APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1444–1450 Vol. 61, No. 4
0099-2240/95/$04.00+0

Detection of Ammonium-Oxidizing Bacteria of the Beta-Subclass of the Class *Proteobacteria* in Aquatic Samples with the PCR

M. A. VOYTEK* AND B. B. WARD

Marine Sciences Program, University of California, Santa Cruz, Santa Cruz, California 95064

Received 7 November 1994/Accepted 27 January 1995

The PCR was used as the basis for the development of a sensitive and specific assay for the detection of ammonium-oxidizing bacteria belonging to the beta-subclass of the class *Proteobacteria*. PCR primers were selected on the basis of nucleic acid sequence data available for seven species of nitrifiers in this subclass. The specificity of the ammonium oxidizer primers was evaluated by testing known strains of nitrifiers, several serotyped environmental nitrifier isolates, and other members of the *Proteobacteria*, including four very closely related, nonnitrifying species (as determined by rRNA sequence analysis). DNA extracts from 19 bacterioplankton samples collected from Lake Bonney, Antarctica, and the Southern California Bight were assayed for the presence of ammonium oxidizers. By using a two-stage amplification procedure, ammonium oxidizers were detected in samples collected from both sites. Chemical data collected simultaneously support the occurrence of nitrification and the presence of nitrifiers. This is the first report describing PCR primers specific for ammonium-oxidizing bacteria and the successful amplification of nitrifier genes coding for rRNA from DNA extracts from natural samples. This application of PCR is of particular importance for the detection and study of microbes, such as autotrophic nitrifiers, which are difficult or impossible to isolate from indigenous microbial communities.

Nitrifiers are gram-negative, obligate aerobic chemolithotrophs which oxidize ammonium to nitrite or nitrite to nitrate as their sole energy source and assimilate carbon dioxide via the Calvin Benson cycle. Beyond the basic distinction between ammonium oxidizers and nitrite oxidizers, genera have been defined by differences in morphology and ultrastructure of the cells, primarily by using the phenotypic characters of cell shape and the arrangement of the intracytoplasmic membranes (38). Nitrifiers are recognized as important agents in the nitrogen cycles of aquatic and terrestrial ecosystems. The ecological importance of ammonium oxidizers and nitrite oxidizers lies in their role in the biological oxidation of reduced nitrogen compounds, often leading to the removal of nitrogen from the environment via denitrification. Although nitrifiers have been isolated from diverse environments and are generally ubiquitous in soils and freshwater and marine environments (14), they account for a very small proportion of the total bacterial population in natural environments. Sensitive and specific methods of detection and identification are necessary to study their ecological importance in natural systems.

To date, detection by immunofluorescence on the basis of serological distinction is the only method available for the identification of single cells and the enumeration of individual strains of nitrifying bacteria in natural samples. Fluorescence detection of polyclonal antibodies has been used to study the serological diversity and distribution of nitrifying bacteria in soils (4, 8, 19), sewage (30, 42), lake sediments (22), and marine environments (32–34). This method requires the isolation and culture of bacterial strains in order to raise antibodies for subsequent detection assays. In natural systems, prior enrichment and cultivation is often not desirable or even possible. Many bacterial cells present in a natural population appear viable and yet cannot be detected by traditional isolation procedures (13, 18). Moreover, for chemoautotrophs, which grow very slowly and therefore require months or even years for isolation and purification, the development of detection methods which do not require culturing would be advantageous.

The PCR, with its capacity to amplify specific sequences of DNA from a few copies of the target DNA, is a tool which overcomes some of the limitations of immunofluorescence. The advantages of molecular techniques over traditional enrichment procedures have been demonstrated in the detection and analysis of bacteria in natural samples from many complex environments from which only a small percentage of the indigenous population can be isolated, such as seawater (9, 20), terrestrial hot spring mats (24, 37, 39), and endosymbiotic associations (2, 7).

The 16S rRNA genes from several species of nitrifying bacteria have been sequenced, and their phylogenetic relationships have been determined (10, 28). Analysis of bacterial systematics using 16S rRNA demonstrated that there are two phylogenetically distinct groups of autotrophic ammonium-oxidizing bacteria, both within the class *Proteobacteria* (10, 28, 40, 41). One group within the gamma-subclass was identified on the basis of the sequence of a single ammonium oxidizer, *Nitrosococcus oceanus*. The other group represents a family within the beta-subclass and contains the following two species clusters as defined by Head et al. (10): *Nitrosomonas* (including *Nitrosomonas europaea* and *Nitrosococcus mobilis*) and *Nitrosospira* (including all of the strains in the genera *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*).

The objective of the present research was to develop a set of PCR primers specific to the 16S rRNA gene for the unambiguous identification and amplification of DNA from ammonium oxidizers in the beta-subclass of the *Proteobacteria*. This approach is of particular usefulness for nitrifiers because they are an ecologically coherent group. The primers, once characterized in terms of their phylogenetic specificity, can be used to detect nitrifiers and lay the foundation for future development of quantitative studies of these bacteria in natural samples without precultivation and with higher sensitivity than is provided by other methods.

