C, optimum salinity 0-0.2%, utilize urea, ammonia oxidation activity can still be detected at a free

407 ammonia concentration of 1592 μM but almost completely inhibited by 2 μM nitrapyrin.

408

409 MATERIALS AND METHODS

410

411 *Culture maintenance*

412 *Ca. Nitrosocosmicus agrestis* was obtained from a vegetable field after the enrichment using a two-step

413 strategy (Liu *et al.*, 2019). The AOA abundance in the enrichment culture was $\geq 95\%$ (symbiotic

414 bacteria mainly belonging to *Rhizobiales* (Fig. S9)).

415 *Ca. Nitrosocosmicus agrestis* was incubated at 30 °C in the dark without shaking, maintaining in a

416 mineral salts medium (MSM) containing (per litre): 1 g NaCl, 0.4 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$,

417 0.2 g KH_2PO_4 , 0.5 g KCl, 1 mL Fe-EDTA solution (49.14 g L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 196.896 g L^{-1} EDTA

418 disodium salt dihydrate), 2.5 mL NaHCO_3 solution (1 M) and 1 mL NH_4Cl solution (1M), 1mL trace

419 element solution (1.5 g L^{-1} $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 190 mg L^{-1} $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg L^{-1} $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$, 70 mg L^{-1}

420 ZnCl_2 , 62 mg L^{-1} H_3BO_3 , 36 mg L^{-1} $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 24 mg L^{-1} $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, and 17 mg L^{-1}
 421 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$). Solutions were filtered using 0.22 μm sterilized filters. To eliminate symbiotic bacteria,
 422 an antibiotic mixture containing with ciprofloxacin (50mg/L) and azithromycin (50mg/L) was added.
 423 The initial pH value was adjusted to 7.0. Ten percent (w/v) of quartz (1 mm diameter) was supplied as
 424 the attachment of archaeal cells during the seed cultivation and transferred into a fresh medium.

425

426 *Physiological characterisation*

427 To prepare AOA culture for physiological characterisation, the quartzs in seed culture were collected
 428 and the AOA cells attaching on quartz were washed out by using MSM; 10% eluate was inoculated into
 429 the fresh MSM without supplying quartz. The effects (tolerances) of ammonium concentration were
 430 determined in MSM added with 1-500 mM NH_4Cl and inhibition by nitrite concentration by
 431 supplementation with 0-100 mM NaNO_2 . The effects of temperature, initial pH and salinity on
 432 ammonia oxidation were also investigated with values in the range of 20-42 $^{\circ}\text{C}$, 6.0-8.0 and 0-2.0%,
 433 respectively. To avoid the rapid pH decrease due to bicarbonate uptake and ammonia oxidation, the
 434 MSM that used for pH characterization was not supplied with NaHCO_3 (the diffused CO_2 is sufficient
 435 for SS growth) and buffered with 10mM MES (2-(N-morpholino)ethanesulfonic acid), HEPES
 436 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Tris (Tris(hydroxymethyl)aminomethane)
 437 between the range of pH 6-7, 7-8, and 8-9, respectively. The effects of organics on ammonia oxidation
 438 were determined in MSM supplemented with 50 mg/L each organic carbon.

439

440 All physiological characteristics were determined by evaluating the ammonia oxidation activity during
 441 batch cultivation. Concentrations of nitrite and ammonium were determined by standard Griess-Ilosvay

442 method and indophenol blue method, respectively (ISO/TS 14256-1:2003, 2003). The mean ammonia
443 oxidation rate was calculated basing on the determination of nitrite concentration.

444

445 The density of AOA in the enrichment was analyzed using absolute quantitative PCR based on
446 16S rRNA gene primers (SS16S-1F / SS16S-1R), following the method described in the previous
447 study (Liu *et al.*, 2019).

