Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea

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Ammonia-oxidizing archaea (AOA), that is, members of the *Thaumarchaeota* phylum, occur ubiquitously in the environment and are of major significance for global nitrogen cycling. However, controls on cell growth and organic carbon assimilation by AOA are poorly understood. We isolated an ammonia-oxidizing archaeon (designated strain DDS1) from seawater and used this organism to study the physiology of ammonia oxidation. These findings were confirmed using four additional *Thaumarchaeota* strains from both marine and terrestrial habitats. Ammonia oxidation by strain DDS1 was enhanced in coculture with other bacteria, as well as in artificial seawater media supplemented with α-keto acids (e.g., pyruvate, oxaloacetate). α-Keto acid-enhanced activity of AOA has previously been interpreted as evidence of mixotrophy. However, assays for heterotrophic growth indicated that incorporation of pyruvate into archaeal membrane lipids was negligible. Lipid carbon atoms were, instead, derived from dissolved inorganic carbon, indicating strict autotrophic growth. α-Keto acids spontaneously detoxify H_2O_2 via a nonenzymatic decarboxylation reaction, suggesting a role of α-keto acids as H_2O_2 scavengers. Indeed, agents that also scavenge H_2O_2, such as dimethylthiourea and catalase, replaced the α-keto acid requirement, enhancing growth of strain DDS1. In fact, in the absence of α-keto acids, strain DDS1 and other AOA isolates were shown to endogenously produce H_2O_2 (up to ∼4.5 μM), which was inhibitory to growth. Genomic analyses indicated catalase genes are largely absent in the AOA. Our results indicate that AOA broadly feature strict autotrophic nutrition and implicate H_2O_2 as an important factor determining the activity, evolution, and community ecology of AOA ecotypes.

H_2O_2 detoxification | mixotrophy | α-keto acid | ammonia-oxidizing archaea

Refining knowledge about the intricacies of the global nitrogen cycle is critical for improving efforts to manage the biosphere (1, 2). Nitrogenous compounds are ubiquitous in aquatic and terrestrial habitats, occurring in both living and deceased biomass (e.g., as amino acids) and in inorganic pools (e.g., ammonia, nitrite, nitrate). They are the naturally occurring microbial communities native to soils and waters that catalyze the cascade of biochemical transformations that constitute the global N cycle [e.g., ammonification, nitrification, denitrification, anaerobic ammonia oxidation, dissimilatory nitrate reduction to ammonia, nitrogen fixation; Canfield et al. (1)].

Ammonia is a key nitrogen-containing compound that occurs in waters and soils. Physiologically, ammonia can act directly as a plant nutrient and is also an energy-rich substrate that is oxidized by naturally occurring chemoautotrophic microorganisms [a physiological group that derives ATP from oxidation of an inorganic compound (in this case, ammonia) and derives cellular carbon from carbon dioxide] that carry out nitrification, a two-step process that oxidizes ammonia to nitrate. Recently, however, *Bacteria* that are capable of complete nitrification (ammonia to nitrate in one step; comammox) were cultivated from an oil exploration well and an anammox reactor (3, 4). The powerful greenhouse gas nitrous oxide (N_2O) is produced as a byproduct of nitrification via intermediary metabolites that include hydroxylamine (NH_2OH), nitroxyl hydride (HNO), and nitrite (NO_2^-) [for details, see Hu et al. (5)].

Traditionally, *Bacteria* have been considered the key agents in ammonia oxidation in terrestrial and aquatic habitats (6, 7). This view has been drastically altered in the last decade with the discovery that *Archaea* are often far more abundant and more active in performing ammonia oxidation (8–11). Understanding the physiological foundations of ammonia oxidation and *N*-cycle biogeochemistry is essential for making predictions about when and in which habitats the process will occur. The traditional view about ammonia oxidation is that it is catalyzed by chemoautotrophs. Originally, AOA were also presumed to be chemolithoautotrophs, similar to their long-characterized bacterial counterparts (12). However, recent reports have suggested that some AOA may use (or even require) organic carbon substrates to achieve ammonia oxidation (13, 14). Mussmann et al. (15) reported the lack of CO_2 fixation by a clade of *Thaumarchaeota* abundant in refinery nitrifying sludges. The controversy surrounding chemoautotrophic versus mixotrophic (autotrophy and heterotrophy combined) paradigms for ammonia oxidation needs to be resolved.

Here we advance fundamental knowledge about the physiology of AOA. We isolated the thaumarchaeotal ammonia-oxidizing

Significance

Ammonia-oxidizing archaea (AOA) are major players in global nitrogen cycling, but the AOA carbon-nutrition paradigm is poorly understood. Once considered strict autotrophs, AOA also have been reported to assimilate organic carbon. We used a marine AOA isolate to test hypotheses about the role of fixed carbon in AOA nutrition. Results were confirmed with tests with four additional marine and terrestrial AOA. We discovered that α-keto acids (pyruvate, oxaloacetate) were not directly incorporated into AOA cells. Instead, the α-keto acids functioned as chemical scavengers that detoxified intracellularly produced H_2O_2 during ammonia oxidation. H_2O_2 toxicity was also counteracted by co-inoculating the AOA with bacteria harboring catalases. Thus, H_2O_2 toxicity in AOA may be an evolutionary force controlling AOA communities and global ammonia cycling.

