Proteomic Response of Three Marine Ammonia-Oxidizing Archaea to Hydrogen Peroxide and Their Metabolic Interactions with a Heterotrophic Alphaproteobacterium

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ABSTRACT Ammonia-oxidizing archaea (AOA) play an important role in the nitrogen cycle and account for a considerable fraction of the prokaryotic plankton in the ocean. Most AOA lack the hydrogen peroxide (H₂O₂)-detoxifying enzyme catalase, and some AOA have been shown to grow poorly under conditions of exposure to H₂O₂. However, differences in the degrees of H₂O₂ sensitivity of different AOA strains, the physiological status of AOA cells exposed to H₂O₂, and their molecular response to H₂O₂ remain poorly characterized. Further, AOA might rely on heterotrophic bacteria to detoxify H₂O₂, and yet the extent and variety of costs and benefits involved in these interactions remain unclear. Here, we used a proteomics approach to compare the protein profiles of three *Nitrosopumilus* strains grown in the presence and absence of catalase and in coculture with the heterotrophic alphaproteobacterium *Oceanicaulis alexandrii*. We observed that most proteins detected at a higher relative abundance in H₂O₂-exposed *Nitrosopumilus* cells had no known function in oxidative stress defense. Instead, these proteins were putatively involved in the remodeling of the extracellular matrix, which we hypothesize to be a strategy limiting the influx of H₂O₂ into the cells. Using RNA-stable isotope probing, we confirmed that *O. alexandrii* cells growing in coculture with the *Nitrosopumilus* strains assimilated *Nitrosopumilus*-derived organic carbon, suggesting that AOA could recruit H₂O₂-detoxifying bacteria through the release of labile organic matter. Our results contribute new insights into the response of AOA to H₂O₂ and highlight the potential ecological importance of their interactions with heterotrophic free-living bacteria in marine environments.

IMPORTANCE Ammonia-oxidizing archaea (AOA) are the most abundant chemolithoautotrophic microorganisms in the oxygenated water column of the global ocean. Although H₂O₂ appears to be a universal by-product of aerobic metabolism, genes encoding the hydrogen peroxide (H₂O₂)-detoxifying enzyme catalase are largely absent in genomes of marine AOA. Here, we provide evidence that closely related marine AOA have different degrees of sensitivity to H₂O₂, which may contribute to niche differentiation between these organisms. Furthermore, our results suggest that marine AOA rely on H₂O₂ detoxification during periods of high metabolic activity and release organic compounds, thereby potentially attracting heterotrophic prokaryotes that provide this missing function. In summary, this report provides insights into the metabolic interactions between AOA and heterotrophic bacteria in marine environments and suggests that AOA play an important role in the biogeochemical carbon cycle by making organic carbon available for heterotrophic microorganisms.
Ammonia-oxidizing archaea (AOA) are a major component of marine microbial communities and represent the dominant ammonia oxidizers in the ocean, carrying out the first and rate-limiting step of nitrification (1–3). AOA are members of the phylum Thaumarchaeota (4, 5) and are particularly abundant in the mesopelagic zone of the open ocean (6) and in oxygen minimum zones (7).

Although more than a dozen strains of autotrophic AOA have been enriched from marine waters and sediments (8–14), their isolation and maintenance on a mineral medium have repeatedly proven difficult. To some extent, this difficulty may be attributed to the dependence of some AOA on the presence of alpha-ketoacids or, alternatively, that of cocultivated heterotrophic bacteria to achieve exponential growth in batch cultures (9, 15, 16). Recently, this dependency has been linked to their sensitivity to hydrogen peroxide (H$_2$O$_2$), which is detoxified by alpha-ketoacids and/or cocultivated heterotrophs (14). The sensitivity of AOA to H$_2$O$_2$ is somewhat surprising as H$_2$O$_2$ appears to be a universal by-product of aerobic metabolism and the vast majority of aerobic organisms encode H$_2$O$_2$-scavenging enzymes, including catalases and peroxidases (17, 18). The H$_2$O$_2$ sensitivity reported for some AOA strains isolated from marine environments correlates with the lack of genes encoding canonical catalase homologs in their genomes (14, 19).

In the ocean, H$_2$O$_2$ is mainly produced by the photooxidation of chromophoric dissolved organic matter (20) but is also introduced via precipitation (21) and metabolic processes (22, 23). The lack of a recognizable form of H$_2$O$_2$ detoxification machinery in AOA was hypothesized to result from their limited exposure to this oxidant under oligotrophic conditions (14). However, in marine surface waters, AOA might be chronically exposed to H$_2$O$_2$, where its concentrations can reach up to 500 nM (24). While the ammonia oxidization activity of the marine archaeon Nitrosopumilus strain DDS1 was completely inhibited after production of ~200 nM H$_2$O$_2$ (14), Nitrosopumilus maritimus SCM1 showed no decrease in ammonia oxidation after additions of H$_2$O$_2$ (up to 1 μmol liter$^{-1}$) to the culture medium (25). Differences in H$_2$O$_2$ sensitivity have also been reported for environmental AOA populations and were suggested previously to be defining features of distinct AOA ecotypes (19). These observations indicate that the degrees of H$_2$O$_2$ tolerance differ across the vast diversity of AOA species, and yet exploration of the entire spectrum of their phenotypic response to H$_2$O$_2$ has just started. Furthermore, it is unclear whether the inability to achieve exponential growth in the absence of an external H$_2$O$_2$ scavenger (i.e., alpha-ketoacids [14–16]) represents a consequence or the absence of a molecular response to H$_2$O$_2$.