* Corresponding author. Phone: (408) 459-3128. Electronic mail address (Internet): voytek@biology.ucsc.edu.

MATERIALS AND METHODS

Bacterial strains and isolates, culture media, and growth conditions. Thirty-three strains of ammonium-oxidizing bacteria isolated from marine and soil environments were grown on Watson medium of appropriate salinity (36) and SW medium (23), respectively. Phenol red was used as a pH indicator. The pH of the cultures was maintained between 7 and 8 by the periodic addition of sterile $CaCO_3$. The eight strains of nitrite-oxidizing bacteria isolated from marine and estuarine environments were grown on N medium of the appropriate salinity (6). All nitrifying cultures were grown at 25°C. All other eubacterial strains were grown on American Type Culture Collection-recommended media under the appropriate temperature and light conditions (5). Samples of *Rhodocyclus purpureus* and *Rhodovivax gelatinosus* were graciously provided by M. T. Madigan. Information regarding the sources, locations, morphologies, and serotypes of the strains used in this study are summarized in Table 1.

Serotyping. Twenty-four of the ammonium oxidizers listed in Table 1 were serotyped previously by the indirect immunofluorescence staining method using antisera raised against a marine *Nitrosomonas* sp. and *N. oceanus* (34). We used the microcentrifuge method (34) for cross-reaction tests to serotype the remaining nine (*Nitrosomonas eutropha*, *Nitrosolobus multiformis*, *Nitrosospira briensis*, *Nitrosovibrio tenuis*, *N. mobilis*, and the environmental isolates TA 921-i-NH4, B19-i-NH4, NOC5, and NOCP). The antisera used were produced by Hazelton-Dutchland Laboratories (Denver, Pa.) and have been fully described by Ward and Carlucci (34). The fluorescent antibody used, fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin G, was obtained from Miles Laboratories, Inc. (Elkhardt, Ind.).

Natural samples. Bacterioplankton samples (approximately 4 liters) were collected in November 1992 in Niskin bottles from nine depths (Table 2) in Lake Bonney, a hypersaline, meromictic lake in the upper Taylor Valley, Victoria Land, Antarctica. Samples were also collected from 10 depths at a station in the Santa Monica Basin of the Southern California Bight in October 1992 (Table 2). The Lake Bonney samples were concentrated 100-fold by ultrafiltration with a Filtron (Northborough, Mass.) open-channel Ultrasette with a 300-kDa nominal molecular mass cutoff membrane, and the concentrate was filtered onto a 47-mm-diameter, 0.2-μm-pore-size Gelman Supor filter. Samples from the Southern California Bight were collected by gentle filtration directly onto a 47-mm-diameter, 0.2-μm-pore-size Gelman Supor filter. Filters were stored frozen in EDTA (0.5 M; 0.5 ml) until total DNA was extracted.

DNA extraction. For the cultured bacteria, cells (750 ml of nitrifying bacterium culture and 10 ml of all other cultured bacteria [Table 1]) were collected by centrifugation. High-molecular-weight, bacterial genomic DNA was extracted and purified from the pelleted cells according to a standard protocol with slight modification (3). Following the protease incubation, the cell lysate was extracted with hexadecyltrimethyl ammonium bromide (approximately 1%), which complexes polysaccharides and residual proteins (3). After isopropanol precipitation the DNA was resuspended in TE2 (10 mM Tris, 0.1 mM EDTA). DNA was extracted in a similar fashion (hexadecyltrimethyl ammonium bromide extraction was omitted) from the frozen filters of bacteria concentrated and collected from the natural samples.

For some cultures (ammonium-oxidizing isolates 3 and 132 [Table 1]) DNA from bacterial cells pelleted from 5 ml of culture was extracted with Chelex 100 chelating resin (Bio-Rad catalog no. 143-2832; procedure modified from that of Walsh et al. [31]). Pellets were transferred to a siliconized Eppendorf tube and resuspended in 50 μl of 5 to 10% Chelex 100 in sterile TE2. After being ground with a micropestle, samples were boiled for 10 to 15 min, vortexed, and then centrifuged to remove resin. Aliquots of the crude extract were used for subsequent PCRs.

Oligonucleotide primers. Using the 16S rRNA sequences of seven species of nitrifying bacteria in the beta-subclass of the *Proteobacteria* (10), we chose PCR primers corresponding to conserved sequences within the 5′ and 3′ regions of the genes coding for 16S rRNAs (16S rDNAs) of these strains (NITA, 5′CTTAAGT GGGGAATAACGCATCG3′, and NITB, 5′TTACGTGTGAAGCCCTACCC A3′, corresponding to positions 137 to 159 and 1214 to 1234 of *Escherichia coli* 16S rDNA, respectively). Amplification with these primers yields a 1,080-bp product. In a comprehensive search of eubacterial 16S rRNA in GenBank and EMBL, we identified only two other eubacteria with greater than 75% identity for both primer sequences: *R. purpureus* and *Spirillum volutans*. We included both species as well as several other phylogenetically related eubacterial species in our amplification assays (see below). The universal eubacterial 16S rRNA primers EUB1 and EUB2, as described by Liesack et al. (16), were used to confirm the PCR quality of the DNA template. These primers correspond to sites at positions 9 through 27 and 1525 through 1542, respectively, of the *E. coli* 16S rRNA, yield a 1,530-bp product, and are highly conserved in all eubacteria. Primers were synthesized commercially (OPERON, Alameda, Calif.).