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Data deposition: The sequences of AOA isolates described in this paper (16S rRNA and *amoA* genes) have been deposited in the GenBank database (accession nos. KR737579, KR737580, KU884942, and KU884943). Genome sequences from the Illumina sequencing have been deposited in the Sequence Read Archive of the National Center for Biotechnology Information (accession no. LGTD00000000). The strains are available from the corresponding author on request.

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strains and show that their growth is stimulated by α-keto organic acids such as pyruvate. Surprisingly, however, pyruvate was not assimilated as a carbon source during ammonia oxidation. Instead, we found that α-keto organic acids served to nonenzymatically detoxify H_2O_2. Our results reveal that previously reported "nutritional requirements" by marine microorganisms for α-keto acids have likely been misinterpreted as indicating "mixotrophic growth." Acceleration of ammonia oxidation by catalase and catalase-positive cocultures grown with strain DDS1 supports the principle that the in situ metabolic activities of many biogeochemically critical microbial populations (involved in the cycling of C and N and other elements) may be regulated by fellow adjacent populations that produce enzymes able to detoxify toxic reactive oxygen species in ocean water, particularly H_2O_2.

Results and Discussion

Isolation of Strain DDS1, a Marine Ammonia-Oxidizing Archaeon. An enrichment culture was obtained from deep water (200-m depth) in the East Sea, Korea. Initial enrichment conditions were designed to stimulate autotrophic ammonia oxidation; the natural seawater was supplemented with ammonia (0.5 mM) as the sole energy source. Once the enrichment showed depletion of ammonia after 2 mo, cells (10% vol/vol) were transferred to artificial seawater medium (ASM) supplied with 0.5 mM ammonia. A highly enriched AOA culture was obtained after ∼2 y of successive transfer of 10% (vol/vol) late-exponential-phase cultures into the fresh ammonia-supplemented ASM.

An axenic culture of an archaeal ammonia-oxidizing strain, designated DDS1, was ultimately obtained by filtration of the enrichment culture through a 0.2-μm syringe filter (∼1% of total cells pass through the 0.2-μm filter) and dilution of the filtrate to extinction. The morphology of strain DDS1 cells is similar to that of other marine AOA including *Nitrosopumilus maritimus* SCM1 (8) and strains HCA1 and PS0 (14): small rods, with a diameter of 0.2–0.4 μm and a length of 0.1–1.8 μm (*SI Appendix*, Fig. S1). Strain DDS1 is phylogenetically affiliated with group I.1a within the *Thaumarchaeota*, showing a 16S rRNA gene sequence similarity >99% to the sequences of strains SCM1, HCA1, and PS0 (Fig. 1). The ammonia monooxygenase (*amoA*) amino acid sequence of strain DDS1 matched the corresponding sequences of strains SCM1, HCA1, and PS0, at >92% (*SI Appendix*, Fig. S2).

Ammonia-Oxidation Activity of Strain DDSI Can Be Enhanced by Added α-Keto Acids. After ∼1 y, the enrichment culture was able to grow on and oxidize 0.5 mM ammonia in less than 20 d (*SI Appendix*, Fig. S3). In contrast, the fully purified DDS1 culture was far less active. Despite an equivalent AOA population size, the pure culture required ∼25 d to oxidize 0.1 mM ammonia in ASM (*SI Appendix*, Fig. S3). To identify the stimulating factor or factors, which were apparently supplied by cocultured bacteria in the enrichment culture, mixotrophic conditions [i.e., those that supply both organic carbon and dissolved inorganic carbon (DIC)] were imposed by growing the pure DDS1 culture in the ammonia-based medium supplemented with various organic substrates (Table 1). Strain DDS1 showed a clear increase in the ammonia oxidation rate and cell growth, with the addition of several organic acids: pyruvate, oxaloacetate, α-ketoglutarate, and glyoxylate. Other organic substrates had no significant stimulatory effect, or even inhibited growth of strain DDS1 (Table 1). Alteration of the proportions of organic acids and ammonia in the growth media provided further insight. In the absence of ammonia, strain DDS1 was unable to grow with any of the growth-stimulating organic acids; this indicated that ammonia was the required energy source. When the ammonia concentration was set at 0.5 mM, the ammonia-oxidation activity responded in a clear quantitative fashion to increasing concentrations of pyruvate (Fig. 2). Growth yields of strain DDS1 normalized on the amount of ammonium consumed were positively correlated with pyruvate concentration up to 0.1 mM, after which they remained constant (*SI Appendix*, Fig. S4).

Hypothesis: The Mechanism of α-Keto Acid-Based Growth Stimulation Is Detoxification of Hydrogen Peroxide. All organic substrates that stimulated ammonia oxidation by strain DDS1 (pyruvate, oxaloacetate, α-ketoglutarate, and glyoxylate; Table 1) were α-keto acids. Interestingly, α-keto acids are known to protect cells against oxidative and nitrosative stress because α-keto acids are nonenzymatically decarboxylated in reactions that simultaneously consume H_2O_2 or peroxynitrite (16–19) [reaction (1)]

(1)

It seems, therefore, likely that α-keto acids detoxify metabolites produced during growth on ammonia. This hypothesis is an alternative to "mixotrophy" for explaining the stimulatory effect of pyruvate, oxaloacetate, α-ketoglutarate, and glyoxylate on