In this study, we compared the growth levels of three strains of marine AOA in the presence and absence of commercial catalase or under conditions of growth in coculture with the heterotrophic alphaproteobacterium Oceanicaulis alexandrii and evaluated the concurrent levels of production and eventual scavenging of H$_2$O$_2$ in the culture medium. The investigated AOA comprised all marine axenic cultures with closed genomes which are currently available, including the first reported AOA isolate, Nitrosopumilus maritimus SCM1 (26), as well as two isolates from coastal surface waters of the Northern Adriatic Sea, Nitrosopumilus Adriaticus NFS and Nitrosopumilus piranensis D3C (27). O. alexandrii was the most persistent contaminant prior to obtaining axenic cultures of N. Adriaticus NFS and N. Piranensis D3C (9). Furthermore, the molecular response of the investigated strains to H$_2$O$_2$ was assessed by comparing the proteomes of cells growing in the presence or in the absence of catalase and/or the alphaproteobacterium O. alexandrii. Additionally, metabolic interactions of the three Nitrosopumilus strains with O. alexandrii were explored using stable isotope probing and comparative proteome analysis.

Collectively, the results of this study provide insights into the molecular and physiological responses of AOA to H$_2$O$_2$ and highlight the potential ecological impor-
tance of their interactions with heterotrophic free-living bacteria in marine environments.

RESULTS AND DISCUSSION
The effect of H$_2$O$_2$ on the growth and ammonia oxidation activity of three Nitrosopumilus strains. Ammonia oxidation activity and growth of three Nitrosopumilus strains (N. adriaticus NF5, N. piranensis D3C, and N. maritimus SCM1) were assessed both in the presence and absence of commercial catalase and under conditions of growth in coculture with the heterotrophic alphaproteobacterium Oceanicaulis alexandrii. When catalase was added to the cultures, all three Nitrosopumilus strains depleted 1 mM ammonium within 5 to 8 days of incubation (Fig. 1A to C), yielding nearly stoichiometric amounts of nitrite as previously described for these strains (9, 25). In cocultures with O. alexandrii, however, nitrite production was consistently slower than in axenic cultures supplemented with catalase. In cocultures, the complete conversion of ammonium to nitrite took 10 to 15 days, possibly due to the proportionally small O. alexandrii cell population size and consequently low H$_2$O$_2$-detoxifying capacity of O. alexandrii relative to purified catalase. Adding both catalase and O. alexandrii did not result in an increased rate of nitrite production relative to those observed for axenic AOA cultures grown in the presence of catalase.

In the absence of catalase and O. alexandrii, all three Nitrosopumilus strains depleted the supplied ammonium only partially and nitrite concentrations remained constant at 150 to 300 μM after 5 to 9 days of incubation (Fig. 1A to C). The stalled production of nitrite in axenic cultures devoid of catalase correlated with a growth arrest of the cultures. Maximum cell abundances ranged between $4 \times 10^6$ and $1 \times 10^7$ cells ml$^{-1}$ after 3 to 6 days of incubation, which was on average 5 to 10 times lower than in cultures containing catalase (see Table S1 in the supplemental material). Growth arrest was observed 2 to 3 days earlier than the arrest of nitrite production in all three strains, indicating that the remaining level of ammonia oxidation was insufficient to meet the energy demands of dividing cells during H$_2$O$_2$ exposure (Fig. 1A to C; see also Table S1).

In the medium of cultures lacking both catalase and O. alexandrii, H$_2$O$_2$ concentrations reached 800 nM to 3 μM after 7 to 9 days of incubation (Fig. 1D to F), which was far above the background levels of H$_2$O$_2$ (~150 nM) in the culture medium (see Fig. S1A in the supplemental material). Concentrations of H$_2$O$_2$ remained fairly stable in the abiotic controls (Fig. S1A), indicating that the investigated Nitrosopumilus strains produced H$_2$O$_2$ as a result of their metabolic activity. Although the fluorescence-based assay used in this study (see Materials and Methods) could potentially detect oxidizing.
agents other than H$_2$O$_2$, complete loss of the signal after catalase addition suggests that H$_2$O$_2$ was the primary oxidant measured. Importantly, the presence of the organic buffer HEPES in the culture medium did not appear to represent a dominant source of H$_2$O$_2$, in contrast to previous reports on phytoplankton cultures (28) (Fig. S1A). Indeed, the HEPES buffer may release H$_2$O$_2$ only under conditions of exposure to light whereas the three *Nitrosopumilus* strains were grown in the dark. After reaching their peak, H$_2$O$_2$ concentrations also declined in axenic cultures devoid of catalase (Fig. 1D to F). This decline in the H$_2$O$_2$ concentration was also observed in the abiotic controls, where H$_2$O$_2$ was added at $\sim$3.5 $\mu$M (Fig. S1B), suggesting that H$_2$O$_2$ was not actively scavenged by the investigated strains. In all cultures containing the purified catalase, H$_2$O$_2$ concentrations never increased above background levels, whereas in cocultures, H$_2$O$_2$ concentrations sporadically reached levels as high as 900 nM in *N. adriaticus-O. alexandrii* cocultures (Fig. 1D).

Comparing the three strains, complete inhibition occurred at lower H$_2$O$_2$ concentrations in *N. piranensis* ($\sim$800 nM; Fig. 1E) than in *N. adriaticus* and *N. maritimus* (2.5 $\mu$M and 2 $\mu$M H$_2$O$_2$, respectively; Fig. 1D and F). Although *N. maritimus* produced more nitrite than *N. adriaticus* prior to inhibition ($\sim$350 $\mu$M nitrite versus $\sim$250 $\mu$M, respectively; Fig. 1A and C), *N. adriaticus* exhibited a higher cell-specific net level of H$_2$O$_2$ production (Fig. 1; see also Table S1), suggesting that the molecular machinery responsible for H$_2$O$_2$ production in distinct AOA strains could have different H$_2$O$_2$ production yields. In contrast to *N. piranensis* and *N. maritimus*, which encode two putative superoxide dismutases, *N. adriaticus* encodes three superoxide dismutases, potentially explaining the higher observed level of H$_2$O$_2$ production by this strain.