PCR amplification. Experiments to optimize the PCR conditions were conducted with *N. europaea* genomic DNA. Here we report the optimal conditions. PCR amplification was performed in a total volume of 100 μl in 0.5-ml Eppendorf tubes under a layer of paraffin oil by using a DNA thermal cycler (GTC-2 Genetic Thermal Cycler, Precision Scientific; or Thermolyne Amplitron I, Barnstead). Reactions were carried out in a solution containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1% Triton X-100, 1.25 mM $MgCl_2$), 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 1 μl of template DNA (0.5 to 1 μg), and 2.5 U of *Taq* DNA polymerase (Promega Biological Research Products, Madison, Wis.). The reactions for our standard assay ran according to the following protocol: initial denaturation of template at 95°C for 3 min, prior to addition of the *Taq* DNA polymerase; 82°C for 2.5 min; 58 and 62°C for 2 min, for nitrifier-specific and eubacterial primers, respectively; and 72°C for 2.5 min; followed by 35 cycles of 94°C for 1 min, 58 or 62°C for 2 min, and 72°C for 2.5 min. A 10-min extension at 72°C was performed at the end of the final cycle. In cases in which amplification did not occur, annealing temperatures were lowered incrementally to 40°C to test the stringency of our assay. Negative controls with no DNA template were run under the same conditions. For DNA extracts from natural samples a two-stage PCR procedure was used. DNA templates were first amplified with the universal eubacterial (EUB) primers. A 1-μl aliquot of the EUB primer amplification product mixture was added to a new reaction mixture and reamplified with the beta-subclass nitrifier-specific (NIT) primers according to the same amplification protocol. PCR-amplified rDNAs were resolved by electrophoresis of 10 μl of the reaction mixture on 1% (wt/vol) horizontal agarose minigels run in 1× TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA).

Southern blotting and 16S rDNA probe hybridization. PCR-amplified products (10 μl) were electrophoresed on 1% (wt/vol) horizontal agarose minigels run in 1× TAE buffer. The rDNAs were transferred to a nylon transfer membrane (Nytran; Schleicher & Schuell, Keene, N.H.) by the procedure for Southern blotting of Ausubel et al. (3). The membrane was dried at 80°C to bind the DNA. Two rDNA probes were used. A general rDNA probe was used to confirm the identity of the product amplified. This probe was derived from the plasmid pNO1301, which contains the complete *E. coli rrnb* gene sequence (12) (a gift from Richard Gourse). By using the eubacterial primers, an approximately 1,500-bp fragment of the gene was labeled during PCR amplification under the following reaction conditions: 200 μM deoxynucleoside triphosphates (50% of the dTTP was replaced with digoxigenin-labeled dUTP) and 100 pM each primer in a 100-μl reaction mixture; amplification cycle, 95°C for 10 min, prior to addition of the *Taq* DNA polymerase; 82°C for 1 min; 62°C for 2 min; and 70°C for 2.5 min; followed by 35 cycles of 94°C for 1 min, 62°C for 2 min, 70°C for 2.5 min. A final 3-min extension at 70°C was performed. The amplified labeled fragment was purified by using the GeneClean kit (Bio 101, San Diego, Calif.). The second probe used was an oligonucleotide complementary to a sequence contained within the amplified products and specific to the beta-subclass ammonium oxidizers (5′AGCTACGTTACCAAGCCAAGCC3′). This probe (NIT-C) was labeled by using the Genius 3 digoxigenin 3′-end-labeling kit (Boehringer Mannheim). Approximately 3 to 6 ng of the labeled probes ml^{-1} was hybridized and detected by using the Genius System (Boehringer Mannheim). The hybridization conditions and the procedures for posthybridization washes and visualization of the probe-target conjugate were those recommended by the manufacturer. Membranes were prehybridized for 3 h and hybridized for 18 h at 65 and 54°C for the general rDNA probe and NIT-C, respectively. Color development proceeded for approximately 6 h.

RESULTS

PCR amplification of known strains and environmental isolates. The results of the PCR amplification assays using the universal EUB primers and the NIT primers are listed in Table 1. In all positive amplification reactions a single product was produced. To evaluate the specificity of the NIT primers, known strains of nitrifiers, several serotyped environmental nitrifier isolates, and other members of the *Proteobacteria*, including two species closely related to the beta-subclass nitrifiers (*R. purpureus* and *S. volutans*) as determined by rRNA sequence analysis (41), were tested. In total, 56 strains were tested, including 33 ammonium oxidizers, 8 nitrite oxidizers, 13 heterotrophic strains of *Proteobacteria* and 2 phototrophic strains of *Proteobacteria*. All strains of eubacteria were first amplified with the EUB primers, and all produced fragments having the correct molecular weight of the targeted sequence (1,530 bp; Fig. 1A). Identification of these as 16S rDNA sequences was confirmed by Southern (Fig. 1B) hybridization with the pNO1301 fragment under high-stringency conditions (Fig. 1B). DNA template from 20 of the ammonium-oxidizing isolates was amplified with the NIT primers and generated a 1,080-bp product (Table 1; Fig. 1A). The identity of these products was confirmed by slot blot hybridization with the NIT-C probe.