448

449 *Microscopy*

450 To prepare samples for the transmission electron microscopy (TEM), AOA cells were collected by the
451 centrifugation at 8000 *g* for 10 min and fixed with 2.5% glutaraldehyde solution at 4 °C for 12 h. After
452 being washed in PBS buffer for 3 times, the cells were fixed with 1% osmic acid solution for 2 h. After
453 being washed in PBS buffer for 2 times, the samples were dehydrated in a graded series of ethanol
454 solutions (50%, 70%, 90%, 100%) at 4 °C for 10 min and transferred onto a copper grid. Each
455 preparation was observed with H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an
456 accelerating voltage of 80 kV.

457

458 To prepare samples for scanning electron microscopy (SEM), glass slides (1 cm²) were placed in the
459 bottom of flask and used as the attachment of AOA cells during cultivation. After the incubation, the
460 slides were directly used for the fixation with glutaraldehyde, the dehydration by a graded series of 100%
461 absolute ethanol, and the critical point dry using an Autosamdri-815 series A (Tousimis, Baltimore,
462 USA). The slides were mounted on an aluminum stub and sputter-coated with platinum using a sputter
463 coater EMS150T (Quorum, East Sussex, UK), then imaged using Merlin field emission scanning

464 electron Microscope (Carl Zeiss, Jena, Germany).

465

466 *Genome sequencing, assembly and annotation*

467 The genomic DNA extraction from 3,000 mL batch cultures was performed according to a previously

468 described protocol (Liu *et al.*, 2019). The purified DNA was fragmented to ~400 bp with the aid of an

469 M220 focused-ultrasonicator (Covaris, Woburn, USA) and subsequently used for library preparation

470 using a TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, USA). Metagenomic sequencing was

471 performed on a HiSeq X Ten sequencing system (Illumina, San Diego, USA).

472

473 The raw data was length- and quality-filtered using Trimmomatic v0.38 (Bolger *et al.*, 2014) and de

474 novo assembled using metaSPAdes v3.13.0 (Nurk *et al.*, 2017) with 127-mer. Then, GapCloser module

475 of SOAPdenovo2 v1.12 (Luo *et al.*, 2012) was used with default parameters to fill a proportion of gaps

476 of the assembled scaffolds. Genome binning of the metagenomic assemblies was conducted using

477 MaxBin2 v2.26 (Wu *et al.*, 2016) with the universal marker gene sets (Wu *et al.*, 2013). Scaffolds with

478 length <1000 bp in assembly were removed from the further analysis. The completeness, contamination

479 and strain heterogeneity of genome were evaluated using CheckM v1.013 (Parks *et al.*, 2015). Gene

480 prediction and annotation were done using JGI-IMG/MER (Chen *et al.*, 2019) and MicroScope

481 platform (Vallenet *et al.*, 2019). KEGG pathways were generated using KASS (Moriya *et al.*, 2007).

482 Putative transport proteins and carbohydrate-active enzymes were identified using TCDB (Saier *et al.*,

483 2014) and dbCAN2 (Zhang *et al.*, 2018), respectively. The average nucleotide identity (ANI) of four

484 *Nitrosocosmicus* genome were calculated using JSpeciesWS (Richter *et al.*, 2016). The genome

485 described in this study has been deposited in NCBI under GenBank accession VUYS000000000.

486

487 *Phylogenomic analysis*

488 A phylogenomic tree was obtained using the automatically generated alignment of 43 concatenated
489 universal marker protein sequences (Table S2), which were identified by CheckM v1.013 (Parks *et al.*,
490 2015). The best-fit model of evolution was selected with ModelFinder (Kalyaanamoorthy *et al.*, 2017)
491 and phylogenomic trees were inferred by Maximum-likelihood with IQ-TREE v1.6.1 (L. T. Nguyen *et*
492 *al.*, 2015).