The three strains investigated in this study appeared to tolerate higher concentrations of H$_2$O$_2$ than *Nitrosopumilus* strain DDS1, which was reported to be completely inhibited at $\sim$200 nM H$_2$O$_2$ (14). While strain DDS1 was isolated from a water depth of 200 m, *N. adriaticus* NF5 and *N. piranensis* D3C were isolated from coastal surface waters where H$_2$O$_2$ concentrations are typically $\sim$10 to 100 times higher than in deeper waters (29, 30). Both strains putatively encode cyclobutane pyrimidine dimer (CPD) photolase (9), an enzyme activated by UV-A radiation to repair DNA damage (31), suggesting that they might be more tolerant of conditions typically found in surface waters (i.e., higher H$_2$O$_2$ concentrations) than strains isolated from deeper water layers. *N. maritimus* was previously observed to be insensitive to additions of H$_2$O$_2$ and was still able to oxidize 1 mM ammonia when H$_2$O$_2$ was added to the culture medium at 5 $\mu$mol liter$^{-1}$ (25), which contrasts with the complete inhibition of *N. maritimus* at 2 $\mu$M H$_2$O$_2$ that we report here (Fig. 1F). Considering the different experimental setup and the lack of H$_2$O$_2$ concentration measurements in the incubations cited above (26), the explanation of the different results that we report here remains currently unclear.

All three *Nitrosopumilus* strains could overcome H$_2$O$_2$-induced growth arrest when initial cell abundances were higher than or equal to $7 \times 10^6$ ml$^{-1}$, and growth of these cultures was similar to those containing catalase (Fig. 2A to F). While H$_2$O$_2$ concentrations appeared to increase linearly with nitrite production in cultures with low initial cell abundances ($\sim$2 $\times$ 10$^5$, $\sim$8 $\times$ 10$^5$, and $\sim$3 $\times$ 10$^6$ ml$^{-1}$; Fig. 2A, C, and G to I), the detected H$_2$O$_2$ concentrations were much lower (<300 nM) in cultures with high initial cell abundances ($7 \times 10^6$ ml$^{-1}$; Fig. 2G to I), indicating either that less H$_2$O$_2$ was produced or that it was scavenged by an unknown mechanism. Similar cell abundance-dependent H$_2$O$_2$ sensitivity patterns have been described in axenic *Prochlorococcus* cultures, which grew well in concentrated but not in dilute cultures (32, 33). In the absence of H$_2$O$_2$ scavengers, growth and ammonia oxidation activity did not show a linear response to the size of the inoculum but rather were induced at a certain cell abundance level in all three *Nitrosopumilus* strains (Fig. 2A to F). Even though knowledge of the exact mechanism of this phenomenon remains elusive, cell abundance-dependent cellular responses are commonly induced by quorum sensing.

While the culture conditions in this study did not reflect the oligotrophic conditions typically found in the ocean, note that all three *Nitrosopumilus* strains also produced large amounts of H$_2$O$_2$ at environmentally relevant cell abundances of $\sim$2 $\times$ 10$^5$ ml$^{-1}$.
Thus, in their natural environment, marine AOA might potentially rely on \( \text{H}_2\text{O}_2 \) detoxification by other microorganisms during periods of high activity and/or in nutrient-rich microniches.

**The proteomic response of three *Nitrosopumilus* strains to \( \text{H}_2\text{O}_2 \).** Comparative proteomics was used to investigate the molecular response of the three *Nitrosopumilus* strains to \( \text{H}_2\text{O}_2 \) exposure relative to cultures grown in the presence of commercial catalase or in coculture with the heterotrophic bacterium *O. alexandrii*. Additionally, we distinguished between axenic cultures that were completely inhibited in their growth (\( \text{H}_2\text{O}_2 \) inhibited) and axenic cultures that were grown at a high initial cell abundance (\( 7 \times 10^6 \text{ ml}^{-1} \)) and were able to grow despite the absence of an \( \text{H}_2\text{O}_2 \) scavenger (\( \text{H}_2\text{O}_2 \) noninhibited).

A total of 1,020 to 1,372 proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and accounted for \( \sim 55\% \) to \( 68\% \) of the predicted coding DNA sequences in the genomes of *N. adriaticus* NF5, *N. piranensis* D3C, and *N. maritimus* SCM1 (Table S2A). Between the four treatments (with catalase, with *O. alexandrii*, \( \text{H}_2\text{O}_2 \) inhibited, and \( \text{H}_2\text{O}_2 \) noninhibited), 1,040, 1,027, and 856 proteins were shared by *N. adriaticus*, *N. piranensis*, and *N. maritimus*, respectively (Table S2A). While the relative abundances of the majority of proteins remained constant between the different treatments, 56 to 109 proteins significantly changed in their relative abundances (increased or decreased; adjusted \( P \) value, \(< 0.05 \)) in the absence of catalase or *O. alexandrii* compared to the results seen with cultures grown in the presence of either catalase or *O. alexandrii* (Table S2B). Of these, 33 proteins were shared among all three strains. Comparing the proteome composition of cultures grown in the presence of catalase to that of cocultures grown with *O. alexandrii*, 12 to 20 proteins changed in their relative abundances, 5 of which were shared by all three strains (Table S2B). However, these 5 shared proteins also changed in relative abundance when *Nitrosopumilus* cells were exposed to \( \text{H}_2\text{O}_2 \), suggesting that they did not represent a specific response to the presence of the heterotrophic bacterium itself. Instead, they might have been induced as a result of the lower capacity of *O. alexandrii* to detoxify \( \text{H}_2\text{O}_2 \) than purified catalase (Fig. 1D to F).
Furthermore, despite the reduced sensitivity to H$_2$O$_2$ in *Nitrosopumilus* cultures with high initial cell abundances (H$_2$O$_2$ noninhibited) (Fig. 2), we did not detect any changes in the proteome composition shared by all three strains relative to cultures that were completely inhibited by H$_2$O$_2$ (Table S2B). This suggests that the proteome composition of cells exposed to H$_2$O$_2$ does not directly indicate whether the cells are active, indicating the existence of further regulatory mechanisms (i.e., noncoding RNAs or posttranslational protein modifications) determining growth. Previous studies have reported that most of the abundant transcripts are relatively invariant across growth phases and environmental conditions in AOA (12, 34).