Of the 10 named and classified strains of ammonium oxidizers tested, target DNA was amplified in all 8 isolates from the beta-subclass: 2 marine isolates, the marine *Nitrosomonas* sp.

TABLE 1. Results of PCR amplification of known strains and environmental isolates with a set of EUB primers and a set of NIT primers

Organism	Source[a]	Type		Serotype(s)[b]	PCR amplification[c]	
		Morphology[d]	Environment(s)		EUB primers	NIT primers
Ammonium oxidizers						
Beta-subclass						
N. europaea	a	R	Soil	Nm	+	+
Nitrosomonas sp. (marine)	b	R	Marine	Nm	+	+
Nitrosomonas sp. strain WH-2	a	R	Soil	Nm	+	+
N. eutropha C-19	a	R	Soil	NR	+	+
N. multiformis C-71	b	L	Soil	NR	+	+
N. briensis C-128	b	S	Soil	NR	+	+
N. tenuis NV-1	b	V	Soil	NR	+	+
N. mobilis NC-2	b	C	Marine	NR	+	+
Gamma-subclass						
N. oceanus	b	C	Marine	No	+	−
N. oceanus	c	C	Marine	No	+	−
Environmental Isolates						
NH4C	d	R	Marine	Nm	+	−
NH4W	d	R	Marine	Nm, No	+	+
URW	d	R	Marine	Nm	+	+
NO3W	d	R	Marine	Nm, Nb	+	+
TT140-89A-2	d	R	Marine	NR	+	+
TT140-098-2	d	R	Marine	NR	+	+
C-17	b	R	Marine, sediment	No, Nm	+	+
C-19	b	C	Marine	No	+	−
C-45	b	R	Marine	No	+	+
C-113	b	R	Marine	Nm	+	+
3	c	C	Marine	No	+	−
5	c	C	Marine	No	+	−
5P	c	C	Marine	No	+	−
24	c	C	Marine	No	+	−
26	c	C	Marine	No	+	−
27	c	C	Marine	No	+	−
36	c	C	Marine	No	+	−
122	c	R	Marine	NR	+	+
132	c	C	Marine	NR	+	−
TA 921-i-NH4	e	R	Estuarine	NR	+	+
B19-i-NH4	e	R	Estuarine	Nm	+	+
NOC5	c	C	Marine	No	+	−
NOCP	c	C or R	Marine	No, Nm	+	+
Nitrite oxidizers						
B50	c	R	Marine	Nb	+	−
26N	c	R	Marine	Ng	+	−
32N	c	R	Marine	Nb	+	−
300B	c	R	Marine	ND	+	−
C-3	b	C	Marine	ND	+	−
TA921-i-NO2	e	C	Estuarine	ND	+	−
TA921-ii-NO2	e	R	Estuarine	ND	+	−
TA921-iii-NO2	e	R	Estuarine	ND	+	−
Other eubacteria						
E. coli LE392	f	R	Enteric	NR	+	−
Thiosphaera pantotropha	g	R	Wastewater	ND	+	−
Paracoccus denitrificans	g	C	Soil	ND	+	−
Pseudomonas aeruginosa	h	R	Unknown	ND	+	−
Pseudomonas stutzeri ATCC 14405	i	R	Marine	ND	+	−
Pseudomonas putida	h	R	Soil	ND	+	−
Pseudomonas atlanticus	h	R	Marine	ND	+	−
Pseudomonas aureofaciens	g	R	River clay	ND	+	−
Pseudomonas fluorescens ATCC 33512	i	R	Soil	ND	+	−
Pseudomonas cepacia ATCC 25416	i	R	Onion	ND	+	−
Vibrio natriegens ATCC 14048	i	V	Marine	ND	+	−
S. volutans ATCC 19554	i	S	Pond	ND	+	+
Alteromonas espejiana ATCC 27025	i	R	Marine	ND	+	−
A. faecalis	g	R	Enteric	ND	+	−
Alcaligenes eutrophus ATCC 17697	i	R	Soil	ND	+	−
Alcaligenes xylosoxidans ATCC 15173	i	R	Soil	ND	+	−
Vitreoscilla stercoraria ATCC 15218	i	V	Cow dung	ND	+	−

Continued on following page

TABLE 1—*Continued*

Organism	Source[a]	Type		Serotype(s)[b]	PCR amplification[c]	
		Morphology[d]	Environment(s)		EUB primers	NIT primers
Thiobacillus denitrificans ATCC 25259	i	R	Soil	ND	+	−
Chromobacterium violaceum ATCC 12472	i	R	Freshwater	ND	+	−
Rhodospirillum rubrum ATCC 33512	i	R	Pond	ND	+	−
R. purpureus	j	RR	Waste pond	ND	+	−
Rhodocyclus tenuis ATCC 25093	i	R	Pond	ND	+	−
R. gelatinosus	j	C	Pond	ND	+	−

