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JT Hollibaugh

HW Ducklow

Virginia Institute of Marine Science

N Bano

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Widespread Distribution in Polar Oceans of a 16S rRNA Gene Sequence with Affinity to *Nitrosospira*-Like Ammonia-Oxidizing Bacteria

James T. Hollibaugh, 1* Nasreen Bano, 1 and Hugh W. Ducklow 2

Department of Marine Sciences, University of Georgia, Athens, Georgia 30602-3636, 1 and Virginia Institute of Marine Sciences, College of William and Mary, Gloucester Point, Virginia 23062-1346 2

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We analyzed the phylogenetic compositions of ammonia-oxidizing bacteria of the β subclass of *Proteobacteria* from 42 Southern Ocean samples. We found a *Nitrosospira*-like 16S rRNA gene sequence in all 20 samples that yielded PCR products (8 of 30 samples from the Ross Sea and 12 of 12 samples from the Palmer Peninsula). We also found this sequence in Arctic Ocean samples, indicating a transpolar, if not global, distribution; however, slight differences between Arctic and Antarctic sequences may be evidence of polar endemism.

Most studies of ammonia-oxidizing bacteria (AOB) have focused on bioreactors, soils, freshwater, estuaries, and coastal sediments. Two groups of chemolithotrophic bacteria (*Nitrosomonas* and *Nitrosospira* spp.) with phylogenetic affinity to the β subclass of the class *Proteobacteria* are the organisms primarily responsible for ammonia oxidation in these environments (19, 29, 34, 39). Relatively little is known about the distribution of AOB in the open ocean, particularly in polar oceans. Since the first isolation of a marine ammonia-oxidizing bacterium, *Nitrosocystis oceanus* (now *Nitrosoccus oceanicus*), from temperate waters by Watson (38), this organism (a member of the γ subclass of the *Proteobacteria*) and *Nitrosomonas* species have been thought to be responsible for ammonia oxidation in the open ocean (32, 35, 37). *Nitrosospira*-like organisms or their rRNA gene (rDNA) sequences, while common in terrestrial and freshwater ecosystems and bioreactors (4, 11, 22), had not been reported to occur in marine environments.

Recently, *Nitrosospira*-like 16S rDNA sequences were detected at a eutrophic coastal site off Scotland (27) and at depths in an area of the northwestern Mediterranean influenced by the Rhone River plume (21). Subsequently, Bano and Hollibaugh (2) detected a closely related 16S rDNA sequence in samples from the Arctic Ocean, a unique oceanic environment. The analyses reported here were conducted to determine whether or not this organism was also present in the Southern Ocean, indicating a transpolar, if not global, distribution. We also wanted to know whether or not Arctic and Antarctic populations exhibited any indication of endemism, which might be expected because of the differences in current environmental conditions in the two oceans and because the polar oceans evolved independently.

Previous investigations of endemism in polar bacteria have focused on heterotrophic assemblages associated with sea ice (reviewed in reference 26) and on cyanobacteria. Gosink and coworkers (9, 10) determined that gas-vacuolate bacteria, a group uniquely adapted to the sea ice habitats they studied, displayed polar endemism. Other groups that they isolated appeared to be more widely distributed, as has been reported for many bacterioplankton (8). Psychrophilic cyanobacteria isolated from Arctic and Antarctic meltwater ponds appeared to be identical, based on 16S rDNA phylogeny, while the phylogeny of psychrotolerant strains was more complex (18). Based on the phylogenetic relationships of polar cyanobacteria, Nadeau and coworkers (18) concluded that these microorganisms originated from more temperate populations. Prior to their isolation by Gosink and coworkers, gas-vacuolate bacteria were known only from freshwater (33), suggesting a more complicated process of radiation for this group because sea ice is a uniquely, and fairly recent (24), polar phenomenon. Chemoautotrophic AOB offer the possibility of gaining another perspective on polar endemism because, unlike for gas-vacuolate bacteria or cyanobacteria, global thermohaline circulation may be an effective dispersal mechanism for AOB.