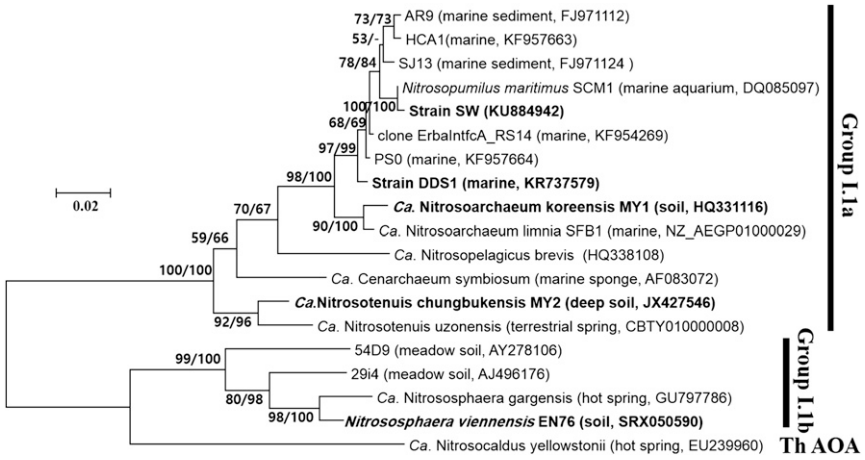

Fig. 1. Phylogenetic placement of strain DDS1 and other AOA isolates based on 16S rRNA gene sequence (ca. 1.3 kb). Numbers at nodes are bootstrap values, expressed as percentages of 1,000 replications, calculated from maximum-likelihood and neighbor-joining methods; only values >50% are shown. The scale bar represents 2% estimated sequence divergence.

Table 1. Effect of a range of substrates on the growth of strain DDS1

Substrate	Growth score
Carbohydrate (0.1 mM)	
Glucose, mannose, lactose, galactose	n
Fructose, sucrose, xylose	–
Organic acid (0.1 mM)	
Pyruvate, oxaloacetate, glyoxylate/ α-ketoglutarate	+
Citrate, propionate, succinate, fumarate, formate	n
Lactate	–
Amino acid (0.1 g/L)	
Amino acid mixture, DL-glutamic acid, DL-alanine, L-methionine, L-leucine, L-asparagine	n
DL-serine, DL-aspartic acid, casamino acid	–
Complex nutrient [0.001% (wt/vol)]	
Yeast extract, algal extract, tryptone, peptone	–
Urea (0.5 mM)	n
Methanol (0.1 mM)	n

+, positive (stimulation); –, negative (inhibition); n, no effect. The default ammonia concentration added to the medium was 0.5 mM.

ammonia oxidation in strain DDS1. To prove our hypothesis, we must disprove the mixotrophy hypothesis while simultaneously documenting both the production of a toxic agent during ammonia oxidation by strain DDS1 and a mechanism for detoxification of this agent.

Strain DDS1's endogenous H_2O_2 production was determined at varying starting concentrations of ammonia in pyruvate-free ASM, using a high-sensitivity chemiluminescence method (20) (detection limit, ~0.02 μM). The initial cell concentration used was within the range observed in situ in the marine environments (10^4–10^5 cells/mL) (11, 21, 22). A time-course experiment showing the link between ammonia oxidation (nitrite formation) and extracellular H_2O_2 concentration is shown in Fig. 3*A*. When this assay was repeated, although varying the initial ammonia concentration, the final H_2O_2 concentrations were clearly proportional to the amount of ammonia oxidized (Fig. 3*B*). In contrast, during complete oxidation of 0.5 mM ammonia in the presence of 0.1 mM pyruvate, no H_2O_2 was formed (Fig. 3*B*); this is consistent with the scavenging of toxic H_2O_2 by pyruvate [reaction (1)]. To verify that pyruvate participated in reaction (1), we used mass spectrometry to measure production of [$^{13}C_2$]-acetate by a culture amended with [2,3-^{13}C]-pyruvate (*SI Appendix*, Fig. S5), proving acetate formation via pyruvate decarboxylation [reaction (1)].

Two additional experiments were conducted to confirm the inhibitory effect of H_2O_2 on ammonia oxidation. The sensitivity of strain DDS1 to exogenous H_2O_2 was tested in pyruvate-free ASM. Under these conditions, ammonia oxidation was completely inhibited at levels of ≥0.2 μM H_2O_2 (Fig. 4*A*). Furthermore, when the H_2O_2-specific scavenging chemical, dimethylthiourea, or the enzyme catalase was added to ASM, pyruvate was no longer required for overcoming inhibition of ammonia oxidation (Fig. 4*B*). Results of assays testing enhancement of ammonia oxidation using other scavengers of reactive oxygen species (ROS) and reactive nitrogen species [superoxide anion (O_2^-), hydroxyl radical (HO·), and peroxynitrite ($OONO^-$); Trion, mannitol, and uric acid, respectively] were unsuccessful (Fig. 4*B*). These data support our hypothesis that the key role of α-keto acids in the stimulation of ammonia oxidation in strain DDS1 is via the detoxification of H_2O_2 by a nonenzymatic decarboxylation reaction [reaction (1)]. As a consequence, we conclude that toxic effects of endogenously produced H_2O_2 are of major importance in understanding the physiological state and ammonia-oxidation activity of strain DDS1.