[a] a, E. L. Schmidt; b, S. W. Watson; c, A. F. Carlucci; d, B. B. Ward; e, S. G. Horigan; f, J. Zyskind; g, D. Castignetti; h, M. Pontius-Brewer; i, American Type Culture Collection; j, M. T. Madigan

[b] According to reference 34. Nm, marine *Nitrosomonas* sp.; No, *N. oceanus*; Nb, *Nitrobacter* sp.; Ng, *N. gracilis*; NR, no reaction; ND, not determined.

[c] +, amplified product of correct size; −, no amplification.

[d] C, coccoid; R, rod; V, vibrio; RR, ring shaped or rod; S, spiral; L, lobular.

and *N. mobilis*; and 6 soil strains, *Nitrosomonas* sp. strain WH-2, *N. eutropha*, *N. europaea*, *N. briensis*, *N. tenuis*, and *N. multiformis* (Table 1). Twelve of the 22 environmental ammonium-oxidizing isolates generated the correct product (Table 1). All cells that tested positive were rod shaped. Seven of the 12 belonged to the marine *Nitrosomonas* sp. serotype, 1 belonged to the *N. oceanus* serotype, and the remainder are unclassified as to serotype. Target DNA was not found in the two strains of *N. oceanus* from the gamma-subclass, and no product was generated for the remaining 10 ammonium-oxidizing strains (Table 1). All but 1 strain (NH4C) of the 10 are coccoid cells, and all but 2 strains (NH4C and 132) cross-react with *N. oceanus* antiserum (34). Target DNA was not found in any of the eight nitrite-oxidizing isolates, and it was found in only one of the other species of *Proteobacteria* assayed (Table 1). Only when the annealing temperature was lowered to 50°C did the NIT primers amplify the 16S rRNA gene in the closely related, phototrophic, nonsulfur species of *Proteobacteria R. purpureus*, but they still did not amplify the 16S rRNA gene in *R. gelatinosus.*

TABLE 2. Results of PCR amplification of DNA extracts from natural samples with a set of EUB primers and a set of NIT primers

Source and sample no.	Depth (m)	PCR amplification[a]	
		EUB primers	NIT primers
Lake Bonney			
EL5-69	5	+	+
EL13-61	13	+	+
EL15-69	15	+	+
EL17-61	17	+	+
EL20-69	20	+	+
EL22-69	22	+	+
EL25-69	25	−	−
EL30-61	30	−	−
EL35-69	35	+	−
Southern California Bight			
37-11	1	+	−
16-07	10	+	−
37-12	11	+	−
37-13	16.5	+	+
37-14	20	+	−
37-15	35.5	+	−
37-16	49	+	+
15-04	80	+	+
16-11	100	+	+
16-15	900	+	+

[a] +, amplified product of correct size; −, no amplification.

PCR amplification of natural samples. Nineteen DNA extracts from bacterioplankton samples collected from aquatic environments were tested. Amplification with the EUB primers yielded the expected 1,530-bp product for DNA templates extracted from all natural samples except the DNA extracts from depths of 25 and 30 m in Lake Bonney (Fig. 3; Table 2). Amplification of the extracted DNA with the NIT primers did not produce the expected product for samples taken at any of the depths at either site. However, reamplification of the EUB primer amplification product with the NIT primers did yield the expected 1,080-bp product for most of the samples taken from Lake Bonney and for samples taken at five of the nine depths sampled in the Southern California Bight (Table 2). The identity of the 16S rDNA sequence generated by the EUB primers and the NIT primers was confirmed by Southern and slot blot hybridization (Fig. 1B and 2). No amplification with the NIT primers occurred in samples taken at depths of 25, 30, and 35 m in Lake Bonney. On the basis of the negative results for both sets of primers, we believe that the DNA extracts from depths 25 and 30 m contain *Taq* polymerase-inhibitory substances (see Discussion). The 16S rRNA gene could be amplified in the 35-m sample, but no nitrifier-specific target was found. Successful amplification of natural samples from both systems with the NIT primers demonstrates the presence of nitrifying bacteria of the beta-subclass at those depths.

DISCUSSION

To define the specificity of the NIT primers, we demonstrated the reproducibility of the PCR assay for target and nontarget representatives of the beta-subclass whose rRNA sequences were known. It was necessary to demonstrate that DNAs from members of different genera and from genotypically related microorganisms did not produce 16S rRNA amplification products from the NIT primers. Several attempts to amplify the 16S rRNA gene by using the NIT primers with sample templates that yielded no product under the optimized conditions were made. We relaxed the conditions of the PCR to allow less-specific annealing of the primers, i.e., by dropping the annealing temperature to 40°C, increasing the primer concentration, and/or adding more enzyme. In all but one case (*R. purpureus*; see above), these attempts resulted in an increase in primer dimer formation and the production of random products, none of which corresponded to the 16S rRNA target sequence.

