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

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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 *Nitrosococcus oceani*), 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′-CTTAAGTGGGGAATAAGCGATGC-3′, and reverse, 5′-TTACGTGTGAAGCCCTACCCA-3′, respectively [32]), which yielded a 1.1-kb fragment (*nitAB*, nominally 1,054 bp,

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TABLE 1. Southern Ocean and Palmer Peninsula sampling dates and locations

Region	Sample date (mo/day/yr)	Location	Station	Latitude	Longitude	Sample depth(s) (m)[a]
Southern Ocean	12/13/95	Southwestern Pacific Ocean	0	50.0°S	174.0°E	0
	12/17/95	Polar Front	1	67.0°S	178.0°E	60
	12/21/95	Ross Sea	3	76.5°S	175°W	0, 50
	12/22/95	Ross Sea	5	76.5°S	171°W	0, 50
	12/23/95	Ross Sea	10	76.5°S	177°W	0, **50**
	12/24/95	Ross Sea	14	76.5°S	177°E	0, 50
	12/26/95	Ross Sea	17	76.5°S	177°E	0, 50
	12/27/95	Ross Sea	20	76.5°S	171°E	0, 50
	12/28/95	Ross Sea	23	76.5°S	168°E	0, **50**
	12/29/95	Ross Sea	26	76.5°S	164°E	0, 50
	12/30/95	Ross Sea	28	76.5°S	171°E	0, **50**
	01/03/96	Ross Sea	40	76.5°S	164°E	0, 50
	01/04/96	Ross Sea	44	76.5°S	164°E	0, **50**
	01/05/96	Ross Sea	47	76.5°S	166°E	**0, 50**
	01/06/96	Ross Sea	54	76.5°S	171°E	0, 50
	01/07/96	Ross Sea	61	76.5°S	171°W	**0, 50**
Coastal waters near Palmer Peninsula	10/1/96	Gerlache Strait	6	64.2°S	61.8°W	**5, 50, 125, 250, 1500**
	08/01/96	Dallman Bay	A	64.1°S	62.9°W	**0, 80, 150**
	09/13/96	Palmer Station	B	64.8°S	64.1°W	**3, 50**
	02/04/97	Palmer Station	B	64.8°S	64.1°W	**3, 50**

[a] The depths of samples that tested positive for AOB are indicated in boldface.

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, 30–32, 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 amplification. This amplification (30 cycles) used primers 356f (5′-CCTACGGGAGGCAGCAG-3′) and 517r (5′-ATTACC GCGGCTGCTGG-3′), directed at the variable 3 (v3) region of the bacterial 16S rDNA (14, 15, 17). Primer 356f included a GC clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGC 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 fluorescein fluorescence as described previously (2). DGGE banding patterns for all samples that yielded *nitAB* amplicons are shown in Fig. 1.

A full-length sequence of the *nitAB* amplicon was obtained from the sample from Palmer Station B taken at a 3-m depth in 1996 (Fig. 1, 4th lane from the left side) by cycle sequencing the amplicon with the nitA and nitB primers. We refer to this as “direct sequencing,” since it does not involve cloning the amplicons. This sample was chosen because it yielded more *nitAB* product than other samples did during preliminary investigations, because we had more of it, and because it produced only one major DGGE band, though a number of minor bands were also present. Most of the minor bands were identical to minor bands obtained even when cloned sequences were used as the template (Fig. 2, compare lanes 3, 7, and 9). We suspect for the following reasons that these bands arose as a result of alternate secondary structures of the amplicon in the major band: they contain the same sequence as the major band; they are obtained even when the major band is excised, amplified, and run on a new DGGE gel; they appear in fingerprints amplified from cloned inserts; and they are highly repeatable (6; J. T. Hollibaugh, unpublished data). However, others have shown that similar patterns can arise from heteroduplex formation (7). The faint band at the top of the PCR and DGGE fingerprints for this sample (seen more clearly in the sample from Palmer Station B, 3 m, 1997 [Fig. 1, 3rd lane from left]) 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* amplicons from a sample (Palmer Station B, 50 m, 1997; we ran out of the template from the sample used for the direct sequence) 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 sample 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 using 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 excising 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). Phylogenetic analyses were conducted by aligning (Genetics Computer Group [Madison, Wis.] package) the 16S rDNA sequences with GenBank sequences with the highest BLAST

