CARBON CYCLE

Major role of nitrite-oxidizing bacteria in dark ocean carbon fixation

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Carbon fixation by chemoautotrophic microorganisms in the dark ocean has a major impact on global carbon cycling and ecological relationships in the ocean's interior, but the relevant taxa and energy sources remain enigmatic. We show evidence that nitrite-oxidizing bacteria affiliated with the Nitrospinae phylum are important in dark ocean chemoautotrophy. Single-cell genomics and community metagenomics revealed that Nitrospinae are the most abundant and globally distributed nitrite-oxidizing bacteria in the ocean. Metaproteomics and metatranscriptomics analyses suggest that nitrite oxidation is the main pathway of energy production in Nitrospinae. Microautoradiography, linked with catalyzed reporter deposition fluorescence in situ hybridization, indicated that Nitrospinae fix 15 to 45% of inorganic carbon in the mesopelagic western North Atlantic. Nitrite oxidation may have a greater impact on the carbon cycle than previously assumed.

Ocean water below the sunlit surface layer (i.e., the dark ocean) constitutes 90% of ocean volume and harbors one of the largest microbiomes on Earth (*1*). These microorganisms have a major impact on global carbon cycling, not only through the remineralization of organic material produced by phytoplankton in sunlit surface waters, but also through the chemoautotrophic fixation of inorganic carbon, as reported in several recent studies (*2*, *3*). Marine group I (MG I) Thaumarchaeota, which perform ammonium oxidation to nitrite, are the most abundant among known chemoautotrophs in the dark ocean (*4*–*6*). However, ammonium oxidation alone is insufficient to support measured carbon fixation rates (*3*). Unknown lineages of bacteria have been found to assimilate inorganic carbon at a faster rate than Thaumarchaeota in the North Atlantic (*7*). An increasing body of evidence suggests that ubiquitous dark ocean Proteobacteria lineages SAR324, SUP05, ARTIC96BD-19, Agg47, and some Oceanospirillales can fix inorganic carbon, using energy from the oxidation of reduced sulfur compounds and other substrates (*8*–*11*). However, all these lineages have genetic signatures for diversified metabolic strategies, including heterotrophy, and their actual contributions to dark ocean autotrophy have not been quantified.

The nitrite-oxidizing bacteria (NOB) constitute a polyphyletic group of potential chemoautotrophs that remain poorly understood in the marine environment. The known marine NOB include a few cultured isolates from the phyla Nitrospinae (former order of Deltaproteobacteria) and Nitrospirae, as well as the genera *Nitrobacter* (Alphaproteobacteria) and *Nitrococcus* (Gammaproteobacteria) (*12*, *13*). Previous field studies indicate that Nitrospinae are abundant in some parts of the dark ocean [e.g., (*14*–*16*)] and that their abundance correlates with nitrite oxidation rates (*15*).

Here, we performed a systematic analysis of the global diversity, abundance, and metabolic potential of dark ocean NOB by combining single-cell genomics and community omics research tools, and we estimated the contribution of this group to the dark carbon fixation. Using polymerase chain reaction of the 16*S* ribosomal RNA (rRNA) gene, we screened 3463 single amplified genomes (SAGs) of bacteria and archaea from 39 samples representing most major oxygenated water masses of the dark ocean, and two samples from a seasonally oxygen-depleted coastal system, Saanich Inlet, British Columbia (table S1). Ninety-eight SAGs were classified as Nitrospinae and four as Nitrospirae; *Nitrococcus* and *Nitrobacter* were not detected. All Nitrospinae SAGs were divergent from the only cultured Nitrospinae isolate for which genomic information is available, *Nitrospina gracilis* (*17*) (Fig. 1 and fig. S1). Of the 98 total Nitrospinae SAGs, 62 clustered with the recently proposed clade 1 or "*Candidatus* Nitromaritima" (*18*). The remaining 36 SAGs clustered with clade 2, which had no sequenced representatives until this study. The four Nitrospirae SAGs formed a clade separate from the marine isolate *Nitrospira marina* (*19*). To investigate the metabolic potential of marine NOB, we sequenced 30 Nitrospinae and four Nitrospirae SAGs spanning the phylogenetic and geographic breath of the SAG library. The resulting genome assemblies reached up to 90% completeness, with an average of 38% (table S2). We also sequenced 240 other SAGs of dark ocean bacteria and archaea representing diverse taxonomic groups, water masses, and geographic locations (table S3). No nitrite oxidoreductase genes were found in any of these SAGs, further indicating that Nitrospinae, and to a lesser extent Nitrospirae, are the predominant NOB in the ocean.