The samples used in our analysis of Antarctic AOB assemblages were collected from stations in two regions in the Southern Ocean (Table 1): one near the Palmer Peninsula (purified DNA kindly provided by A. E. Murray) and the other along a transect of the Ross Sea. These stations and the sample collection methodologies are described more fully by Murray et al. (16) and Carlson et al. (5); however, it is important to note that Palmer Peninsula samples were filtered through Gelman GF/A glass fiber filters (1.6-μm nominal pore size) to remove phytoplankton prior to the collection of prokaryotic DNAs. DNA extraction, PCR amplification, denaturing gradient gel electrophoresis (DGGE), cloning, sequencing, and phylogenetic analysis were done as described previously (2). Briefly, 16S rDNA fragments of AOB of β subclass Proteobacteria were selectively amplified by PCR (35 cycles) from genomic DNAs extracted from cells collected on filters. To lessen the potential for PCR bias, we did not use nested amplifications (universal primer set followed by an AOB-specific primer set [21, 32]).

For the PCR, we used primers nitA and nitB (forward, 5′-CTTAAGTGGGGAATAGCGATGC-3′, and reverse, 5′-TTACGTGTGAAAGCCTACCCCA-3′, respectively [32]), which yielded a 1.1-kb fragment (nitAB, nominally 1,054 bp, 5′-CTTAAGTGGGGAATAGCGATGC-3′, and reverse, 5′-TTACGTGTGAAAGCCTACCCCA-3′, respectively [32]), which yielded a 1.1-kb fragment (nitAB, nominally 1,054 bp,
excluding primer sites) encompassing Escherichia coli positions (3) 136 to 1233. These primers have been tested extensively
and found to be specific for ammonia oxidizers of β-subclass
 Proteobacteria (22, 30, 37). Nitrosomonas cryotolerans
genomic DNA and filters through which no water was passed served
as the positive and negative PCR controls, respectively.

When nitAB amplicons were obtained, their diversity was
assessed by PCR and DGGE by use of a second, nested am-
plification (30 cycles) used primers 356f (5'-CCTACGGAGGCAGCAG-3')
and 517r (5'-ATTACC CGCCCCCGCCCCGCCCCGCCCCGCCCCGCCG
CGCCCCCGCCCC-3'), while the 517r primer was 5'-labeled
with fluorescein. The products (nominally 233 bp, including primer sites and clamp) were resolved by DGGE, and
the banding pattern was recorded with an FM-BIO II laser-based
gel scanner (Hitachi) set to measure banding pattern was recorded with an FM-BIO II laser-based
gel scanner (Hitachi) set to measure

We obtained an unambiguous sequence by this approach;
however, to further verify that the direct sequence we obtained
was not contaminated, we also cloned the nitA and nitB am-
plicons from a sample (Palmer Station B, 50 m, 1997; we ran
out of the template from the sample used for the direct se-
quence) that gave the same banding pattern (Fig. 1, compare
left and right) suggests that another sequence was present but in low
relative abundance. This band was not found in subsequent
amplifications of the sample.

We obtained an unambiguous sequence by this approach;
however, to further verify that the direct sequence we obtained
was not contaminated, we also cloned the nitA and nitB am-
plicons from a sample (Palmer Station B, 50 m, 1997; we ran
out of the template from the sample used for the direct se-
quence) that gave the same banding pattern (Fig. 1, compare
the 2nd and 4th lanes from left), screened the clone libraries
(by PCR and DGGE; clones were run on the same gel with the
to verify that v3 region fragments amplified from clones
had the same mobility as that of the fragment amplified from
the original sample), and sequenced representative clones us-
ing the Sp6 and T7 plasmid primers. This approach was also used
to obtain sequences from Arctic samples that yielded
more than one major band during PCR and DGGE screening
(2). Finally, short sequences (nominally 161 bp, excluding primer sites) were obtained from major DGGE bands by ex-
cising bands from the gel, eluting the DNA they contained, and
then using this DNA as the template for cycle sequencing with
unmodified 356f and 517r primers. The short sequences were
used to verify the inferred association, based on similar melting
points, between the DGGE bands in the original sample and
DGGE bands from cloned nitAB inserts (Fig. 2).