The Role of α-Keto Acids in the Nitrification of Other AOA. Intriguingly, α-keto acids (pyruvate and α-ketoglutarate) have also been reported to stimulate the growth of other marine AOA strains [HCA1 and PS0 (14)] and *Nitrososphaera viennensis* (13), an AOA falling in the soil thaumarchaeotal group I.1b. This suggests that the role of α-keto acids, as reported here for strain DDS1, applies generally to AOA. To test this, we isolated taxonomically diverse pure strains of AOA from enrichment cultures derived from both soil and oceanic sources (*SI Appendix*, Table S1 and Fig. 1); *N. viennensis* EN76 (from garden soil) (13) was among the four additional AOA tested. All of the AOA strains tested required pyruvate for growth enhancement, and the requirement could be replaced by the addition of H_2O_2 scavengers (*SI Appendix*, Fig. S6). Furthermore, all the AOA strains were shown to endogenously produce H_2O_2 in the absence of α-keto acids (*SI Appendix*, Fig. S7). In particular, H_2O_2 production by *N. viennensis* was pronounced.

Strain DDS1 and *N. viennensis* Are Not Mixotrophic. According to our experiments, strain DDS1 appeared to be strictly chemoautotrophic: in the absence of ammonia, strain DDS1 was unable to grow with any of the growth-stimulating α-keto acids. To further explore chemoautotrophic growth of strain DDS1, we designed an experiment to examine whether the α-keto acid, pyruvate (0.1 mM), can be used as a carbon source during growth on ammonia. Incorporation of ^{13}C from fully labeled [$^{13}C_3$]-pyruvate, [2,3-^{13}C]-pyruvate, and [1-^{13}C]-pyruvate into the membrane lipids of strain DDS1 was examined. This resulted in only minor labeling of the lipids (Fig. 5*A* and *SI Appendix*, Table S2). In contrast, the degree of labeling observed in the experiment with ^{13}C-labeled DIC and unlabeled pyruvate was much more extensive (Fig. 5*A*). The degree of labeling indicates that with the latter experiment, all the carbon of the membrane lipids was derived from DIC, whereas in case of the experiments with fully labeled pyruvate and [1-^{13}C]-pyruvate, <1% of the carbon of the membrane lipids was potentially derived from labeled pyruvate. In fact, for the labeled pyruvate experiments, indirect incorporation of ^{13}C is expected because oxidation by H_2O_2 will release ^{13}C-DIC via pyruvate decarboxylation [reaction (1)]. Production of ^{13}C-labeled CO_2 by decarboxylation of pyruvate was confirmed by measuring an increase of ^{13}C-DIC in the culture broth amended with the fully labeled [$^{13}C_3$]-pyruvate or with [1-^{13}C]-pyruvate (Fig. 5*A*). In these experiments, the degree of labeling of the DIC matched that of the lipids, indicating that the membrane lipids were primarily derived from DIC fixation. As expected, DIC

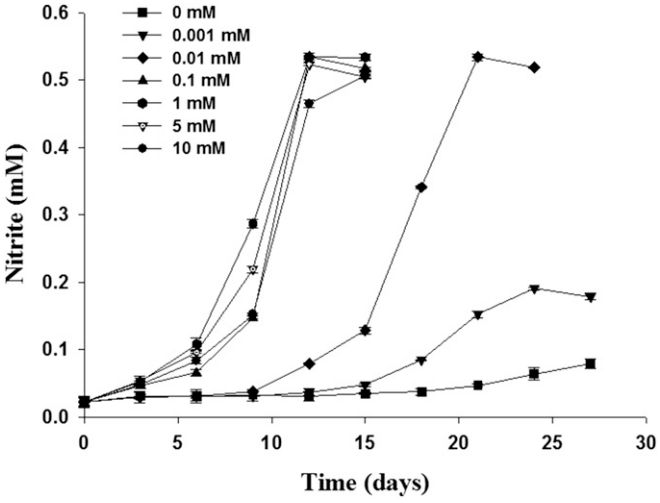

Fig. 2. Influence of pyruvate concentration upon ammonia oxidation (nitrite production) by the pure culture of strain DDS1. Initial cell density was ~10^6 cells·mL^{-1}. Error bars represent the SDs from triplicate experiments.