Several strain-specific changes in the proteome composition were identified across the four treatments in each of the three strains (Fig. S2A to C; see also Text S1 in the supplemental material); however, we focus in the following sections on the proteomic features shared by all three strains.

(i) Thaumarchaeal homologs of proteins involved in canonical oxidative stress defense. Microbial cells use various intracellular scavenging and repair mechanisms to limit and repair damage caused by reactive oxygen species (ROS). In bacteria, the basal scavenging system for O$_2^-$ is superoxide dismutase, whereas H$_2$O$_2$ is scavenged by catalase and peroxidases (18, 35). Genetic responses to H$_2$O$_2$ stress in Gram-negative and Gram-positive bacteria are controlled by the transcriptional regulons OxyR and PerR, respectively (36–38), which regulate the expression of catalase, alkyl hydroperoxide reductase (Ahp), and proteins involved in disulfide reduction, heme synthesis, iron scavenging (ferritin and related proteins), and iron import control, as well as proteins involved in divalent cation import (mostly manganese) (see reference 39 and references therein). ROS scavenging systems in archaea, although much less extensively studied, have been shown to be similar to those in bacteria (40, 41).

Although the genomes of all cultured marine AOA do not encode canonical catalase homologs, genes encoding putative catalases have recently been reported from two single-cell genomes obtained from AOA cells sampled from Antarctic surface waters (42). These genes were predicted to be horizontally acquired genes (42). We reanalyzed these single-cell genomes and observed that catalase-encoding genes are part of short contigs that harbor only genes of presumed bacterial origin (due to their similarity to known bacterial homologs), suggesting that they represent contaminating genome fragments that originated from bacterial genomes (see Data Set S2A in the supplemental material). Although there is tangible evidence of the presence of catalase-encoding genes in AOA isolated from terrestrial environments (43, 44) and from a wastewater treatment plant (45), thus far, there is no evidence suggesting that marine AOA encode catalases.

In spite of the notable absence of catalase homologs, we identified homologs of known ROS-scavenging enzymes and, more generally, of proteins involved in canonical pathways of oxidative stress defense in the genomes and proteomes of the three *Nitrosopumilus* strains that we investigated (Data Set S1D). Surprisingly, however, the relative abundances of most proteins assumed to play a role in oxidative stress defense did not change in response to H$_2$O$_2$ exposure. These also included the five putative Ahp proteins, which raises the issue of whether these putative candidates are functional H$_2$O$_2$-detoxifying enzymes as previously contested (14) or are generally not regulated on the gene expression level in members of the *Nitrosopumilus* genus. Alternatively, intracellular H$_2$O$_2$ concentrations might not have been high enough to induce a response.

Nevertheless, more than one-third (36%) of the proteins that showed a significant response to H$_2$O$_2$ exposure were assigned to KEGG orthologous groups (OGs) associated with genetic information processing and nucleotide metabolism (Fig. 3; see also Data Set S1C). These included DNA polymerase I and ATP-dependent helicase, which are key enzymes of DNA replication, as well as two subunits of DNA-directed RNA polymerase, which is involved in RNA synthesis (Fig. 3). Additionally, ribonucleotide reductase, which converts ribonucleotides into deoxyribonucleotides, was identified at high relative abundance. While ribonucleotide reductase expression is necessary for
DNA replication, it has also been shown to be induced in response to DNA damage and replication blocks (46, 47). Furthermore, proteins putatively involved in the repair of DNA damage, including excinuclease ABC subunit A (UvrA) and DNA helicase Hel308, were identified at high relative abundance during H$_2$O$_2$ exposure (Fig. 3). The latter is suggested to play a role in the repair and start of replication forks (48), whereas UvrA is part of the nucleotide excision repair mechanism (49). Nucleotide excision repair initiation involves a two-step mechanism in which UvrA initially scans the genome and locates DNA damage prior to recruiting UvrB and UvrC, which are needed for DNA damage verification, excision, and, ultimately, repair (49, 50). However, we did not detect an increase in the relative abundance of UvrBC, suggesting that H$_2$O$_2$ concentrations in Nitrosopumilus cells might not have been high enough to induce extensive DNA damage.