The NOB made up 4.6% of SAGs on average in the mesopelagic samples and up to 8.5% in the North Atlantic (fig. S2). The bathypelagic samples averaged 2.6%, whereas the relative abundance of NOB dropped to 1.6% in the abyssopelagic SAG libraries. No NOB SAGs were identified in any of the epipelagic and hadopelagic samples (table S1). We then recruited the reads from publicly available metagenomes against NOB genomes, using a >95% nucleotide identity threshold (Fig. 2, table S4, and fig. S3). As shown for other lineages (*8*, *18*), we found that cultivated strains recruit only a small number of reads, or none, and thus are not good representatives of the predominant NOB in the ocean. The recruitment by most NOB SAGs was higher (by a factor of ~10 for Nitrospirae and a factor of ~100 for Nitrospinae) and displayed distinct patterns (Fig. 2). Recruitment by all open-ocean Nitrospinae SAGs demonstrated a cosmopolitan distribution but different depth preferences; SAGs from clade 1.a recruited predominantly from the mesopelagic metagenomes, whereas open-ocean clade 2.a SAGs recruited predominantly from the bathypelagic, abyssopelagic, and hadopelagic metagenomes. The metagenomes from the euphotic layers showed poor or no recruitment, with a few exceptions (clade 2.b SAGs are well represented in metagenomic data sets 108 to 120; table S4). The phylogenetically diverse Nitrospinae from the suboxic Saanich Inlet recruited best from their source environment (metagenomes from Saanich Inlet), suggesting adaptations to low-oxygen environments