Sequences were checked for chimeras using the Ribosomal
Database Project’s Check_Chimera program (12) and were
then compared to known sequences with BLAST (1). Phylo-
genetic analyses were conducted by aligning (Genetics Com-
puter Group [Madison, Wis.] package) the 16S rDNA se-
quencies with GenBank sequences with the highest BLAST

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### Table 1. Southern Ocean and Palmer Peninsula sampling dates and locations

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample date (mo/day/yr)</th>
<th>Location</th>
<th>Station</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Sample depth(s) (m)</th>
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</thead>
<tbody>
<tr>
<td>Southern Ocean</td>
<td>12/13/95</td>
<td>Southwestern Pacific Ocean</td>
<td>0</td>
<td>50.0°S</td>
<td>174.0°E</td>
<td>0</td>
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<td>12/17/95</td>
<td>Polar Front</td>
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<td>178.0°E</td>
<td>60</td>
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<tr>
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<td>12/21/95</td>
<td>Ross Sea</td>
<td>3</td>
<td>76.5°S</td>
<td>175°W</td>
<td>0, 50</td>
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<tr>
<td></td>
<td>12/22/95</td>
<td>Ross Sea</td>
<td>5</td>
<td>76.5°S</td>
<td>171°W</td>
<td>0, 50</td>
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<tr>
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<td>12/23/95</td>
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<td>10</td>
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<td>177°E</td>
<td>0, 50</td>
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<td>12/24/95</td>
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<td>177°E</td>
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<td>12/26/95</td>
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<td>76.5°S</td>
<td>177°E</td>
<td>0, 50</td>
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<tr>
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<td>20</td>
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<td>171°E</td>
<td>0, 50</td>
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<tr>
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<td>164°E</td>
<td>0, 50</td>
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<td>76.5°S</td>
<td>164°E</td>
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<td>76.5°S</td>
<td>166°E</td>
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<tr>
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<td></td>
<td>01/07/96</td>
<td>Ross Sea</td>
<td>61</td>
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<td>171°W</td>
<td>0, 50</td>
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<tr>
<td>Coastal waters near</td>
<td>10/1/96</td>
<td>Gerlache Strait</td>
<td>6</td>
<td>64.2°S</td>
<td>61.8°W</td>
<td>5, 50, 125, 250, 1500</td>
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<tr>
<td>Palmer Peninsula</td>
<td>08/01/96</td>
<td>Dallman Bay</td>
<td>A</td>
<td>64.1°S</td>
<td>62.9°W</td>
<td>0, 80, 150</td>
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<tr>
<td></td>
<td>09/13/96</td>
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<td>B</td>
<td>64.8°S</td>
<td>64.1°W</td>
<td>3, 50</td>
</tr>
<tr>
<td></td>
<td>02/04/97</td>
<td>Palmer Station</td>
<td>B</td>
<td>64.8°S</td>
<td>64.1°W</td>
<td>3, 50</td>
</tr>
</tbody>
</table>

*The depths of samples that tested positive for AOB are indicated in boldface.
similarity values. Phylogenetic trees were constructed by using Jukes-Cantor distances and the neighbor-joining method (Phylogenetic Inference Package [PHYLIP], version 3.5; University of Washington, Seattle). Tree robustness was tested by bootstrap analysis (100 replicates). Sequences have been deposited in GenBank under the following accession numbers: AF142412 and AF203524 to AF203526.

We obtained nitAB PCR products from 8 of 30 (27%) Ross Sea samples and 12 of 12 (100%) Palmer Peninsula samples (20 of 42, or 48% of all, Antarctic samples) (Table 1). Most (six of eight) of the nitAB-positive Ross Sea samples were not from the surface layer (Table 1), consistent with our findings for samples from the Arctic Ocean (2). As reported previously (2), we obtained nitAB PCR products from 212 of 287 (74%) Arctic Ocean samples.