Significantly, with the exception of *S. volutans*, the NIT primer pair did not find targets in any of the related strains of

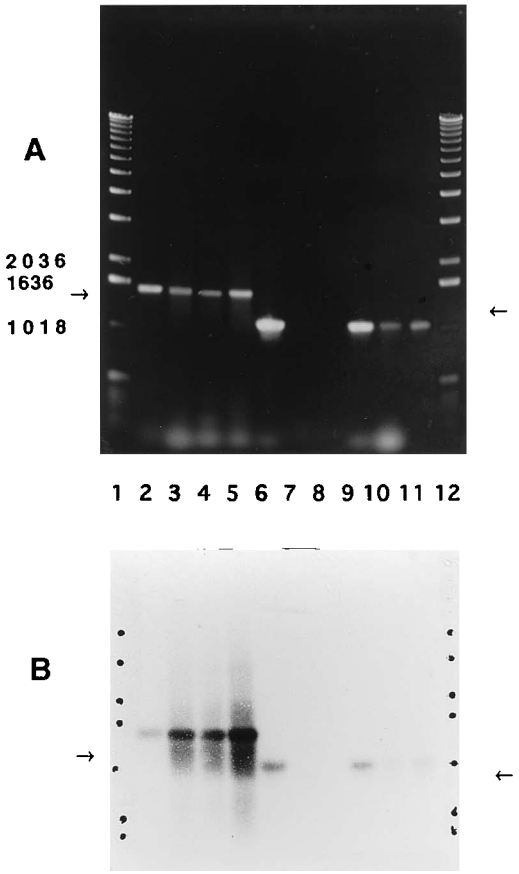

FIG. 1. Agarose gel (A) and Southern blot hybridization (B) analysis of PCR products amplified from DNA extracts of ammonium-oxidizing bacteria from cultures of known nitrifier isolates and environmental samples with EUB (lanes 2 through 5) and NIT (lanes 6 through 11) primers. Lanes: 1 and 12, 1-kb DNA size standard (Gibco BRL); 2, *N. europaea*; 3, *N. oceanus*; 4, *A. faecalis*; 5, *E. coli*; 6, *N. europaea*; 7, *N. oceanus*; 8, *A. faecalis*; 9, *R. purpureus*; 10, isolate URW; 11, bacterioplankton sample from Lake Bonney, Antarctica (13 m). Numbers on the left indicate sizes of DNA fragments in base pairs. The arrows indicate the expected EUB (1,530 bp) and NIT (1,080 bp) primer products. Lanes in the Southern blot correspond to those in the gel (A). The Southern blot was probed with digoxigenin-labeled pNO1301, carrying the *rrnB* gene of *E. coli*. The black dots correspond to the following 1-kb DNA ladder size markers: 4,072, 3,054, 2,036, 1,636, 1,018, 506 or 517, and 396 bp (top to bottom).

Proteobacteria tested. Although their 16S rRNA sequence is quite closely related to the beta-subclass nitrifiers and the NIT primer corresponding to the 3′ region is complementary to this region, DNA extracted from *R. purpureus* was amplified only under conditions less stringent than those of the assay and *Alcaligenes faecalis* DNA was not amplified. These results are consistent with the results of a computer-assisted comparison of the 16S rDNA sequences available for the strains tested (National Center for Biotechnology Information, Experimental GENINFO BLAST Network Service). The NIT primer pair could not discriminate between target DNA from *R. purpureus* (at annealing temperatures of less than 51°C), *S. volutans*, and the beta-subclass nitrifiers. Additionally, it is possible that nonspecific amplification products could be produced by untested bacterial strains with the primers described here. However, keeping the annealing temperature high (58°C) should minimize nonspecific amplification. Furthermore, any nonspecific products obtained could be distinguished from the desired amplification products on the basis of size and hybridization

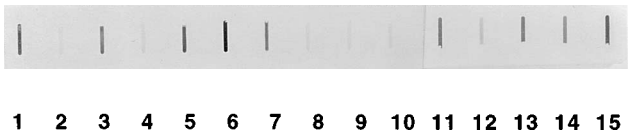

FIG. 2. Specificity of the NIT-C probe. Unless otherwise indicated, approximately 5 to 20 ng of DNA was blotted in each slot blot. Slot positions: 1, *N. europaea*; 2, sheared herring sperm DNA; 3, *N. tenuis*; 4, 1:20 dilution of marine *Nitrosomonas* sp. DNA; 5, marine *Nitrosomonas* sp. 6, *N. eutropha*; 7, *N. briensis*; 8, *R. purpureus*; 9, *S. volutans*; 10, *N. oceanus*; 11, *N. multiformis*; 12, sheared herring sperm DNA; 13, isolate URW; 14, Southern California Bight sample 15-04; 15, Lake Bonney sample EL13-61.

analyses utilizing additional nitrifier-specific oligonucleotide probes internal to the primers. In this study, we were able to distinguish between products generated from ammonium oxidizers and the products generated from *R. purpureus* and *S. volutans* (Fig. 2) by using another specific oligomer sequence (NIT-C) internal to the amplified region.