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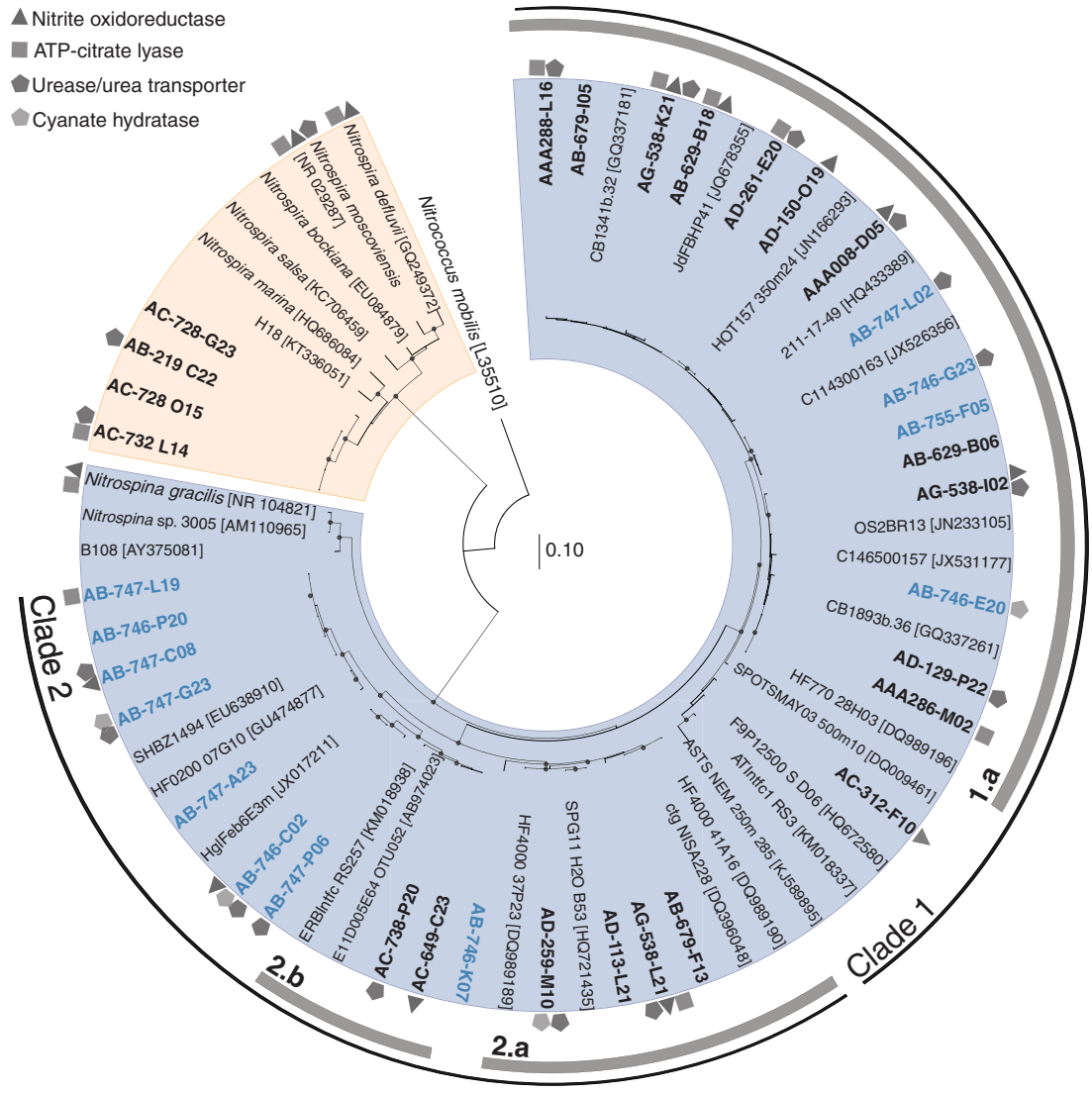

Fig. 1. Phylogenetic tree of Nitrospirae (orange background) and Nitrospinae (blue background) partial 16*S* rRNA gene sequences. Genomically sequenced SAGs from this study (in bold), environmental sequences, and cultivars reported in prior studies are included. Open-ocean oxygenated water column SAGs are indicated in black; Saanich Inlet SAGs are in blue. Nodes with bootstrap support of at least 75% are indicated as solid circles. The symbols on the periphery indicate the presence of the four key genes discussed in the text. The scale bar (center) represents the estimated phylogenetic distance. Because the isolates *Nitrospira bockiana*, *Nitrospira salsa*, and *Nitrospira marina* have not been genomically sequenced, the presence of the four key genes cannot be inferred.

that either evolved independently in multiple lineages or spread horizontally among lineages. Metagenomic fragment recruitment by the four Nitrospirae SAGs indicated a patchy geographic distribution with no obvious spatial or vertical pattern. Using metagenomic fragment recruitment on SAGs, Swan *et al.* (*8*) observed strong latitudinal patterns in the biogeography of bacterioplankton in the surface ocean. By contrast, our study, which uses similar tools, indicates no latitudinal patterns in the global distribution of dark ocean NOB. A plausible explanation is the lack of major latitudinal differences in water temperature below the epipelagic layer. Instead, dark ocean NOB display vertical biogeography that is likely driven by differential adaptations to nutrient and oxygen availability and hydrostatic pressure.

Partial genome assemblies of multiple Nitrospinae and Nitrospirae SAGs contained genes encoding adenosine triphosphate (ATP) citrate lyase and other proteins indicative of the capacity for carbon fixation through the reductive tricarboxylic acid cycle (rTCA) (Fig. 1). By contrast, carbohydrate transporter genes were not recovered in any of these genomes, providing no evidence for heterotrophy. Genes for glyconeogenesis and glycolysis were detected (table S5), but they may be involved in the production and utilization of glycogen, a known storage compound in NOB (*12*). These genomic findings agree with prior experimental work on cultured NOB from wastewater treatment plants, which showed that organic substrates supported neither mixotrophic nor heterotrophic growth of *Nitrospina gracilis* and *Nitrospira moscoviensis* (*12*, *20*). If confirmed by further field studies, the finding of marine Nitrospinae and Nitrospirae being obligate chemolithotrophs would set them apart from the growing number of marine bacterial and archaeal lineages recently reported to encode mixotrophic metabolisms (*2*, *21*).