493

494 *Relative expression of genes*

495 To extract total RNA from liquid culture, archaeal cells in the enrichments were collected from
496 biological triplicates by filtering through a 0.22µm cellulose filter; the filter was cut into pieces and
497 placed in a grinding tube containing 0.5 g quartz sand. RNA extraction was performed using the
498 RNAiso Plus (Takara, Dalian, China), and the first strand of cDNA synthesis was performed using the
499 PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). All quantitative PCR were
500 performed in triplicate on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Carlsbad,
501 USA) using TransStart Tip Green qPCR SuperMix (Transgen, Beijing, China). The reaction condition
502 was as follows: 1 min at 94 °C; 40 cycles of 10 s at 94 °C and 34 s at 60 °C. The $2^{-\Delta\Delta Ct}$ method was
503 used to estimate fold change in gene expression, normalized to the endogenous control 16S rRNA.
504 Primer sets (Table S9) were designed on the function genes from *Ca. Nitrosocosmicus agrestis*
505 genome.

506

507 *Determination of protein content*

508 To measure the total protein in AOA cells, about 20 mL of enrichment culture was collected and
509 filtered through a 0.22 µm cellulose filter; the filter retaining with cells was collected and cut into
510 pieces and placed in a 2 mL grinding tube containing 0.5 g quartz sand. 500 µL of lysis buffer (TE
511 buffer containing 1% Triton X100) was added into the tube and mixed for 5 min using the Vortex
512 Adapter (13000-V1-24, QIAGEN, Germany). After the centrifugation at 5,000 g for 5 min, the pellet
513 was collected and used for the protein measurement by BCA Protein Assay Kit (Sangon Biotech,
514 Shanghai, China).

515

516 *Quantification of polyamines and amino acids in supernatant*

517 After the centrifugation, 800 µL of the supernatant was collected and filtered through a 0.22 µm
518 cellulose filter. The filtrate was mixed with 200 µL of sulfosalicylic acid (10%) and incubated at
519 4 °C for 1 hour, following a centrifugation at 14,000 g for 15 min. The supernatant was filtered
520 through a 0.22 µm cellulose filter again and used for the quantitative analysis of amino acids or
521 polyamines.

522

523 Using LC-20A HPLC system (shimadzu, Japan) coupled with API 4000 Quantiva triple
524 quadrupole tandem mass spectrometer (SCIEX, USA) to analyze putrescine, cadaverine,
525 spermidine, and spermine in the supernatant (HPLC–MS/MS experimental conditions of each
526 polyamine were listed in Table S10) (Gosetti *et al.*, 2013). HPLC separation was performed at
527 0.30 mL/min and the column compartment to 40 °C. The stationary phase was an Inertsil® ODS-3
528 column (2.1×100 mm, 3 µm), and the mobile phase was an isocratic mixture (A: B=20:80) of 5
529 mmol/L CH₃COONH₄ in H₂O (A) and 0.2% HCOOH in H₂O (v/v) (B). Using amino acid analyser

530 A300-advanced (membraPure, Berlin, Germany) to analyze seventeen dissolved amino acids (Cys,
531 Asp, Met, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys, Arg) in the supernatant.

532

533 **Compliance with ethical standards**

534 Conflict of interest: The authors declare that they have no conflict of interest.

535

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794 **Figure and Table legends**

795

796 **Fig. 1. Phylogenomic analysis of “*Candidatus Nitrosocosmicus agrestis*”.** The tree was inferred on
797 43 concatenated universal marker proteins from thaumarchaeota (Table S2) by maximum likelihood
798 with IQ- TREE using a LG+F+R4 model and 1000 replicates.

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800 **Fig. 2. Micrographs of *Ca. Nitrosocosmicus agrestis* cells.** (A) TEM observation of *Ca.*
801 *Nitrosocosmicus agrestis* cell. Scale bar = 500 nm; (B) SEM observation of *Ca. Nitrosocosmicus*
802 *agrestis* aggregates. Scale bar = 1 μ m. (C) SEM observation of *Ca. Nitrosocosmicus agrestis*
803 aggregates. Scale bar = 200 nm.