We detected AOB in a higher percentage of our samples than Voytek and Ward (32) reported for Southern California Bight waters or Phillips et al. (21) reported for northwest Mediterranean waters, where no AOB were detected unless a nested-amplification procedure was used. Our PCR conditions were similar to theirs. The βAMoF and βAMoR primers used by Phillips et al. (21) have somewhat broader specificities than the nitA and nitB primers we used (30), but we used lower template concentrations and we did not use nested reactions to increase sensitivity. Although mixed-template PCR amplifications are subject to bias (23, 28), the high percentage of

FIG. 1. PCR and DGGE analysis of all Antarctic samples that yielded nitAB products and thus were presumed to be positive for the presence of AOB. Lane designations refer to sampling data in Table 1. Pal B, Palmer Station B; Dal Bay, Dallman Bay; Ross Sta, Ross Station; Ger Str, Gerlache Strait. Two Arctic samples (SCICEX) were included for comparison. Samples were run on three different DGGE gels as indicated by boxes. Lanes marked “Standard” contained amplicons from a mixture of Clostridium perfringens and Bacillus thuringiensis genomic DNAs (Sigma).

FIG. 2. Denaturing gradient gel comparing representative Antarctic and Arctic samples and clones. Lanes: 1, Nitrosomonas cryotolerans; 2, sample from Palmer Station B, 50 m, 4 February 1997; 3, clone A-2 from Palmer Station B, 50 m, 4 February 1997; 4, sample from Palmer Station B, 3 m, 13 September 1996 (this nitAB product was also sequenced directly); 5, sample SCICEX 96B, 55 m, from 83°38′/N, 131°16′E (this nitAB product was also sequenced directly); 6, sample SCICEX 96A, 132 m, from 80°28′/N, 156°53′W; 7, clone 96A-8 from sample SCICEX 96A; 8, sample SCICEX 95B, 55 m, from 72°34′/N, 155°47′/W; 9, clone SCICEX 95B-10 from sample 95B; 10, sample SCICEX 95A, 55 m, from 72°16′/N, 154°24′/W; 11, clone SCICEX 95A-44 from sample SCICEX 95A.
positive samples we detected compared to the percentages detected by Voytek and Ward (32) and Phillips et al. (21) suggests that AOB are relatively more abundant in polar oceans than in the Southern California Bight or the northwestern Mediterranean. In contrast, Ward et al. (37) obtained nitAB products in 12 of 22 direct amplifications of DNAs from samples collected from natural environments (primarily lakes) in Germany, and Stephen et al. (27) detected AOB in all direct amplifications of samples from polluted Scottish coastal sediments using the /H9252AMOf and /H9252AMOr primer set, suggesting comparable relative abundances of AOB at these sites.

The DGGE fingerprints of all Antarctic samples from which we obtained nitAB PCR products are shown in Fig. 1, along with two representative Arctic Ocean samples. All PCR and DGGE fingerprints of nitAB products contained the same major band (shown as band A in Fig. 2); two samples (Palmer Station B, 3 m, 1997 and Palmer Station B, 50 m, 1997) contained additional bands near the top of the gel. Attempts to determine the nucleotide sequences of DNAs in these bands were unsuccessful. Figure 2 compares DGGE banding patterns of fragments amplified from the nitAB products of samples with banding patterns amplified from cloned inserts of nitAB products. The same band was found in both Arctic and Antarctic samples, and all eight of the Antarctic clones we screened (one is shown in Fig. 2) expressed band A, as did the Arctic clones (Fig. 2). A second band that was common in Arctic samples (band B in Fig. 2) and that corresponded to a Nitrosomonas-like sequence (2) was not detected in our Antarctic samples, possibly due to prefiltering.

Band A was the most frequently encountered ribotype (found in 68% of 122 clones) in Arctic Ocean clone libraries (2). It was also the strongest band in DGGE gels containing more than one nitAB product (2). Phillips and coworkers (21) detected Nitrosospira-like sequences in 5 of the 52 AOB clones they screened. They did not find Nitrosospira-like sequences in Mediterranean surface water samples, and they reported that these sequences were associated with free-living bacteria (passing through a 0.8-μm-pore-size filter). Stephen et al. (27) reported finding Nitrosospira-like sequences in 31 of the 40 AOB clones they screened.