In agreement with studies of cultured Nitrospinae and Nitrospirae (*17*, *22*, *23*), we detected nitrite oxidoreductase genes, indicative of the potential for energy production through nitrite oxidation (Fig. 1), in Nitrospinae and Nitrospirae SAGs. Ten marine NOB SAGs contained a single operon encoding the three subunits of the key enzyme, nitrite oxidoreductase (NxrABC), with additional copies of NxrC located separately (fig. S4). The phylogeny of the NxrA gene indicates that marine NOB possess a high-affinity type of this enzyme (fig. S5). Experimental evidence has shown that NOB carrying the high-affinity NxrA are repressed by >20 mM nitrite and can only be cultivated in media with low nitrite concentrations (*24*). This may provide an advantage in the generally oligotrophic marine environment and may explain the numeric predominance of Nitrospinae over *Nitrococcus* and *Nitrobacter*; the latter were found to encode the low-affinity NxrA and to require higher nitrite concentrations (*18*, *24*). We found no genomic evidence for other energy production pathways in any of the sequenced NOB SAGs, such as ammonium oxidation, hydrogen oxidation (observed in *Nitrospira moscoviensis*), or oxidation of sulfur compounds. Metaproteomic analyses of microbial communities from the North and South Atlantic revealed that subunits of Nxr were among the most abundant proteins that could be attributed to NOB (fig. S6 and