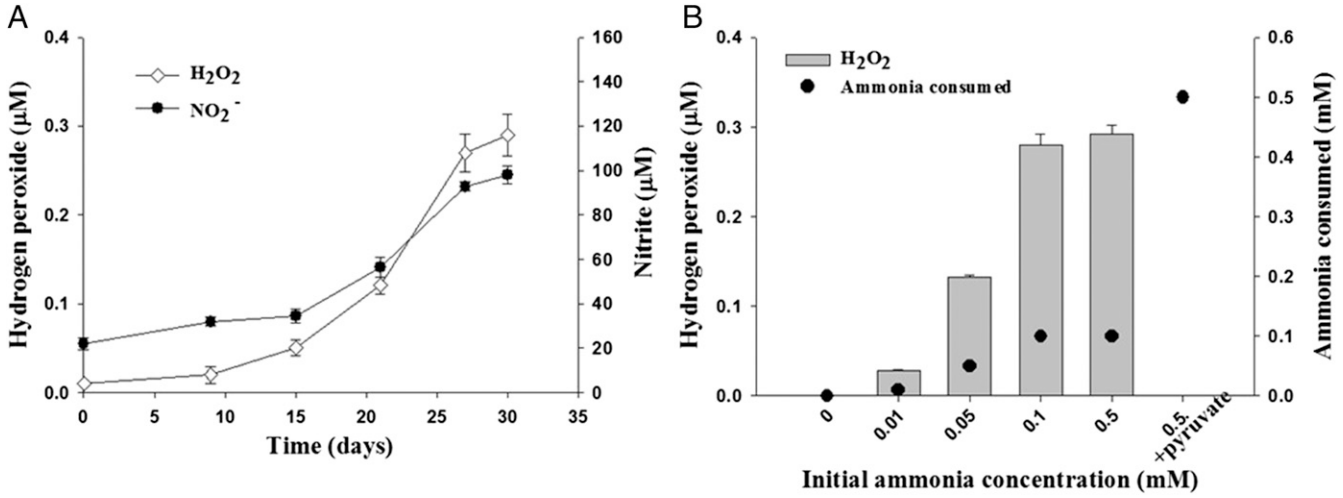

Fig. 3. Hydrogen peroxide production during ammonia oxidation by the pure culture of strain DDS1. (*A*) Time course production of H_2O_2 concomitant with oxidation of ammonia. (*B*) Quantitative relationship between ammonia concentration and H_2O_2 production. Strain DDS1 was incubated at an initial inoculum, cell density was ~10^5 cells·mL^{-1}, which is close to in situ AOA density in marine environments. In *B*, H_2O_2 was determined after complete ammonia oxidation (0.01–0.1 mM initial ammonia without pyruvate and 0.5 mM ammonia with 0.1 mM pyruvate) or after 1 mo (0.5 mM initial ammonia without pyruvate). Error bars represent the SDs from triplicate experiments.

did not become labeled in the experiment with [2,3-^{13}C]-pyruvate (Fig. 5*A*); the membrane lipids were slightly labeled, most probably through some incorporation of labeled acetate. When catalase was amended to the experiment with [1-^{13}C]-pyruvate, the degree of labeling of DIC and lipids dropped substantially, in agreement with the proposed H_2O_2 scavenging role of pyruvate.

When the same set of labeling experiments was applied to *N. viennensis*, the overall pattern of incorporation was similar to that observed for strain DDS1 (Fig. 5*B*). However, the degree of labeling with fully labeled [$^{13}C_3$]-pyruvate and [1-^{13}C]-pyruvate was much higher than for strain DDS1 (Fig. 5). This can readily be explained by the substantially increased production of H_2O_2 by *N. viennensis* (*SI Appendix*, Fig. S7), leading to a higher rate of decarboxylation of pyruvate and subsequent incorporation of labeled DIC. This is supported by a higher amount of acetate production by *N. viennensis* than strain DDS1 (*SI Appendix*, Fig. S5).

Overall, we conclude that incorporation of C originating from pyruvate into biomass of strain DDS1 and *N. viennensis* was negligible, and thus there is no evidence of mixotrophic growth by these strains. We conclude that the physiological effect of pyruvate (and other α-keto acids) on strain DDS1 and other AOA strains arises from the protection of the cells from H_2O_2. This result also indicates that the positive correlation of growth rate (Fig. 2) and growth yield (*SI Appendix*, Fig. S4) with the initial pyruvate concentration is not explained by the use of pyruvate as a carbon source, but by protection against the stress caused by the production of H_2O_2.