Ward and Carlucci (34) reported concerning the specificity of antisera to five genera of nitrifying bacteria in terms of their cross-reactivity with many of the isolates used in this study. Fourteen of the isolates that were amplified with the NIT primers were rod shaped, reacted with the antiserum to the marine *Nitrosomonas* sp., and did not react with the *N. oceanus* antiserum. These results confirm that the NIT primers amplify nitrifiers morphologically and serologically similar to those in the beta-subclass and do not amplify nitrifiers in the gamma-subclass. The 16S rRNA gene of the marine *Nitrosomonas* sp. has not been sequenced, but on the basis of serotype and amplification with the NIT primers, we expect that this isolate belongs to the beta-subclass. Conversely, those nitrifiers that were not amplified with the NIT primers generally were coccoid, cross-reacted with the *N. oceanus* antiserum or the antisera of two nitrite oxidizers, *Nitrospina gracilis* and a marine *Nitrobacter* sp., and did not react with the antiserum to the marine *Nitrosomonas* sp. The two exceptions, NH4C and C-45, are uncharacterized isolates. This assay is the first step towards genotypically characterizing them, and further study may resolve the genotypic-phenotypic discrepancy. These results may also indicate changes to or loss of the original isolates.

Samples were collected at Lake Bonney, Antarctica, because

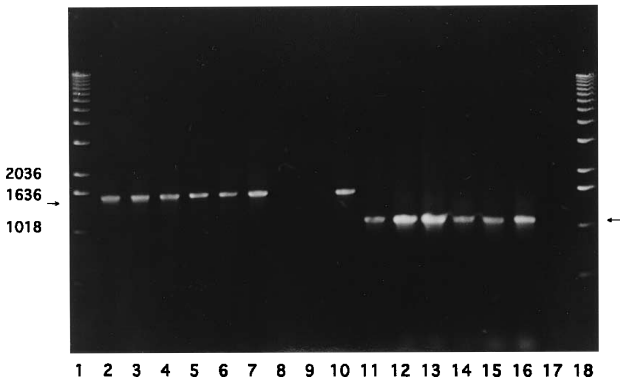

FIG. 3. Agarose gel analysis of PCR 16S rDNA amplification of DNA extracts from bacterioplankton samples collected at various depths in Lake Bonney, Antarctica, with EUB (lanes 2 through 10) and NIT (lanes 11 through 17) primers. Lanes: 1 and 18, 1-kb DNA ladder size standard (Gibco BRL); 2, 5 m; 3, 13 m; 4, 15 m; 5, 17 m; 6, 20 m; 7, 22 m; 8, 25 m; 9, 30 m; 10, 35 m; 11, 5 m; 12, 13 m; 13, 15 m; 14, 17 m; 15, 20 m; 16, 22 m; 17, 35 m. Numbers on the left indicate sizes of DNA fragments in base pairs. The arrows indicate the expected EUB (1,530 bp) and NIT (1,080 bp) primer products.

the chemistry of this lake implies that nitrification is an important process controlling the distribution of dissolved nitrogen compounds, including nitrous oxide. A strong chemocline occurs at about 17 m, above which aerobic, low-nutrient-concentration conditions exist. Below the chemocline, there is a rapid increase in the concentration of oxidized nitrogen compounds, even though the deep waters (down to the bottom at 40 m) are virtually anoxic. An extremely high concentration of nitrous oxide occurs between 20 and 30 m (17), suggesting the occurrence of nitrification at low oxygen concentrations. The presence of nitrifying bacteria was detected by the PCR assay from 6 depths above 25 m in Lake Bonney. This distribution is consistent with the occurrence of nitrification and the presence of nitrifiers in this depth range. Data concerning the rate of nitrification obtained by performing ^{15}N tracer experiments (16a) also support the occurrence of nitrification in this interval. All attempts so far to isolate nitrifying strains from this environment have failed. The PCR assay, however, demonstrates the presence of the organisms, which appear to be too fastidious to culture.

Nitrification and nitrifying bacteria in the Southern California Bight have been studied previously. The rate of nitrification in these waters (measured by using ^{15}N tracers) generally shows a maximum near the bottom of the euphotic zone, in the region of the primary nitrite maximum (33). At the station where these DNA samples were collected in the Santa Monica Basin, the primary nitrite maximum was found between 70 and 80 m (31a). In two earlier studies, the abundance of ammonium-oxidizing bacteria had been estimated to be in the range of 10^5 cells per liter by using immunofluorescence and antisera to two ammonium-oxidizing bacteria (33, 34). PCR amplification detected beta-subclass nitrifier sequences at all depths between 49 and 900 m and at one shallower depth (16.5 m). The two techniques are expected to detect overlapping subsets of the total ammonium oxidizer population (Table 1). The distribution of PCR-positive samples does not simply reflect the abundance of total bacteria, which shows a maximum above the primary nitrite maximum (35); rather, the PCR assay detected ammonium oxidizers at depths where the highest activity of these organisms is expected. We did not detect nitrifier sequences in surface water, although nitrifiers have been enumerated there by immunofluorescence (33, 34) and have been isolated from similar samples (11). This failure to detect nitrifier sequences may be due to interferences from nontarget DNA (see below), which would be most severe in surface waters. The PCR assay as applied here cannot be interpreted in terms of the in situ abundance of nitrifiers, but further refinements may make this possible.