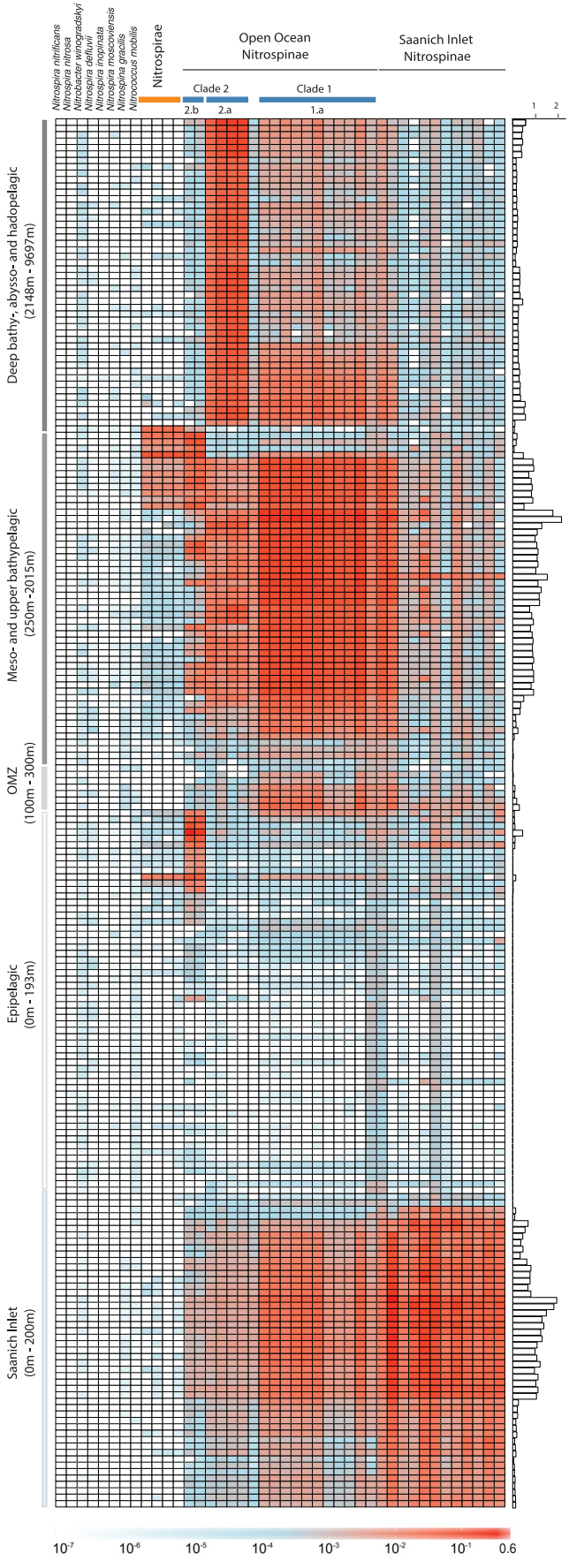

Fig. 2. Global distribution of NOB SAGs and cultivars, as determined by metagenomic fragment recruitment. Reference genomes (SAGs and cultivars) are listed along the *x* axis (order and sources in table S2); metagenomes are listed along the *y* axis (order and sources in table S4). The color scale indicates the proportion (%) of metagenome bases recruited per megabase of each reference genome. Percentages of aligned bases from each metagenome to all SAGs are presented as a bar plot on the *y* axis.

table S6). A similar finding has been observed in the suboxic waters of Saanich Inlet (*25*). Likewise, metatranscriptomic analyses showed that Nxr subunits are among the most highly expressed transcripts in a North Atlantic sample from 2000 m depth, as well as in publicly available metatranscriptomes from the eastern Mediterranean, Gulf of Dolce, and subarctic Pacific Ocean (table S7). Collectively, this evidence suggests strict specialization among the predominant marine NOB in nitrite oxidation as the sole energy source.

Several marine Nitrospinae and Nitrospirae SAGs contained genes for urease and cyanate hydratase/lyase (Fig. 1), which respectively convert urea and cyanate to ammonium and CO_2. Ureases have been previously reported in nonmarine Nitrospirae, whereas cyanate hydratase/lyase genes are present in sequenced NOB cultures (*22*, *23*). The urease operon is highly conserved among Nitrospinae SAGs and includes transporters for urea, branched amino acids, and oligopetides, suggesting co-regulation (fig. S7). The synteny of the operon and the phylogeny of the large urease subunit suggest horizontal acquisition by Nitrospinae from Gammaproteobacteria, whereas the Nitrospirae UreC is deeply branching (fig. S8). Interestingly, the Nitrospinae cyanase genes cluster with cyanases of eukaryotic origin (fig. S9). The fact that the two predominant marine NOB lineages acquired and maintained mechanisms for urea utilization independently is an example of convergent evolution and an indication of the importance of urea utilization for marine NOB. Our finding of ureases and urea transporters in predominant NOB lineages suggests that reciprocal feeding on urea between NOB and ammonium oxidizing MG I Thaumarchaeota (Fig. 3A) may be feasible in the dark ocean, although urease genes have also been found in SAGs of some marine Thaumarchaeota (*26*). On the basis of the finding of genes that encode cyanate hydratase/lyase and transporters for amino acids and oligopeptides in NOB, we speculate that similar processes may involve other nitrogen-rich substrates. Cyanate concentrations are comparable to those of ammonium in oligotrophic marine environments (*27*), and all marine Thaumarchaeota analyzed to date lack cyanate hydratase/lyase genes. Thus, reciprocal feeding on diverse substrates may be an overlooked component of the marine nitrogen cycle and deserves further investigation.