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805 **Fig. 3. Relationships between ammonia depletion, nitrite production, and archaeal growth in the**
806 **enrichment culture of strain SS (A), and influences of temperature (B), salinity (C) and**
807 **nitrification inhibitors (D) on the ammonia oxidation activity of *Ca. Nitrosocosmicus agrestis*.**
808 Influence was evaluated by the relative mean ammonia oxidation rates of 8 days cultivation;
809 thaumarchaeotal 16S rRNA genes were measured by qPCR; ATU: allylthiourea; DCD: dicyanodiamide;
810 DMPP: 3,4-dimethylpyrazole phosphate; NP: nitrapyrin; error bars indicate the standard error of the
811 mean for biological triplicates.

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813 **Fig. 4. Influences of the initial ammonium (A), nitrite (B), pH (C) and ammonia (D) on the**
814 **ammonia oxidation activity of “*Ca. Nitrosocosmicus agrestis*”.** Influence was evaluated by the
815 relative mean ammonia oxidation rates of 8 days cultivation; The profile of nitrite accumulation with

time of ammonia and pH are placed in Fig. S7. (M: MES; H: HEPES; error bars indicate the standard error of the mean for biological triplicates. Free ammonia concentration, calculated according to total ammonia, pH and temperature, estimating by Emerson et al.'s (Emerson *et al.*, 1975) formulas.

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Fig. 5. 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: carbonic 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 *Nitrosocosmicus* clade are marked in red color. Candidate enzymes, gene accession numbers and transporter classes are list in the supporting information.

Fig. 6. Phylogeny of DME gene (A), relative gene expressions in response to high ammonia (B), and schematic mechanism of ammonia tolerance of *Ca. Nitrosocosmicus agrestis* (C). The functionally identified DME in bacteria and yeast are marked in blue color; the codes in parentheses are accession number of UniProt or NCBI. *amoA*: ammonia monooxygenase subunit A; Amt: ammonium transporter; CPS: carbamoylphosphate synthase large subunit; OTC: ornithine carbamoyltransferase; ASS: argininosuccinate synthase; ASL: argininosuccinate lyase; ADC: arginine decarboxylase; AUH: agmatinase; DHS: deoxyhypusine synthase; PA: putrescine aminotransferase; GDH: glutamate dehydrogenase; GS: glutamine synthetase; DME: drug/metabolite exporter (DME) family; CA: carbonic anhydrase; error bars indicate the standard error of the mean for technical triplicates; the red arrows represent the flow of ammonia nitrogen.

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Fig. 7 After adding different concentrations of ammonia nitrogen for two days, the accumulation of nitrite (A), protein synthesis (B), and accumulation of polyamine (C) by *Ca. Nitrosocosmicus* *agrestis*. Put: putrescine; Cad: cadaverine; Spd: spermidine; error bars indicate the standard error of the mean for biological triplicates.

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FIGURE S1 Heat maps showing pairwise ANI values inferred from the available AOA genomes.

FIGURE S2 Transmission electron micrograph of *Ca. Nitrosocosmicus* *agrestis* cells in pairs (A) (scale bar = 500 nm) and aggregates (B) (scale bar = 2 μ m). (C) Scanning electron micrograph of strain SS cells incubating with 50mg/L ciprofloxacin and azithromycin. Scale bar = 500nm.

FIGURE S3 Effects of temperature (A) and salinity (B) on the ammonia oxidation activity of *Ca. Nitrosocosmicus* *agrestis*. Error bars indicate the standard error of the mean for biological triplicates.

FIGURE S4 Effects of nitrification inhibitors ATU (A), DCD (B), DMPP (C) and NP (D) on the ammonia oxidation activity of *Ca. Nitrosocosmicus* *agrestis*. Error bars indicate the standard error of the mean for biological triplicates.

FIGURE S5 (A) Variation of nitrate nitrogen concentration with time at different initial nitrite concentration; (B) the average ammonia oxidation rate of homogenization at different initial nitrite concentration. Error bars indicate the standard error of the mean for biological triplicates.