The concentration of H$_2$O$_2$ inside cells is dependent on the rate of its endogenous formation and influx, balanced against the rate of H$_2$O$_2$ scavenging and efflux (51). A few studies have suggested previously that extent of passage of H$_2$O$_2$ through biological membranes is limited (51–53). In Escherichia coli, endogenous H$_2$O$_2$ is rapidly

### FIG 3
Heat map of proteins that showed a significant change in relative abundance in three Nitrosopumilus strains (N. adriaticus NF5, N. piranensis D3C, and N. maritimus SCM1) comparing different culture conditions (with catalase, with O. alexandrii, H$_2$O$_2$ inhibited, and H$_2$O$_2$ noninhibited). Columns and rows were clustered based on Euclidean distances corresponding to differences between treatments and relative protein abundances, respectively. The presence of a signal peptide is indicated in dark gray.
scavenged by Ahp (and to only a lower extent by catalase) to levels below 20 nM and does not persist long enough to penetrate the membrane, with less than 10% escaping the cell (51, 54). Hence, there is typically no measurable accumulation of H$_2$O$_2$ in the culture medium (54), in contrast to the range of H$_2$O$_2$ concentrations of ~1 to 2.5 μM measured in the *Nitrosopumilus* culture medium in this study. Additionally, putative Ahp proteins have been identified in the proteomes of all three *Nitrosopumilus* strains at similar abundance levels in all treatments as mentioned above. On the basis of these observations, we hypothesize that production of H$_2$O$_2$ in AOA possibly takes place at the outer side of the membrane (e.g., in the pseudoperiplasmic space).

(ii) Increase of relative abundances of membrane-associated proteins in response to H$_2$O$_2$. Among the proteins that showed a significant change in relative abundance in AOA cultures exposed to H$_2$O$_2$ (H$_2$O$_2$ inhibited and noninhibited treatments), ~30% contained a signal peptide indicating secretion and/or localization on the outer side of the cytoplasmic membrane (Fig. 3). Concomitantly, tRNA synthetases for branched-chain amino acids, essential components of membrane spanning helices and thus of membrane bound/surface proteins (55), were identified at higher relative abundances (Fig. 3). Moreover, the high level of representation of the signal recognition particle (SRP) receptor protein FtsY, which likely mediates the delivery of SRP-nascent chain complexes to the cell membrane (56), suggests an increased rate of transport of proteins to the membrane.

Both putative membrane-bound S-layer proteins were among the 10 to 50 most abundant proteins detected in axenic cultures devoid of catalase (Fig. 3). The putative S-layer proteins were on average 4 to 30 times more abundant in the proteomes of axenic cultures grown in the absence of catalase than in those of cultures grown in the presence of catalase or in coculture with *O. alexandrii*, potentially indicating an increased renewal or restructuring of the S-layer coat under conditions of H$_2$O$_2$ exposure. In addition, three PEFG-CTERM domain-containing proteins were detected at 4 to 10 times higher relative abundance in axenic cultures without addition of catalase than cultures grown with catalase or *O. alexandrii* (Fig. 3). Proteins containing TT3R motifs in AOA show sequence similarity to hypothetical proteins of the myxobacterial thrombospondin-like gene cluster, which has been suggested to play a role in the construction of the cell surface matrix (58). However, besides the well-known calcium-binding capacities of TT3R motifs, knowledge of their exact functions in prokaryotes remains elusive (59). Nevertheless, we detected putative structural proteins (OG1004 and OG0954) which share homology with proteins known to interact with thrombospondins in eukaryotes at high relative abundance levels in all three *Nitrosopumilus* strains under conditions of exposure to H$_2$O$_2$ (Fig. 3) (further discussed in Text S1 in the supplemental material).

The increase in the relative abundance of putative components of the extracellular matrix of AOA is reminiscent of the protective barrier formed by the eukaryote *Saccharomyces cerevisiae* to limit the influx of H$_2$O$_2$ into its cells (60). Furthermore, cell aggregation, exopolysaccharide (EPS) production, and, ultimately, biofilm formation represent common physiological responses of bacteria exposed to H$_2$O$_2$ and may promote survival (61, 62). AOA species, including members of the *Nitrosopumilus* genus, contain the genomic repertoire for exopolysaccharide production and cell surface modifications (63). Formation of some small aggregates was observed in H$_2$O$_2$-exposed cultures (Fig. S3A and B), indicating that members of the *Nitrosopumilus* genus potentially remodel their membrane and/or extracellular matrix, including cell-to-cell attachment properties, in response to H$_2$O$_2$. However, the response of secreted/membrane-bound proteins observed for the three AOA strains investigated here could also
represent a result of the renewal of damaged proteins in close proximity to the \( \text{H}_2\text{O}_2 \) production site (further discussed in Text S1 in the supplemental material).

Furthermore, a putative membrane-bound copper transport protein was detected at significantly higher relative abundance in the proteomes of the three \textit{Nitrosopumilus} strains grown under conditions of exposure to \( \text{H}_2\text{O}_2 \) than in the proteomes derived from cultures containing catalase or \textit{O. alexandrii} (Fig. 3). The N-terminal side of this protein exhibits homology with CopC family proteins, which are periplasmic copper binding proteins suggested to primarily play a role in bacterial copper homeostasis (64). \textit{E. coli} cells containing excess copper were shown to be less sensitive to \( \text{H}_2\text{O}_2 \)-induced DNA damage (65). Furthermore, reactions of Cu(I) and Cu(II) with \( \text{H}_2\text{O}_2 \) have recently been suggested to be involved in the formation of Cu(III) and O\(_2^-\), respectively, instead of \( \text{OH}^- \) (66). Hence, while classical Fenton reactions might induce oxidative damage and inactivation of iron-containing enzymes (67), copper import could represent a strategy to reduce or even prevent damage to macromolecules induced by \( \text{OH}^- \). Nevertheless, the function of this protein in AOA remains to be confirmed and requires further investigations.