Our bulk dissolved inorganic carbon (DIC) assimilation experiments demonstrated dark chemoautotrophy rates of 0.03 to 10.37 μmol C m^{-3} day^{-1} (table S8), in agreement with prior studies in the eastern and subtropical North Atlantic (*3*). To estimate the specific contribution of NOB to dark carbon fixation, we incubated samples from six stations (Fig. 4A) with radiolabeled DIC ($DI^{14}C$), followed by microautoradiography linked with catalyzed reporter deposition fluorescence in situ hybridization (MAR-CARD-FISH), targeting Nitrospinae. We found that Nitrospinae constituted 14 to 35% of cells fixing $DI^{14}C$ in the mesopelagic (Fig. 4B). Earlier studies have shown that the

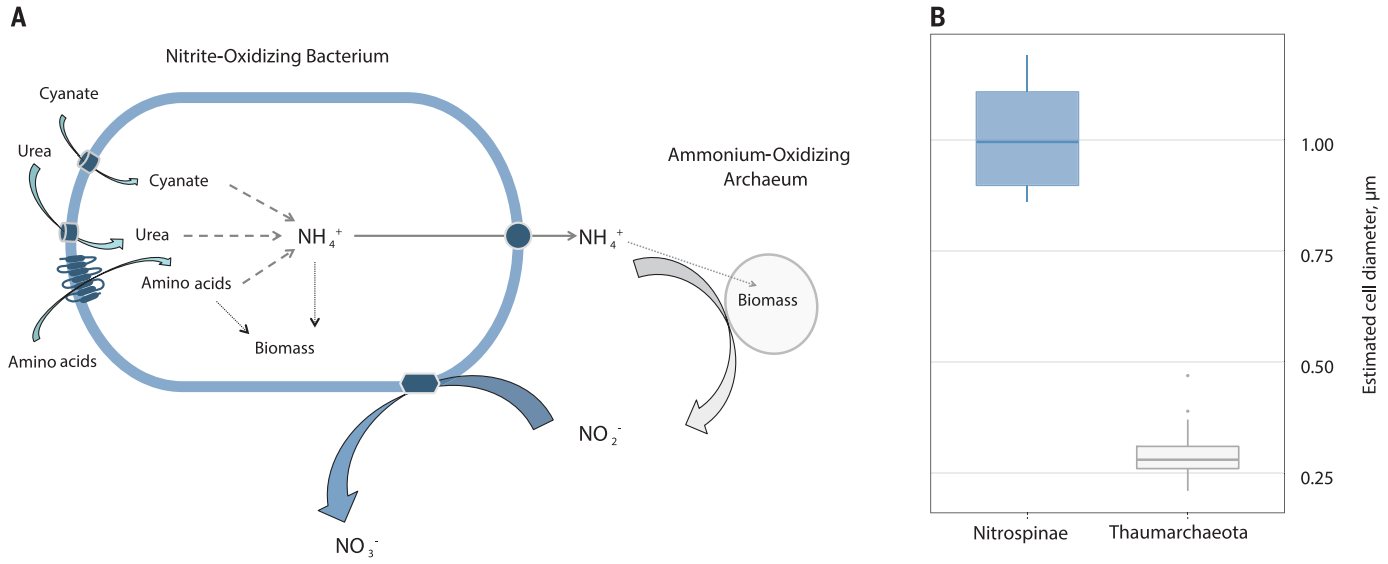

Fig. 3. Potential interaction between nitrite-oxidizing bacteria and ammonium-oxidizing archaea. (**A**) Schematic illustration of the hypothesized reciprocal feeding between marine nitrite-oxidizing bacteria and ammonium-oxidizing archaea. (**B**) Cell diameter of Nitrospinae (four cells) and Thaumarchaeota (58 cells) estimated from calibrated-index cell sorting of SAG microplate AG-538 (table S1).