FIGURE S6 Effect of 1mM (A), 10mM (B), 50mM(C), 100mM (D) and 200mM (D) concentration of ammonium on ammonia oxidation activity of strain SS at different pH. Error bars indicate the standard error of the mean for biological triplicates.

FIGURE S7 Effect of urea on nitrite (A) or ammonia (B) concentration in ammonia oxidation process, and relative gene expressions in response to urea (C). 1A, 0.1A, 0A: 1mM, 0.1mM, 0mM NH_4Cl ; 1U: 1mM urea. Amt: Amt ammonium transporter; UreC: urease subunit alpha; UT: urea transporter. Error bars indicate the standard error of the mean for biological triplicates.

FIGURE S8 Growth of *Ca. Nitrosocosmicus* *agrestis* amended with organic carbon and incubated with ciprofloxacin- azithromycin (A) or no antibiotics (B). The initial ammonia concentration is 1mM. Error bars indicate the standard error of the mean for biological triplicates.

867 **FIGURE S9** Classification and coverage of metagenome contigs (A) and composition of bins from the
868 *Ca. Nitrosocosmicus agrestis* cultures (B); bin2 is the genome of *Ca. Nitrosocosmicus agrestis*.
869 **FIGURE S10** Gene arrangement around 16S/23S operons in the genomes of *Ca. Nitrosocosmicus*. SS:
870 *Ca. Nitrosocosmicus agrestis* SS; G61: *Ca. Nitrosocosmicus exaquare* G61; MY3: *Ca.*
871 *Nitrosocosmicus oleophilus* MY3.
872 **FIGURE S11** Evolution Analysis of Amt amino acid sequences in the different AOA genomes.
873 Software: iqtree; Method: maximum likelihood; Model: LG+F+R3; bootstrap value: 1000.
874 **FIGURES 12** Evolution analysis of β (A) or γ (B)-carbonic anhydrase based on amino acid sequence.
875 Software: iqtree; Method: maximum likelihood; Model: LG+I+G4; bootstrap value: 1000.
876 **FIGURE S13** Detection of dissolved free amino acid in the supernatant of the enrichments under
877 different concentrations of ammonium. Except for (Cys)₂ of 50 nmol / mL, all other standards are
878 100 nmol / mL.
879 **Table 1. Ammonia, nitrite and pH tolerances of AOA strains**
880 **Table 2 Half-maximal effective concentration [IC₅₀ (μM)] of nitrification inhibitors to ammonia**
881 **oxidation activity of *Ca. Nitrosocosmicus agrestis***
882 **Table 3 Genome features of *Ca. Nitrosocosmicus agrestis* and other AOA**
883 **TABLE S1** Evaluation of genomes by CheckM software.
884 **TABLE S2** 43 concatenated universal marker proteins for phylogenomic analysis.
885 **TABLE S3** Genes of energy metabolisms in *Ca. N. agrestis* genome.
886 **TABLE S4** Genes of carbon metabolisms in *Ca. N. agrestis* genome.
887 **TABLE S5** Genes of nitrogen metabolisms in *Ca. N. agrestis* genome.
888 **TABLE S6** Genes of transporters in *Ca. N. agrestis* genome.
889 **TABLE S7** Genes of carbohydrate-active enzymes in *Ca. N. agrestis* genome.
890 **TABLE S8** DME family transporters of AOA and polyamine exporters
891 **TABLE S9** Primers used in this study
892 **TABLE S10** HPLC–MS/MS experimental conditions of polyamines
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894 Table 1. Ammonia, nitrite and pH tolerances of AOA strains