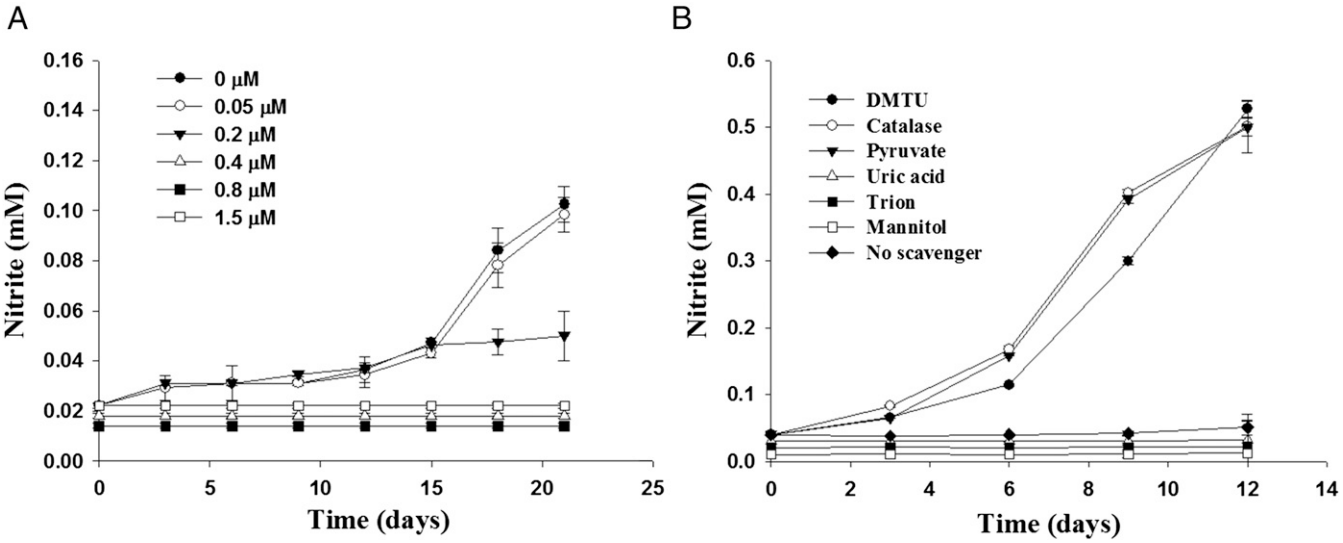

Fig. 4. Influence of initial H_2O_2 concentration (*A*) and various ROS scavengers (*B*) on ammonia oxidation (nitrite production) by the pure culture of strain DDS1 in pyruvate-free medium. Error bars represent the SDs of triplicate experiments. In *A*, initial cell density was ~10^5 cells·mL^{-1} which is close to in situ AOA density in marine environments. Ammonia oxidation was negligible, with initial H_2O_2 concentrations of >0.2 μM. To avoid overlapping symbols, (*A*) the value was shifted −0.004 or 0.008 for the experiments with 0.8 and 1.5 μM H_2O_2, respectively, and (*B*) the value was shifted −0.01, 0.02, or 0.03 for the experiments with uric acid, Trion, and mannitol, respectively. In *B*, initial cell density was ~10^6 cells·mL^{-1}.

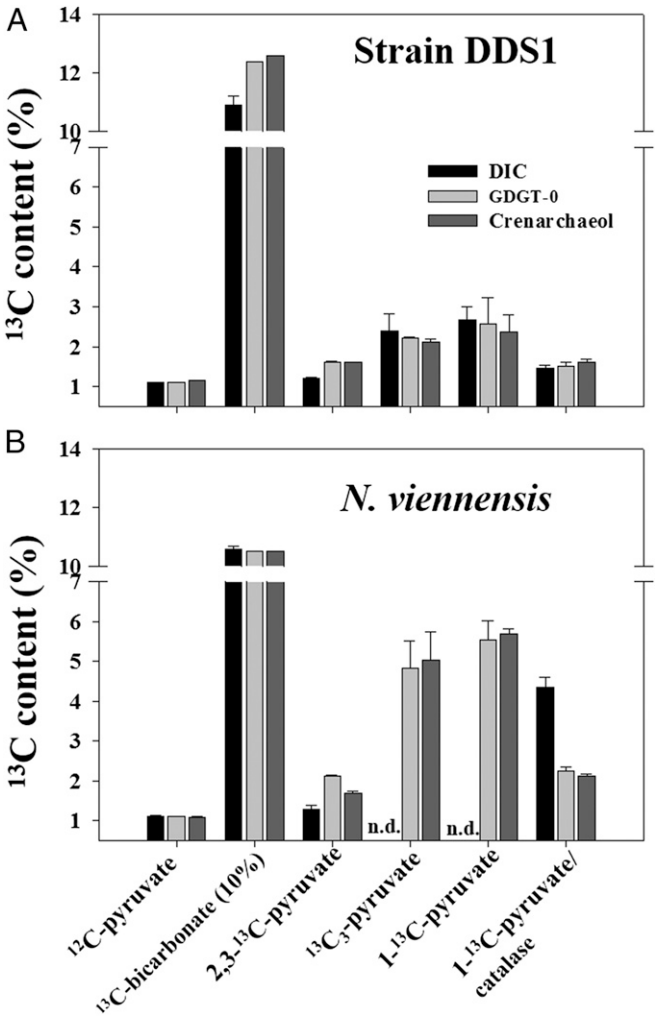

Fig. 5. The ^{13}C content of DIC and thaumarchaeotal membrane lipids at the end of experiments designed to monitor incorporation of ^{13}C from pyruvate for (*A*) strain DDS1 and (*B*) *N. viennensis*. For all pyruvate labeling experiments, 0.5 mM ammonia, 0.1 mM pyruvate, and 0.1 mM DIC were used. In the ^{13}C DIC experiment, a 10-fold higher concentration of DIC was used because background DIC values posed analytical problems. Mean values with SD from duplicate experiments are indicated. GDGT-0 and crenarchaeol are the main glycerol dibiphytanyl glycerol tetraether (GDGT) membrane lipids of strain DDS1 and *N. viennensis*. In the case of *N. viennensis*, ^{13}C incorporation into the regioisomer of crenarchaeol was measured because crenarchaeol was coeluting with GDGT-4. The elevated ^{13}C incorporation measured in the 1-^{13}C-pyruvate/catalase experiment with *N. viennensis* was probably caused by the high H_2O_2 concentration produced by *N. viennensis*, and a higher concentration of catalase would be required to completely remove H_2O_2.

Peroxidase Activity by Bacteria Explains the High Rate of Ammonia Oxidation in the Enrichment Culture. Many types of marine and other bacteria are known to be efficient scavengers of exogenous H_2O_2 (20). Thus, H_2O_2 toxicity during growth by strain DDS1 on ammonia may be neutralized by peroxidase-producing bacteria. To test this, we performed coinoculation assays in pyruvate-free ASM. Five well-recognized, catalase-positive marine heterotrophic bacteria representing five different clades were selected for the coinoculation assays (*SI Appendix*, Table S3). When coinoculated with strain DDS1 in pyruvate-free ASM, all bacterial strains supported active growth of strain DDS1 in a manner that mimicked the protection delivered by α-keto acids (*SI Appendix*, Table S3). During these coinoculation assays, as expected, ambient H_2O_2 concentrations remained below the level of detection. Thus, peroxide scavenging via peroxidase activity is most likely the mechanism that prevented H_2O_2 toxicity in these coinoculation experiments.