**Metabolic interactions between \textit{Nitrosopumilus} and \textit{Oceanicaulis alexandrii}**.

The peak concentrations of \( \text{H}_2\text{O}_2 \) in cocultures of the heterotrophic alphaproteobacterium \textit{Oceanicaulis alexandrii} with \textit{Nitrosopumilus} strains were on average 2 to 3 times lower than those measured in axenic \textit{Nitrosopumilus} cultures (Fig. 1D to F), suggesting that \textit{O. alexandrii} is capable of reducing the \( \text{H}_2\text{O}_2 \) concentration in AOA cultures. The initial description of \textit{O. alexandrii} noted that strains of this bacterium are catalase positive (68), and, accordingly, the genome of the type strain \textit{O. alexandrii} DSM 11625\(^T\) encodes two homologs of heme-containing catalase peroxidases belonging to the class I catalases (69). Amino acid residues forming the catalytic site of biochemically characterized heme-containing catalases are conserved in catalase homologs of \textit{O. alexandrii} (Data Set S2B), suggesting that these homologs are bona fide catalases. One of these two homologs is characterized by an N-terminal signal peptide (Data Set S2B), suggesting that it is addressed to the periplasm and might be further secreted into the culture medium. Additionally, we detected proteotypic peptides of these catalase homologs in protein extracts prepared from cocultures of \textit{O. alexandrii} and \textit{Nitrosopumilus} spp., further suggesting that reduced concentrations of \( \text{H}_2\text{O}_2 \) in cocultures of \textit{O. alexandrii} and AOA could result from \textit{O. alexandrii} catalase activity.

\textit{O. alexandrii} was able to grow in coculture with all three \textit{Nitrosopumilus} strains, as well as in the supernatant of \textit{Nitrosopumilus} cultures (Fig. S4A; see also Table S1). RNA stable isotope probing (RNA-SIP) was used to directly confirm the transfer of organic carbon from autotrophic \textit{Nitrosopumilus} cells to \textit{O. alexandrii}. After two consecutive passages in medium containing \(^{13}\text{C}\)-labeled bicarbonate, 16S rRNA sequences of \textit{N. piranensis} showed a clear enrichment in the “heavy” (\(^{13}\text{C}\)) fraction (approximately 25\% of the RNA was labeled) relative to control incubations (Fig. S5). When \textit{O. alexandrii} was subsequently grown in coculture with \textit{N. piranensis} cells or in \textit{N. piranensis} culture supernatant, 16S rRNA sequences of \textit{O. alexandrii} showed an enrichment in the “heavy” (\(^{13}\text{C}\)) fraction (approximately 17\% in coculture and 8\% on supernatant) compared to the control incubations (Fig. 4). Furthermore, no incorporation of \(^{13}\text{C}\) directly from bicarbonate via anaplerotic reactions was observed in the control treatment (i.e., \textit{O. alexandrii} cells growing in yeast extract-peptone medium supplemented with \(^{13}\text{C}\)-labeled bicarbonate) (Fig. 4).

While the \(^{13}\text{C}\) enrichment clearly confirms the incorporation of \textit{Nitrosopumilus}-derived organic carbon into the biomass of \textit{O. alexandrii}, these results do not clarify the identity of organic carbon compounds that mediate the metabolic interaction between AOA and \textit{O. alexandrii}. The alphaproteobacterium generally showed the highest growth rates during exponential growth of the AOA strains (Table S1; see also Fig. S4B), indicating growth on compounds released by active \textit{Nitrosopumilus} cells rather than subsistence on dead cell material. We showed in another study that all investigated strains indeed released organic matter, including labile compounds such as amino acids and thymidine (70). However, the possibility of proteolytic growth on \textit{Nitrosopumilus}
cells cannot be completely excluded. Nevertheless, growth on released, soluble substances is also supported by the ability of *O. alexandrii* to grow on *Nitrosopumilus* culture supernatant, suggesting a rather unspecific interaction. And yet, *O. alexandrii* cells often appeared to be attached to *Nitrosopumilus* cells during growth in coculture (Fig. S3C and D). The highest growth yields of *O. alexandrii* were observed in *Nitrosopumilus* cultures with added catalase (Table S1), suggesting either a higher level of release of organic matter by *Nitrosopumilus* under optimal conditions or growth of *O. alexandrii* on the purified catalase itself. Alternatively, the presence of a purified catalase could provide a growth advantage to *O. alexandrii* by reducing the amount of catalase it needs to produce. Furthermore, *O. alexandrii* achieved higher cell abundances in coculture with *N. piranensis* (composing up to 25% of the cells during the incubation period, Table S1) than were seen with *N. adriaticus* or *N. maritimus*. Hence, the levels of quantity and/or quality of organic carbon released by different *Nitrosopumilus* strains potentially differ under the same culture conditions.

**Conclusions.** Our results, combined with the results of previous studies, suggest that sensitivity to H$_2$O$_2$ is common among different members of the *Nitrosopumilus* genus and contribute to the understanding of the physiological and molecular responses of AOA to H$_2$O$_2$. The extent of the sensitivities of marine AOA to H$_2$O$_2$ appears to differ between strains, which may lead to niche differentiation.

The absence and/or loss of a specific function (i.e., H$_2$O$_2$ detoxification) has been suggested to provide a selective advantage by conserving an organism’s limiting resources (71). The ocean’s most abundant free-living prokaryotes, including *Prochlorococcus*, “Candidatus Pelagibacter” (SAR11 clade), and AOA, can grow axenically only when such missing metabolic functions are provided (14, 33, 72). Our results suggest that marine AOA rely on H$_2$O$_2$ detoxification during periods of high activity and release organic compounds, thereby attracting heterotrophic prokaryotes that provide the missing catalase function.