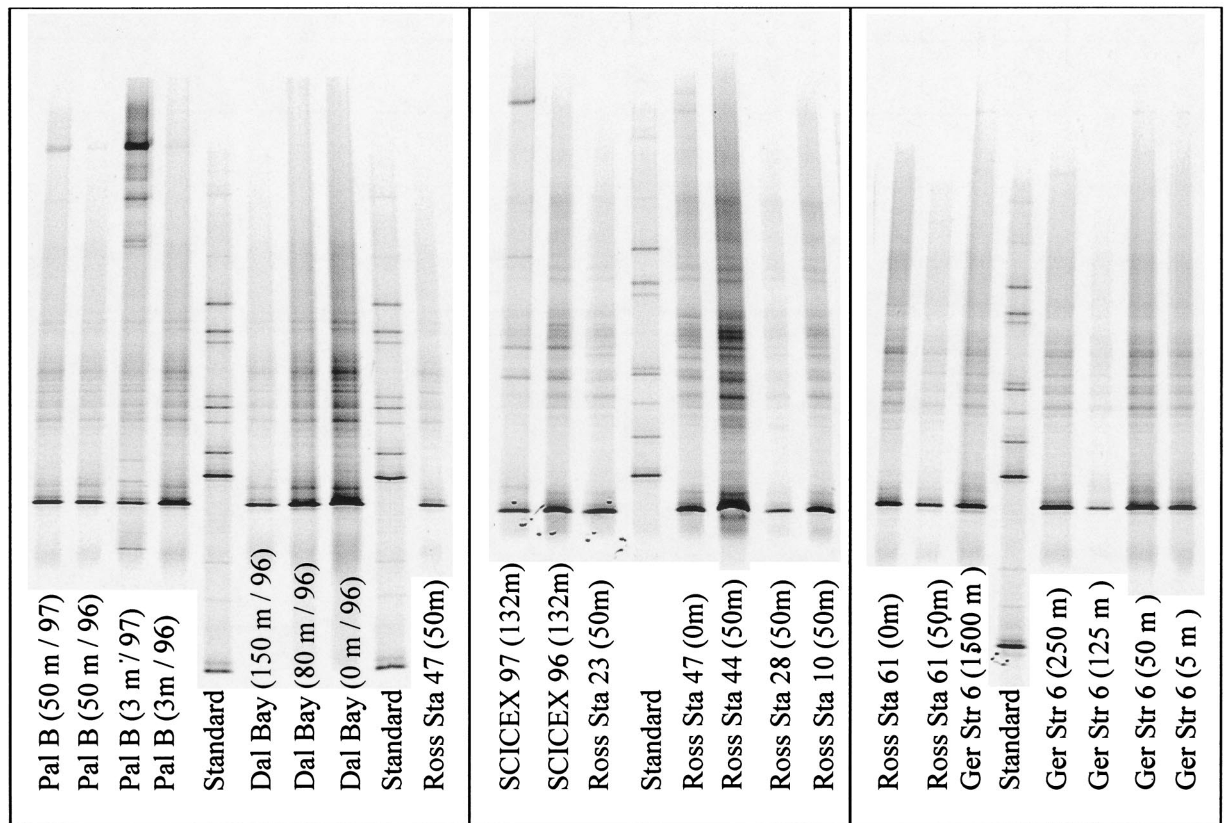

FIG. 1. PCR and DGGE analysis of all Antarctic samples that yielded *nitAB* products and thus were presumed to be positive for the presence of AOB. Lane designations refer to sampling data in Table 1. Pal B, Palmer Station B; Dal Bay, Dallman Bay; Ross Sta, Ross Station; Ger Str, Gerlache Strait. Two 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).