Peroxidase Genes Across AOA Genomes. All aerobic organisms need defense systems that prevent cellular damage caused by endogenous and exogenous oxidative stress. The genomic repertoire of microorganisms must reflect such physiological and evolutionary pressures. We searched for these types of genes in the genome of strain DDS1 involved with ROS/reactive nitrogen species and compared this with genomes of other AOA, ammonia-oxidizing bacteria (AOB), and other selected ecologically important marine microorganisms (phototrophs, *Prochlorococcus*; heterotrophs, *Pelagibacter*; *SI Appendix*, Table S4). All genomes of the AOA (both from group 1.1a and group 1.1b) contain genes encoding for superoxide dismutase (SOD) for removing super oxide. Strain DDS1 even contains three copies of the SOD gene. Importantly, however, AOA (groups 1.1a and 1.1b) with sequenced genomes generally lack genes encoding catalase (*SI Appendix*, Table S4). This observation is consistent with the idea that AOA are unable to perform detoxification of H_2O_2; we note that *Nitrososphaera gargensis* is annotated as containing a putative Mn-catalase gene that is truncated. In contrast, all known AOB contain putative catalase genes in their genomes (23, 24), and diverse AOB showed catalase activities (25). The absence of catalase genes in AOA is unlikely to be related to a broad trend such as reduced genome size, because the sizes of AOA of group I.1b genomes are close to those of AOB (24, 26). In contrast to the catalase gene, genes encoding for alkyl peroxide reductase, also referred to as peroxiredoxin, which has also been implicated in the removal of H_2O_2 (27), were detected in the genomes of strain DDS1 and other AOA. The types of alkyl peroxide reductase and number of copies differed for each phylotype (*SI Appendix*, Fig. S8). However, on the basis of the hydrogen peroxide toxicity data (Fig. 4*A*), we conclude that alkyl peroxide reductase is not effective in protecting strain DDS1, and probably other AOA isolates, from peroxide-induced cell damage.

Sequenced representatives of *Prochlorococcus* (a photosynthetic autotroph) and *Pelagibacter* (a heterotroph) also lack the gene encoding for catalase. This fits with the observation that cultivated representatives of these groups also require α-keto acids (especially pyruvate) for growth (28). Growth of a nitrite-oxidizing bacterium, *Nitrospira marina* ATCC 43039, was also shown to be stimulated by pyruvate (29), and some nitrite-oxidizing bacterium lack putative catalase genes in their genome (30, 31). It would be interesting to assess, across all environmentally important prokaryotes, the degree to which growth enhancement by α-keto acid amendment is caused by mixotrophy versus detoxification of H_2O_2.

It is important to emphasize that genetic and physiological diversity among AOA is likely quite high. Thus, exceptions to the trends in ROS sensitivity among AOA strains reported here should be expected among both currently characterized AOA and ones isolated in the future. For example, growth of *N. maritimus* in pure culture has been found to not be strictly dependent on keto acid addition (8) [as opposed to other strains (14)]; also, the degree of H_2O_2 production by *N. viennensis* was higher than what we report here for strain DDS1.

Ecological Relevance of H_2O_2 Sensitivity of AOA. Throughout the meso- and bathy-pelagic zones of the ocean, group 1.1a *Thaumarchaeota* make up a large fraction of total marine picoplankton, equivalent in cell numbers to total bacteria (21). In this setting, these AOA perform nitrification (32, 33), often under oligotrophic conditions (ammonia concentrations <5 nM) (21). In these environments, the H_2O_2 concentration is also extremely low (i.e., in the 5–10-nM range) (34). Therefore, over evolutionary time, many autotrophic AOA may have dispensed with their burden of ROS-detoxification genes (such as those encoding catalase) to streamline their genome for efficient use of rare resources. There may be H_2O_2-tolerant AOA ecotypes featuring traits adapted to distinct habitats. For

example, in the epipelagic zone, photochemical reactions can boost H_2O_2 concentrations by orders of magnitude (i.e., up to ~0.4 μM) (20, 34). Furthermore, single-cell genome analysis of epipelagic AOA ecotypes has revealed that they do harbor putative catalase genes (35). This observation suggests that H_2O_2 sensitivity may be an important physiological trait contributing to the AOA ecotype distribution in the marine environment. A similar adaptation likely applies the common marine genera, *Prochlorococcus* and *Pseudoaltermonas*, whose genetically distinct ecotypes exhibit different sensitivities to oxidative stress (20, 36).

Taken together, our results indicate that prior studies reporting a "requirement" by AOA for α-keto acids have likely been misinterpreted as an indication of organic-carbon assimilation and, therefore, as an indication of mixotrophy. The fundamental paradigm for marine carbon metabolism, especially as it applies to the nutrition of marine autotrophic populations, needs to be refined: When the metabolic role of α-keto acids is detoxification of H_2O_2, carbon assimilation should not be implicated. H_2O_2 detoxification may thus be an important factor in marine microbial ecology because in situ metabolic activities of many biogeochemically relevant microbial populations (i.e., those involved in the cycling of C and N and other elements) may be regulated by other members of the microbial communities that produce and release enzymes able to detoxify ocean water ROS, particularly H_2O_2. Future work is required to identify sources and sinks of ROS and their interactions with AOA in situ. Differential sensitivity of AOA ecotypes to ROS toxicity needs to be investigated to understand AOA function and ecotype distribution in marine environments.