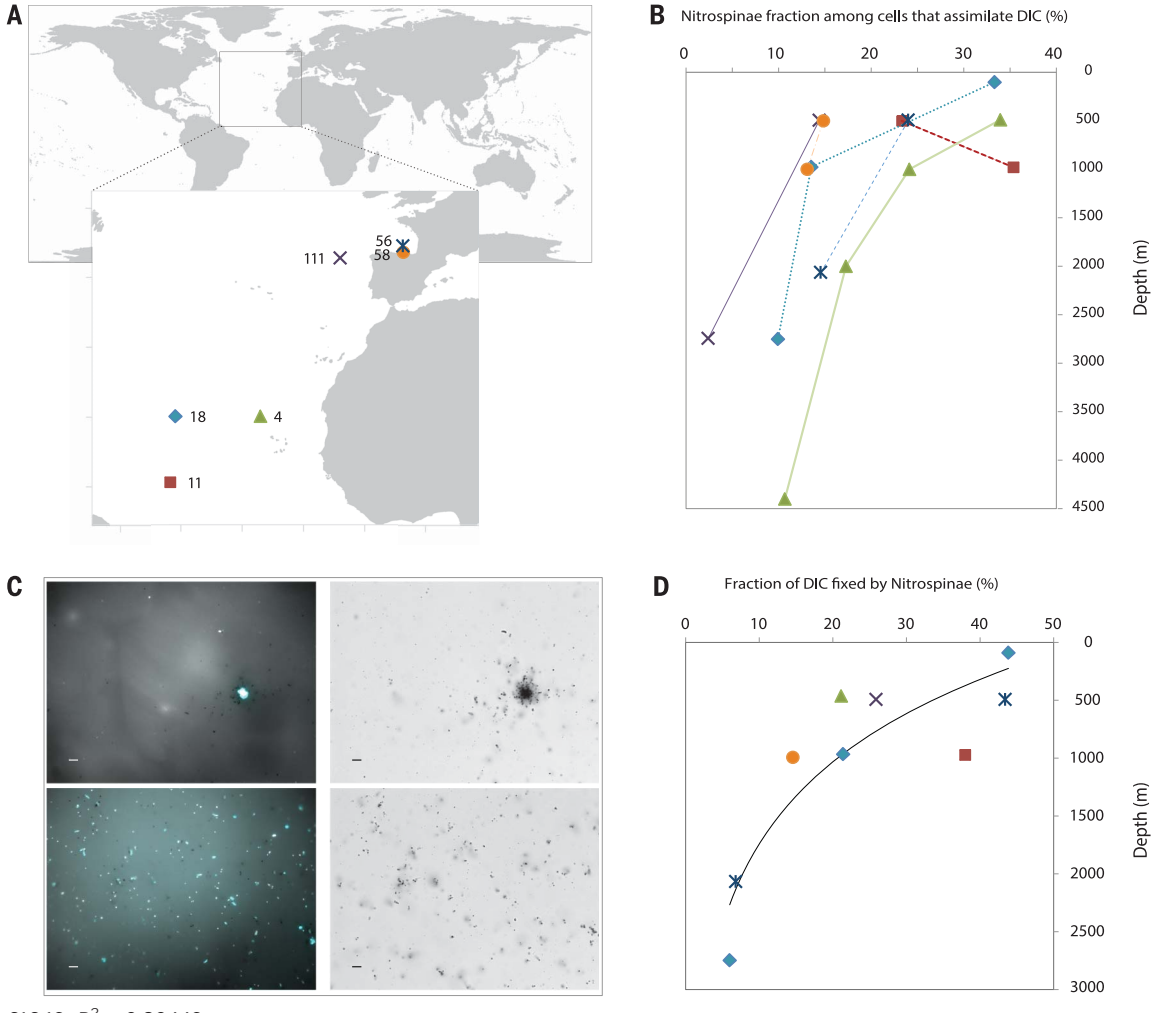

Fig. 4. Aphotic bicarbonate assimilation by Nitrospinae in field experiments. (**A**) Locations of sample collection sites. (**B**) Fraction of Nitrospinae among cells that assimilate labeled bicarbonate. (**C**) Images on the left are overlays of transmitted light and epifluorescence micrographs showing Nitrospinae cells labeled by CARD-FISH (cyan), other cells labeled with 4′,6-diamidino-2-phenylindole (white), and silver grains indicating bicarbonate uptake (dark gray spots); images on the right are single-layer transmitted-light micrographs for better visibility of the silver grains. Scale bars, 5 µm. (**D**) Estimated contribution of Nitrospinae to the aphotic bicarbonate assimilation in the studied samples. The best fitted curve is plotted: $y = -1092 \ln(x) - 616.19$; $R^2 = 0.80449$.