Consistent with PCR and DGGE banding patterns, the nitAB fragments amplified from Antarctic samples contain the same 16S rRNA gene sequence. Identical sequences (100% similarity over 1,040 bp) were obtained by directly sequencing nitAB fragments amplified from Antarctic and Arctic samples. The DGGE fingerprints of these two samples are compared in Fig. 2, lanes 4 and 5. Sequences of cloned nitAB fragments were essentially identical, with differences of ≤6 bp among Arctic and Antarctic clone sequences and the direct sequences (Table 2). These minor discrepancies may be artifacts of clon-
FIG. 3. Phylogenetic relationship of sequences from Arctic and Antarctic samples to sequences from closely related AOB from β-subclass Proteobacteria and environmental clones; accession numbers follow the isolate or clone name. Polar sequences are indicated in boldface type, sequences labeled “SCICEX” are from the Arctic, and sequences labeled “Palmer” are from the Antarctic (this study). Directly sequenced nitAB products are indicated by an asterisk (*); the remaining sequences are from clones. The tree was constructed using 1,040 bp of the 16S rDNA sequence. Bootstrap values that were greater than 50 from 100 samplings are shown. The tree is unrooted and was constructed by using E. coli (accession number J01695) as the outgroup. The bar indicates a Jukes-Cantor distance of 0.05.
ing (20, 25); however, they may also represent microdiversity (13) because the substitutions in Arctic sequences are different from those in Antarctic sequences (Table 2). Sequences obtained from DNA in DGGE band A exactly matched the expected sequence from the longer nitAB fragment, indicating that the ubiquitous band A does in fact represent the Nitrosospiro-like sequence obtained from clones. Overall, the Arctic and Antarctic ribotypes of this AOB have sequence similarities of ≥99.3%.

Phylogenetic analysis (Fig. 3) indicates that the Antarctic AOB sequences are distantly related to sequences from isolates in group 1 AOB of Stephen et al. (27). The sequences that we obtained were most closely related to a recently reported environmental sequence (400 FREE Z14) in a clone library generated from a sample collected at a depth of 400 m at a station off the mouth of the Rhone River in the northwestern Mediterranean (21). The next most closely related sequences (EnvB1-17 and EnvC2-23) were obtained from clone libraries generated from sediment samples collected around salmon-rearing pens in Scottish coastal waters (27).

Our sequences differed only slightly from sequences retrieved from the mesopelagic zone of the northwestern Mediterranean, but they differed significantly from sequences retrieved from Scottish coastal sediments (Fig. 3). This was somewhat surprising given that the Scottish coast is in more direct contact with Arctic Ocean water than is the Mediterranean Sea. The Scottish and Mediterranean samples were analyzed by the same research team using the same approach, so differences in the sequences they obtained are unlikely to be due to variability between labs. The Scottish sequences may thus represent a sediment-associated strain of the Nitrosospiro-like organism that is distinct from the pelagic form.

Band B in Fig. 2 corresponds to a Nitrosomonas-like sequence and was common in Arctic samples (2), yet it was not detected in our Antarctic samples. Phillips and coworkers (21) found that similar Nitrosomonas-like sequences were predominately associated with particles. Samples from the Palmer Peninsula region were filtered (Gelman GF/A glass fiber filters, 1.6-μm nominal pore size) to remove phytoplankton before bacteria were collected (16). This filtration step would have removed other particles and particle-associated bacteria, including particle-associated Nitrosomonas-like organisms, if they had been present. The Ross Sea samples are from a deeper, central region of the Southern Ocean, less influenced by coastal water than are the Palmer Peninsula sampling locations. Based on our Arctic Ocean experience, where this sequence seemed to be associated with waters flowing from the eutrophic Chukchi Shelf into the central Arctic Ocean (2), we do not expect to find the Nitrosomonas-like organism in Ross Sea surface waters.

The broad distribution of the Nitrosospira-like ribotype in Arctic and Antarctic samples suggests that it is a ubiquitous and important marine AOB, certainly in cold oceans and possibly globally. These findings do not preclude the presence of Nitrosococcus-like sequences in our samples; however, they were not detected in preliminary screenings of Arctic samples ( Hollibaugh, unpublished) with the NOC1 and NOC2 primer set (31, 36).

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