Materials and Methods

All materials and methods are described in detail in *SI Appendix, SI Materials and Methods*.

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1. Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. *Science* 330(6001):192–196.
2. Galloway JN, et al. (2008) Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* 320(5878):889–892.
3. Daims H, et al. (2015) Complete nitrification by *Nitrospira* bacteria. *Nature* 528(7583): 504–509.
4. van Kessel MA, et al. (2015) Complete nitrification by a single microorganism. *Nature* 528(7583):555–559.
5. Hu HW, Chen D, He JZ (2015) Microbial regulation of terrestrial nitrous oxide formation: Understanding the biological pathways for prediction of emission rates. *FEMS Microbiol Rev* 39(5):729–749.
6. Arp DJ, Sayavedra-Soto LA, Hommes NG (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch Microbiol* 178(4):250–255.
7. Koops HP, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M (2006) The lithoautotrophic ammonia-oxidizing bacteria. *The Prokaryotes: Volume 5: Proteobacteria: Alpha and Beta Subclasses*, eds Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (Springer, New York), pp 778–811.
8. Könneke M, et al. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437(7058):543–546.
9. Leininger S, et al. (2006) *Archaea* predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442(7104):806–809.
10. Wuchter C, et al. (2006) Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* 103(33):12317–12322.
11. Santoro AE, Casciotti KL, Francis CA (2010) Activity, abundance and diversity of nitrifying archaea and bacteria in the central California Current. *Environ Microbiol* 12(7):1989–2006.
12. Wuchter C, Schouten S, Boschker HT, Sinninghe Damsté JS (2003) Bicarbonate uptake by marine Crenarchaeota. *FEMS Microbiol Lett* 219(2):203–207.
13. Tourna M, et al. (2011) *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc Natl Acad Sci USA* 108(20):8420–8425.
14. Qin W, et al. (2014) Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation. *Proc Natl Acad Sci USA* 111(34):12504–12509.
15. Mussmann M, et al. (2011) Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not obligate autotrophic ammonia oxidizers. *Proc Natl Acad Sci USA* 108(40):16771–16776.
16. Kao KK, Fink MP (2010) The biochemical basis for the anti-inflammatory and cytoprotective actions of ethyl pyruvate and related compounds. *Biochem Pharmacol* 80(2):151–159.
17. Varma SD, Hegde KR (2007) Lens thiol depletion by peroxynitrite. Protective effect of pyruvate. *Mol Cell Biochem* 298(1-2):199–204.
18. Vásquez-Vivar J, Denicola A, Radi R, Augusto O (1997) Peroxynitrite-mediated decarboxylation of pyruvate to both carbon dioxide and carbon dioxide radical anion. *Chem Res Toxicol* 10(7):786–794.
19. Desagher S, Glowinski J, Prémont J (1997) Pyruvate protects neurons against hydrogen peroxide-induced toxicity. *J Neurosci* 17(23):9060–9067.
20. Morris JJ, Johnson ZI, Szul MJ, Keller M, Zinser ER (2011) Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. *PLoS One* 6(2):e16805.
21. Karner MB, DeLong EF, Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* 409(6819):507–510.
22. Agogué H, Brink M, Dinasquet J, Herndl GJ (2008) Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic. *Nature* 456(7223):788–791.
23. Klotz MG, et al. (2006) Complete genome sequence of the marine, chemolithoautotrophic, ammonia-oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. *Appl Environ Microbiol* 72(9):6299–6315.
24. Chain P, et al. (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J Bacteriol* 185(9): 2759–2773.
25. Wood NJ, Sørensen J (2001) Catalase and superoxide dismutase activity in ammonia-oxidising bacteria. *FEMS Microbiol Ecol* 38(1):53–58.
26. Norton JM, et al. (2008) Complete genome sequence of Nitrosospira multiformis, an ammonia-oxidizing bacterium from the soil environment. *Appl Environ Microbiol* 74(11):3559–3572.
27. Seaver LC, Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* 183(24):7173–7181.
28. Carini P, Steindler L, Beszteri S, Giovannoni SJ (2013) Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* 7(3):592–602.
29. Watson SW, Bock E, Valois FW, Waterbury JB, Schlosser U (1986) *Nitrospira marina* gen. nov. sp. nov.: A chemolithotrophic nitrite-oxidizing bacterium. *Arch Microbiol* 144(1):1–7.
30. Lücker S, Nowka B, Rattei T, Spieck E, Daims H (2013) The genome of *Nitrospina gracilis* illuminates the metabolism and evolution of the major marine nitrite oxidizer. *Front Microbiol* 4:27.
31. Lücker S, et al. (2010) A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proc Natl Acad Sci USA* 107(30):13479–13484.
32. Mincer TJ, et al. (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ Microbiol* 9(5):1162–1175.
33. Ingalls AE, et al. (2006) Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon. *Proc Natl Acad Sci USA* 103(17):6442–6447.
34. Baltar F, Reinthaler T, Herndl GJ, Pinhassi J (2013) Major effect of hydrogen peroxide on bacterioplankton metabolism in the Northeast Atlantic. *PLoS One* 8(4):e61051.
35. Luo H, et al. (2014) Single-cell genomics shedding light on marine Thaumarchaeota diversification. *ISME J* 8(3):732–736.
36. Qin QL, et al. (2011) Comparative genomics reveals a deep-sea sediment-adapted life style of *Pseudoalteromonas* sp. SM9913. *ISME J* 5(2):274–284.