AOA Strain	Source	Inhibitory NH_4^+ (mM) ^a / NH_3 (μM) ^b	Inhibitory NO_2^- (mM) ^c	pH ^d	Reference
Ca. Nitrosopumilales					
<i>Nitrosopumilus maritimus</i> SCM1	Tropical marine aquarium	2 / 49.49	N/A	6.8-8.2	(Martens-Habbena <i>et al.</i> , 2009)
<i>Ca. Nitrosopumilus koreensis</i> AR1	Marine sediment	4 / 399.04	N/A	N/A	(Park <i>et al.</i> , 2010)
<i>Ca. Nitrosoarchaeum koreensis</i> MY1	Agricultural soil	20 / 112.71	10 (7.0)	6.0-8.0	(Jung <i>et al.</i> , 2011)
<i>Ca. Nitrosotenuis chungbukensis</i> MY2	Agricultural soil	20 / 112.71	2 (7.0)	6.5-8.5	(Jung <i>et al.</i> , 2014)
<i>Ca. Nitrosotenuis cloacae</i> SAT1	Wastewater treatment plant	5 / 11.82	5 (6.5)	5.0-7.0	(Li <i>et al.</i> , 2016)
<i>Nitrosopumilus cobalaminigenes</i> HCA1	50 m depth marine water	10 / 176.06	N/A	6.8-8.1	(Qin <i>et al.</i> , 2017)
<i>Nitrosopumilus ureiphilus</i> PS0	Marine surface sediment	20 / 377.35	N/A	5.9-8.1	(Qin <i>et al.</i> , 2017)
<i>Nitrosopumilus oxycloinae</i> HCE1	17 m depth marine water	1 / 17.61	N/A	6.4-7.8	(Qin <i>et al.</i> , 2017)
<i>Ca. Nitrosotenuis aquariensis</i> AQ6f	Freshwater aquarium biofilter	3 / 607.18	N/A	N/A	(Sauder <i>et al.</i> , 2018)
Ca. Nitrosotaleales					
<i>Ca. Nitrosotalea devanaterrea</i> Nd1	Acid soil	50 / 1.11	0.04 (4.5)	4.0-5.5	(Lehtovirta-Morley <i>et al.</i> , 2011)
Nitrososphaerales					
<i>Ca. Nitrososphaera gargensis</i> Ga9.2	Hot spring	3 / 161.78	N/A	N/A	(Hatzenpichler <i>et al.</i> , 2008)
<i>Nitrososphaera viennensis</i> EN76	Garden soil	20 / 686.11	10 (7.5)	6.0-8.5	(Tourna <i>et al.</i> , 2011; Stieglmeier <i>et al.</i> , 2014)
<i>Ca. Nitrososphaera</i> sp. JG1	Agricultural soil	20 / 80.71	10 (6.5)	6.0-8.0	(Kim <i>et al.</i> , 2012)
<i>Ca. Nitrosocosmicus oleophilus</i> MY3	Coal tar-contaminated sediment	50 / 398.02	N/A	5.5-8.5	(Jung <i>et al.</i> , 2016)
<i>Ca. Nitrosocosmicus franklandus</i> C13	Arable soil	100 / 4689.43	20 (7.5)	6-8.5	(Lehtovirta-Morley <i>et al.</i> , 2016)
<i>Ca. Nitrosocosmicus exaquare</i> G61	Wastewater treatment plant	20 / 1485.64	30 (8.0)	N/A	(Sauder <i>et al.</i> , 2017)
<i>Ca. Nitrososphaera</i> sp. OTU8	Wastewater treatment plant	3 / 23.88	N/A	7.0-8.0	(Chen <i>et al.</i> , 2017)
<i>Ca. Nitrosocosmicus agrestis</i> SS	Vegetable soil	200 / 1592.07	50 (7.0)	6.0-8.0	This study

895 Inhibitory NH_4^+ (mM)^a: Ammonium chloride concentration that completely or almost inhibited AOA ammonia oxidization. NH_3 (μM)^b: Free ammonia concentration, calculated
 896 according to total ammonia, pH and temperature, estimating by Emerson *et al.*'s (Emerson *et al.*, 1975) formulas. Inhibitory NO_2^- (mM)^c: Nitrite concentration that completely or
 897 almost inhibited AOA ammonia oxidization. The number in parentheses represents the pH of the nitrite concentration inhibition experiment. pH^d: The range of pH that AOA can grow.