Interactions between *Nitrosopumilus* spp. and the alphaproteobacterium *O. alexandrii* are reminiscent of interactions between heterotrophic bacteria and phytoplankton cells (i.e., within the “phycosphere”), and the importance of these microscale interactions for aquatic ecosystems is widely acknowledged (73). Similarly, metabolic interactions within the immediate surroundings of AOA cells might represent a successful ecological strategy for heterotrophic bacteria, especially in locations below the euphotic layer of the ocean. Microbial radiocarbon signatures indicate that chemolithoautotrophic production can supply up to 95% of the organic carbon incorporated by free-living microbial communities in mesopelagic waters (74). AOA are the most abundant chemolithoautotrophic microbes in the global ocean, suggesting that they could play a crucial role in the production of reduced carbon compounds from

**FIG 4** Proportion of *O. alexandrii* 16S rRNA gene copies recovered from RNA-SIP gradient fractions. *O. alexandrii* was grown in coculture with *N. piranensis* in medium containing 13C-labeled bicarbonate (solid black line) and on *N. piranensis* supernatant (dashed black line). Growth in medium containing unlabeled yeast extract-peptone and 13C-labeled bicarbonate served as a control (solid gray line).
inorganic carbon and therefore in the provision of labile organic matter for heterotrophic prokaryotes.

MATERIALS AND METHODS

Cultivation procedures and H2O2 sensitivity experiments. Axenic cultures of *Nitrosopumilus adriaticus* NIFS, *Nitrosopumilus piranensis* D3C, and *Nitrosopumilus maritimus* SCM1 were routinely grown in synthetic *Crenarchaeota* medium (SCM) in the dark as previously described (27, 75) with the addition of catalase (Sigma catalog no. C1345) (5 units ml⁻¹ final concentration). Cultures were maintained in 30-ml polypolyene plastic bottles, and growth was monitored via flow cytometry (described in Text S1 in the supplemental material) and by measuring nitrite production levels (76).

Prior to establishing different culture treatments, *Nitrosopumilus* cultures were grown without the addition of catalase for one passage (initial cell abundances in the preculture were ≈7 × 10⁸ ml⁻¹) to ensure exclusion of the remaining catalase activity and culture carryover to the culture medium. Subsequently, each AOA strain was grown under four distinct sets of conditions and triplicate cultures were prepared for each growth condition and strain. The tested growth conditions were as follows: (i) no H2O2 scavenger, (ii) supplementation with catalase, (iii) inoculation with *Oceanicaulis alexandrii*, and (iv) both inoculation with *O. alexandrii* and supplementation with catalase. To establish cocultures, *O. alexandrii* was grown in SCM medium with 0.01% yeast extract-peptone and cells were harvested via centrifugation (10,000 × g, 10°C, 15 min) after 3 days, washed three times with SCM culture medium, and added to freshly inoculated *Nitrosopumilus* cultures (5% *O. alexandrii* and 95% *Nitrosopumilus* spp. [based on cell abundance measurements]). Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) was performed on cocultures to differentiate between bacterial cell abundance and archaeal cell abundance (described in Text S1 in the supplemental material).

Furthermore, the effect of the AOA inoculum size on their cellular response to H2O2 was tested by establishing duplicate cultures of *Nitrosopumilus* spp. with various initial cell abundances (−2 × 10⁷, −8 × 10⁷, −3 × 10⁸, and 7 × 10⁹ cells ml⁻¹) without catalase addition. H2O2 concentrations were measured with a fluorescence-based assay (Sigma-Aldrich, catalog no. MAK165) according to the manufacturer’s protocol.

Proteomics and differential protein expression analysis. Triplicate cultures of *Nitrosopumilus* spp. were grown in 250-ml Schott bottles for each of the following treatments: with addition of catalase, in coculture with *Oceanicaulis alexandrii*, without catalase and *O. alexandrii* (H2O2 inhibited), and without catalase and *O. alexandrii* at a high (7 × 10⁹ cells ml⁻¹) initial cell abundance (H2O2 noninhibited). Culture conditions were established as described in the section above. Cells were harvested during exponential growth via centrifugation (18,500 × g, 4°C, 1.5 h), and cell pellets were immediately frozen at −80°C until whole-cell protein extraction was performed.

Proteins were extracted from cell pellets and subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) followed by overnight trypsin in-gel digestion (described in detail in Text S1 in the supplemental material). Desalted peptides were resuspended in an aqueous solution containing 2% acetonitrile and 0.1% formic acid to a concentration of 0.2 μg μl⁻¹ (1 μg total) prior to loading onto an Easy-spray column (Thermo Fisher Scientific PepMap RSL) (C18; 500 mm by 75 m; pore size of 2.0 μm). Peptides were separated during a 270-min gradient step using a flow rate of 300 nl min⁻¹ and a one-dimensional (1D) nano-LC instrument (Dionex UltiMate 3000; Thermo Fisher Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) (see Text S1 in the supplemental material). Each of the 36 protein extracts was analyzed twice (resulting in a total of 72 proteomic profiles), and the two technical replicates were combined for bioinformatic analysis (resulting in 36 combined proteomes). Acquired MS/MS spectra were analyzed using the SEQUEST-HT algorithm implemented in Proteome Discoverer 2.2 software (Thermo Fisher Scientific), and spectra were searched against the entire set of translated coding sequences of *Nitrosopumilus adriaticus* NIFS (2627854092), *Nitrosopumilus piranensis* D3C (2627853696), and *Nitrosopumilus maritimus* SCM1 (641228499), downloaded from the Integrated Microbial Genomes (IMG) database (77). Protein matches were accepted if they were identified by at least one unique peptide and with high confidence (details can be found in Text S1 in the supplemental material), and proteins were quantified using the normalized spectral abundance factor (NSAF) approach (78) as follows:

\[
NSAF_k = \left( \frac{PSM_k}{L} \right) \left( \sum_{i=1}^{N} \left( \frac{PSM_i}{L} \right) \right)^{-1}
\]

where the total number of spectral counts for the matching peptides from protein k (PSM) was divided by the protein length (L) and then divided by the sum of PSM/L for all N proteins.