similarity values. Phylogenetic trees were constructed by using Jukes-Cantor distances and the neighbor-joining method (Phylogeny 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 andβAMOr 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

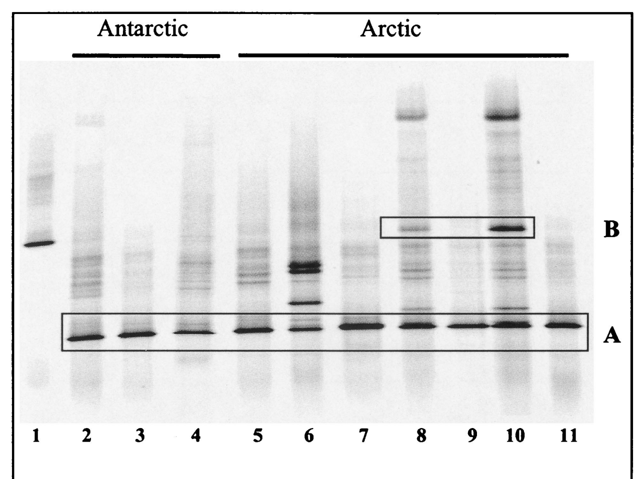

FIG. 2. Denaturing gradient gel comparing representative Antarctic and Arctic samples and clones. Lanes: 1, *Nitrosomonas cryotolerans*; 2, sample from Palmer Station B, 50 m, 4 February 1997; 3, clone A-2 from Palmer Station B, 50 m, 4 February 1997; 4, sample from Palmer Station B, 3 m, 13 September 1996 (this *nitAB* product was also sequenced directly); 5, sample SCICEX 96B, 55 m, from 83°38′N, 131°16E (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.

TABLE 2. Presumed nucleotide substitutions in sequences of cloned *nitAB* 16S rDNA fragments amplified from Arctic and Antarctic samples, relative to the sequence obtained directly from *nitAB* amplicons[a]

Position[b]	Direct sequence	Arctic clone												Antarctic clone		
		95A-44	95B-3	95B-4	95B-7	95B-10	95B-22	96A-4	96A-8	96A-11	96A-17	96A-19	96B-3	Pal 6-2	Pal 6-8	Pal 6-13
182	T									C						
208	C			T		T										
249	T			**A**									**A**			
264	A			G												
272	A														**T**	
280	G							**T**		**T**	A					
299	A													G		
327	C							T		T	T					
388	*A*													T		
412	*G*												T			
456	*T*			A												
470	*C*							*T*		*T*	*T*					
475	*T*		*C*									*C*				
481	*A*									*G*						
486	*A*									*G*						
500	*C*						*T*									
511	*T*				*C*											
521	*C*	*T*														
578	C							**G**		**G**	**G**					
586	T							**G**		**G**	**G**					
609	G						**T**	**T**		**T**	**T**					
636	C							T		T	T					
729	G														A	
761	G							**C**		**C**	**C**					
837	G											A				
947	T														C	
1036	A		**T**													
1093	A								G							
1157	G														A	
1170	A								G							
1175	G															A
1178	G													A		

[a] Transversions are indicated in boldface. The area with italicized nucleotides corresponds to the portion of the sequence probed with DGGE primers.
[b] Nucleotide positions correspond to *E. coli* 16S rRNA positions (accession number J01695).