898 **Table 2 Half-maximal effective concentration [IC₅₀ (μM)] of nitrification inhibitors to ammonia oxidation activity of *Ca. Nitrosocosmicus agrestis***

Nitrification inhibitors	Average IC ₅₀ (μM)	Concentrations tested (μM)
Allylthiourea (ATU)	445.1	0, 50, 100, 200, 500, 1000
Dicyanodiamide (DCD)	947.1	0, 500, 1000, 2000
3, 4-Dimethylpyrazole phosphate (DMPP)	488.0	0, 200, 500, 1000, 2000
Nitrapyrin (NP)	0.599	0, 0.2, 0.5, 1, 2, 5

899 The IC₅₀ were calculated by GraphPad Prism7 refer to shen et al. (Shen *et al.*, 2013).

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913 **Table 3 Genome features of *Ca. Nitrosocosmicus agrestis* and other AOA**

AOA strain	Genome size (Mb)	GC (%)	Protein coding genes	coding region (%)	rRNA operons	tRNA genes	Motility	Urease	β -CA	γ -CA-Cam	γ -CA-CamH	Amt-1	Amt-2	DME
<i>Ca. N. agrestis</i> SS	3.22	33.41	3524	70.74	2 ^a	45	-	+	1	1	1	-	1	4 (3) ^b
<i>Ca. N. exaquare</i> G61	2.99	33.94	3196	77.32	2	36	-	+	-	2	1	-	1	4 (3)
<i>Ca. N. oleophilus</i> MY3	3.43	34.14	3722	73.96	3	38	-	+	1	3	1	-	1	2 (2)
<i>Ca. N. franklandus</i> C13	2.84	34.07	3025	72.94	2	39	-	+	-	1	1	-	1	3 (3)
<i>Ca. N. arcticus</i> Kfb	2.64	34.00	2970	75.83	3	33	-	+	1	1 ^c	1	-	1	3 (1)
<i>N. viennensis</i> EN76	2.53	52.72	3123	87.32	1	39	+	+	-	1	-	2	1	1
<i>Ca. N. gargensis</i> Ga9.2	2.83	48.35	3562	81.53	1	37	+	+	-	-	-	2	1	1
<i>Ca. N. evergladensis</i> SR1	2.95	50.14	3499	83.60	1	39	+	+	-	1	-	2	1	1
<i>Ca. N. devanaterre</i> Nd1	1.81	37.07	2067	90.99	1	41	+	-	-	1	-	1	2	1
<i>Ca. N. aquarius</i> AQ6f	1.70	42.18	2008	92.90	1	42	+	+	-	-	-	1	1	1
<i>Ca. C. symbiosum</i> A	2.05	57.37	2017	91.67	1	45	-	+	-	-	-	2	-	-
<i>Ca. N. brevis</i> CN25	1.23	33.16	1469	94.48	1	42	-	-	-	-	-	1	1	-
<i>Ca. N. limnia</i> SFB1	1.74	32.46	2038	84.83	1	45	+	-	-	-	-	1	1	2
<i>Ca. N. catalina</i> SPOT01	1.36	31.44	1677	92.48	1	43	-	+	-	-	-	1	1	-
<i>N. maritimus</i> SCM1	1.65	34.17	1797	90.82	1	40	-	-	-	-	-	1	1	2
<i>Ca. N. adriaticus</i> NF5	1.80	33.41	2206	90.61	1	40	+	-	-	-	-	1	1	1
<i>Ca. N. piranensis</i> D3C	1.71	33.82	2140	90.37	1	41	-	+	-	-	-	1	1	3
<i>Ca. N. islandicus</i> 3F	1.62	41.54	1746	87.67	1	39	+	+	-	-	-	2	1	-

914 a: The SS genome has two 16S/23S operons, one of which is intact and the other lacks a partial sequence due to technical reasons for assembly (Fig. S10).

915 b: The number in parentheses indicates the number of copies of the putative polyamine exporter gene.

916 c: The complete gene sequence cannot be obtained by gene prediction.

A

C