In order to adequately match genes shared by the three *Nitrosopumilus* strains and subsequently compare their individual proteomic responses, orthologous groups (OGs) were constructed on the basis of their entire set of coding sequences using OrthoFinder (version 1.0.8) with standard settings (79). The complete list of assigned OGs and their annotations can be found in Data Set S1A and B in the supplemental material. Differential levels of expression of proteins recovered for each strain and treatment were tested with the DESeq2 Bioconductor package (version 1.20.0) (80) in the R software environment (version 3.5.0) using default parameters and spectral counts as input data based on the recommendations of Langley and Mayr (81). All possible (i.e., all six) pairwise comparisons between the four different treatments, (i.e., with catalase, with O. alexandrii, H2O2 inhibited, and H2O2 noninhibited) were performed separately for each *Nitrosopumilus* strain. Each test included three biological replicates per treatment, with the exception of the *N. maritimus* SCM1 "H2O2 inhibited" treatment, where one
biological replicate was excluded from all analyses because of apparent problems during MS analyses that resulted in poor identification of the proteins. Probability values (P values) were adjusted using the Benjamini-Hochberg correction method as previously described (80, 81). The following filter criteria were applied in DESeq2: adjusted P value, < 0.05; log 2-fold difference between treatments: greater than or equal to 2 and less than or equal to −2; mean of normalized counts, ≥3. Proteins that showed significant pairwise correlations were visualized with the heatmap package (version 1.0.12) (82) in the R software environment (83). Columns and rows were clustered based on Euclidean distances corresponding to differences between treatments and relative protein abundances, respectively, as implemented in the heatmap package. Curation of the annotations of proteins showing significant changes in relative abundance was performed by sequence similarity searches using BLAST (84) and the RefSeq (release 92) and UniprotKB/Swissprot (release 2019_01) databases (85, 86), and protein domain searches were performed using InterProScan (release 72.0) (87). Signal peptides were identified with PRED-Signal (88) and SignalP 5.0 (89) to determine if proteins were potentially targeted to the membrane and/or released to the (pseudo) periplasmic space, and additional homology modeling of proteins and functional predictions were carried out with Phyre2 (90).

13C-RNA-stable isotope probing (13C-RNA-SIP). The three AOA strains investigated in this study were grown in SCM medium containing 13C-labeled bicarbonate (2 mM final concentration) for two consecutive passages, each lasting for 5 to 7 days. Subsequently, *O. alexandrii* was grown with each AOA in separate cocultures and axenically on the culture supernatant of the three AOA strains. Cocultures were established as described above in the cultivation procedure section. Culture supernatants were obtained via centrifugation (10,000 x g, 10°C, 30 min) and gentle serial filtration through 0.2-µm-pore-size filters (Durapore, Millipore; 47 mm) and 0.1-µm-pore-size filters (Durapore, Millipore; 33 mm). Cells of *O. alexandrii* grown in yeast extract-peptone medium containing 13C-labeled bicarbonate (2 mM) served as a control to evaluate potential labeling of *O. alexandrii* RNA via anaplerotic reactions. After 5 days of incubation, cultures were harvested by filtration through 0.2-µm-pore-size polycarbonate filters (Millipore; 47 mm) which were immediately frozen at −80°C. RNA was extracted according to the protocol of Angel (91) with some modifications for the use of filters (described in detail in Text S1 in the supplemental material), and samples of late-exponential-phase cultures of *N. piranensis* were selected for tracing the incorporation of AOA-derived organic matter into *O. alexandrii* 16S rRNA.

Subsequently, heavy (13C-labeled) RNA was separated from light (natural 13C/12C isotope ratio) RNA by isopycnic centrifugation. Approximately 300 ng of RNA was mixed with cesium trifluoroacetate (CsTFA; GE Healthcare), HiDi formamide (Thermo Fisher Scientific), and gradient buffer (0.1 M Tris-HCl [pH 8.0], 0.1 M KCl, 1 mM EDTA) as described previously (92). Samples were centrifuged at 130,000 x g at 20°C for at least 65 h in an Optima L-100 XP ultracentrifuge with a VTi 90 rotor (Beckman Coulter), and the resulting CsTFA density gradients were fractionated into 20 equal (250-µl) fractions. Fractions accounting for densities ranging between 1.760 and 1.875 g ml−1 were used for downstream analysis. RNA was precipitated at −80°C after addition of 2.5 volumes of 100% ethanol, 0.5 volumes of 5 M NH4-acetate, and 2 µl glycerol (molecular biology grade; Thermo Fisher Scientific) and pelleted by centrifugation at 20,000 x g for 30 min at 4°C. The RNA pellets were washed with ice-cold 75% ethanol, air-dried, and subsequently resuspended in 10 µl RNA storage solution (Ambion). cDNA was synthesized using SuperScript III reverse transcriptase and random hexamer primers (both from Thermo Fisher Scientific) according to the manufacturer’s protocol. 16S rRNA copies from individual SIP fraction were quantified by quantitative PCR (qPCR) (Bio-Rad) (see Text S1 in the supplemental material), and results are expressed as a proportion of the total number of 16S rRNA copies from all SIP fractions.

Data availability. All acquired raw spectrum files and proteomic result files, including identified peptides, relative protein abundances, and DESeq outputs, are available on MassIVE (https://massive.ucsd.edu) under accession number MSV000083517 (ftp://massive.ucsd.edu/MSV000083517).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/msystems.00181-19.

TEXT S1, DOCX file, 0.1 MB.

FIG S1, PDF file, 0.2 MB.

FIG S2, PDF file, 0.8 MB.

FIG S3, PDF file, 2.6 MB.

FIG S4, PDF file, 0.2 MB.

FIG S5, PDF file, 0.2 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.01 MB.

DATASET S1, XLSX file, 0.1 MB.

DATASET S2, DOCX file, 0.2 MB.

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We declare that we have no conflict of interest.

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