area of MAR-CARD-FISH silver grains correlates linearly with the ^{14}C fixed by the chemoautotrophic cells (*7*). By using silver grain area (Fig. 4C) as a proxy for cell-specific DI^{14}C fixation rates (*28*), we estimated that Nitrospinae performed 25 to 43%, 15 to 23%, and <10% of the DI^{14}C uptake in the upper mesopelagic, lower mesopelagic, and bathypelagic samples (Fig. 4D). The Nitrospinae cell-specific DIC fixation rates ranged from 0.002 to 0.734 fmol C cell^{-1} day^{-1}. These rates were up to an order of magnitude higher than DIC fixation rates by ammonium-oxidizing MG I Thaumarchaeota, determined by Varela *et al.* (*7*) using similar techniques and samples from the same geographic region in an earlier study.

Our findings contradict the prevailing assumption that Thaumarchaeota dominate dark ocean chemoautotrophy but are consistent with several prior reports of unidentified groups of bacteria being more important contributors to DIC fixation than archaea in the mesopelagic layer of the North Atlantic (*5*, *7*). The prevailing assumption stems from the numeric predominance of Thaumarchaea in the dark ocean (*5*). However, our measurements of cell sizes in the dark ocean suggest that the biovolume of marine Nitrospinae is larger than the biovolume of Thaumarchaeota by a factor of ~50 (Fig. 3B), in agreement with prior studies of cultured isolates (*12*, *29*). Thus, the cumulative biovolume of NOB and Thaumarchaea may be comparable in some parts of the ocean. Furthermore, although the theoretical energy yield per reaction is greater for ammonium oxidation than for nitrite oxidation by a factor of 3.7 (*30*), Nitrospinae may have a greater metabolic efficiency, owing to their less costly DIC fixation through the rTCA cycle (2 ATP equivalents per pyruvate molecule), as compared with the 3-hydroxypropionate/4-hydroxybutyrate cycle of Thaumarchaeota (6 to 9 ATP equivalents per pyruvate molecule) (*31*). Finally, NOB may have access to complementary sources of nitrite, such as partial denitrification, which was recently identified in SAR11, the most abundant bacterioplankton clade in the ocean (*32*). A contributing factor to the underappreciation of NOB in dark ocean chemoautotrophy to date may be the typically very low concentrations of nitrite, which probably are a result of effective utilization by NOB.

Averaging the mesopelagic DIC fixation rates by Nitrospinae in our incubations and extrapolating this value to the entire volume of the mesopelagic layer gives a rough estimate of ~1 Pg C year^{-1} being fixed by marine Nitrospinae globally. This is similar to prior estimates of the total carbon fixation in the dark ocean, ranging 0.4 to 1.1 Pg C year^{-1} (*4*, *33*), and is only an order of magnitude below the estimated global export production from the epipelagic layer (*34*, *35*). For a comparison, estimates of dark ocean carbon fixation based solely on ammonium oxidation are lower by an order of magnitude (*36*). Although a geographically broader set of experiments is required to refine our estimates, they strongly suggest that NOB, primarily Nitrospinae, have a greater impact on the carbon flux in the ocean's interior than previously considered.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/358/6366/1046/suppl/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 to S8
References (*37*–*66*)

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Dissolved inorganic carbon fixers revealed

Most of the ocean is dark. Yet it is in this darkness, away from photosynthesizing sunlight, that most planetary carbon cycling occurs. Pachiadaki *et al.* show that nitrite-oxidizing bacteria in one phylum are the predominant fixers of dissolved inorganic carbon in the mesopelagic ocean. The authors sequenced thousands of single amplified genomes of marine prokaryotes. They identified more than 30 nitrite-oxidizing obligate chemoautotrophic bacteria that were unable to transport carbohydrate and that expressed nitrite oxidoreductase. This enzyme provides electrons to drive a reverse tricarboxylic acid cycle that fixes the carbon. Many of the genomes also suggest organisms that have the capacity to produce ammonium and other substrates, possibly to feed nitrite-producing metabolic partners.

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