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 βAMOf and βAMOr 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-

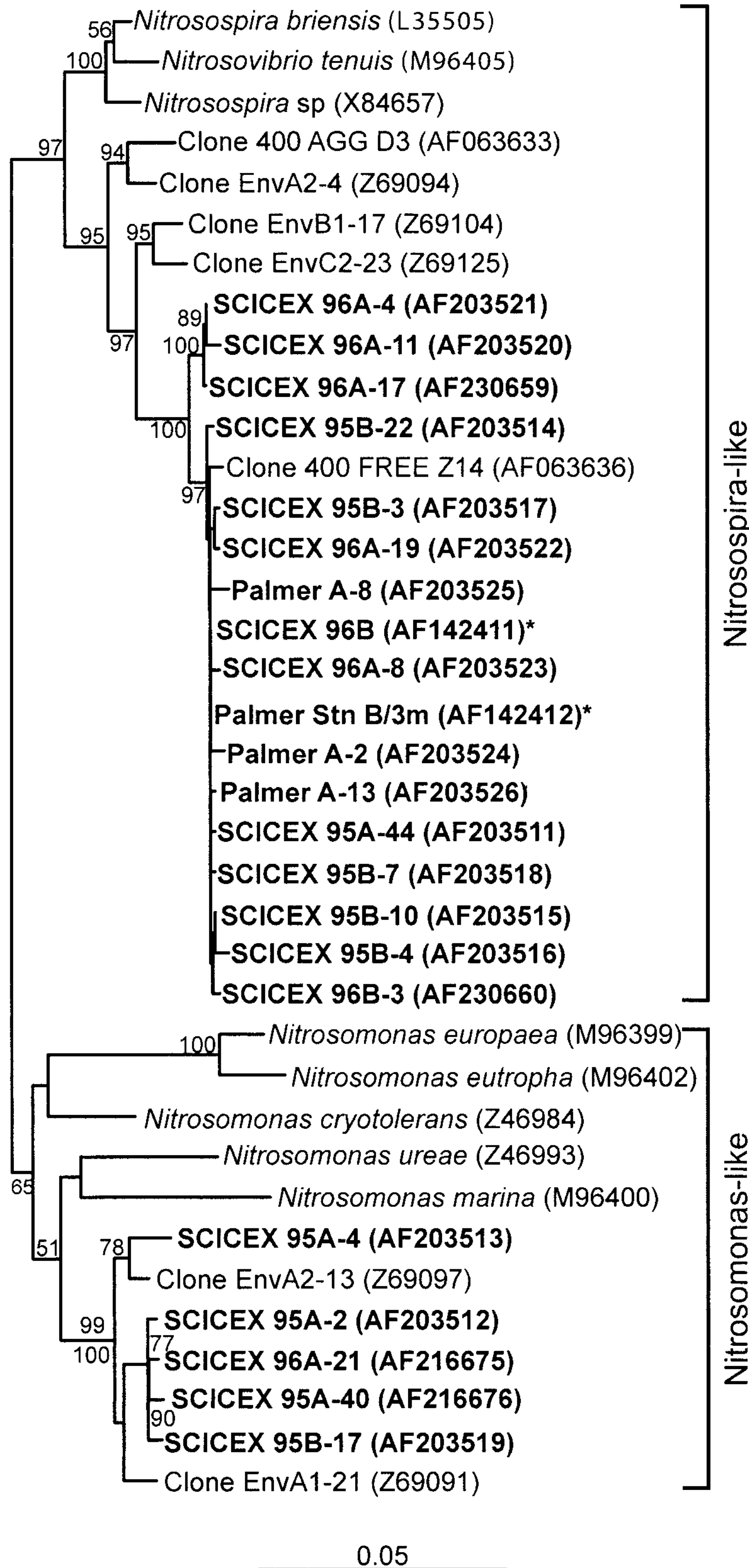

FIG. 3. Phylogenetic relationship of sequences from Arctic and Antarctic samples to sequences from closely related AOB from β-subclass *Proteobacteria* and environmental clones; accession numbers follow the isolate or clone name. Polar sequences are indicated in boldface type, sequences labeled "SCICEX" 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 *Nitrosospira*-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 *Nitrosospira*-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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