Amplification of 16S rDNA from the DNA extracts of natural samples with the NIT primers did not produce the target fragment, and yet subsequent amplification of the EUB primer product with the NIT primers did produce the NIT primer fragment. Although nitrifiers may have been relatively abundant, they were undoubtedly a very small fraction of the total cells present. Annealing efficiency is apparently reduced in the presence of large amounts of nontarget DNA (21, 27). Target DNA in extracts from our natural samples was diluted to an unknown and possibly quite large degree with nontarget DNA, and this may have affected the results reported here. A two-stage PCR strategy with nested primers effectively decreases the ratio of competing DNA to target DNA and enables amplification of the target sequence.

DNA from samples taken at 25 and 30 m in Lake Bonney could not be amplified. A control template (1 μl of *N. europaea* DNA) was added to PCR mixtures containing aliquots of DNA from extracts of samples taken at 25 and 30 m, and still no amplification was detected. The inhibition of PCR by humics and other compounds expected to be in environmental extracts has been previously reported (25, 26, 29). Although impurities often copurify, attempts to further purify the DNA extracts from these depths should be made (1, 15).

This is the first report describing PCR primers specific for nitrifying bacteria in the beta-subclass of the *Proteobacteria* and the successful amplification of nitrifier rDNA from natural aquatic samples. Although the detection limit in environmental samples has yet to be determined, these primers offer a rapid and specific identification method that can detect the presence of organisms which are suspected to be a very small part of the total natural assemblage (34). The PCR assay can be used specifically to detect small numbers of ammonium-oxidizing bacteria of the beta-subclass of the *Proteobacteria*, without the need of prior enrichment or cultivation. Its application is of particular importance in the detection of microbes that are difficult or impossible to culture, such as those in aquatic environments where less than 0.1% of the community is cultivable. Moreover, unlike detection by immunofluorescence, detection by PCR amplification relies on genotypic characteristics of the specific organisms and therefore may be more reliable. Antigenic features are phenotypic characteristics whose expression may be regulated by environmental factors and thus may affect the sensitivity and specificity of immunological techniques. Another advantage of using PCR is its flexibility in terms of specificity. Depending on the primer sequences selected, individual species, genera, families, and even kingdoms can be preferentially amplified. This is of particular usefulness for nitrifiers because they are an ecologically coherent group. To detect the presence of ammonium oxidizers by using species-specific primers would require multiple reactions, and this would be possible only if the sequences of all nitrifiers in a system were known. By using genus- or family-specific primers, the presence of nitrifiers can be detected with a single reaction. Furthermore, use of these primers should enhance the functional investigation of the process of nitrification in any environment.

Many questions regarding the distribution, abundance, and role of nitrifying bacteria in natural environments, particularly in environments less eutrophic than sewage, remain unanswered to date partly because of the lack of a simple, rapid, and sensitive detection and quantification procedure. The presence of nitrifying bacteria in the environment is often inferred from chemical profiles and rate measurements, but it is rarely empirically determined. Substrate concentration, cell number, temperature, and light may all moderate the relationship between nitrification rates and the presence of nitrifiers. The NIT primers described here will be useful for investigating these aspects of the ecology and the environmental importance of nitrifiers.

ACKNOWLEDGMENTS

We are indebted to John Priscu for inviting us to work in Lake Bonney and to J. C. Priscu, R. D. Bartlett, K. Wing, A. R. Cockcroft, and C. Woolston for logistical and technical help in Antarctica. A. R. Cockcroft and M. T. Geissler assisted in the collection of Southern California Bight samples.

This work was supported by the National Science Foundation (OCE-9115040 and DPP-9117907).

REFERENCES

1. **Abbaszadegan, M., M. S. Huber, C. P. Gerba, and I. L. Pepper.** 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl. Environ. Microbiol. **59:**1318–1324.
2. **Amann, R., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer.** 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts.

Nature (London) **351:**161–164.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York.
4. **Besler, L. W., and E. L. Schmidt.** 1978. Serological diversity within a terrestrial ammonia-oxidizing population. Appl. Environ. Microbiol. **36:**584–593.
5. **Carlucci, A. F., and D. Pramer.** 1957. Factors influencing the plate method for determining abundance of bacteria in sea water. Proc. Soc. Exp. Biol. Med. **96:**392–394.
6. **Carlucci, A. F., and J. D. H. Strickland.** 1968. The isolation, purification and some kinetic studies of marine nitrifying bacteria. J. Exp. Mar. Biol. Ecol. **2:**156–166.
7. **Distel, D. L., E. F. DeLong, and J. B. Waterbury.** 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. Appl. Environ. Microbiol. **57:**2376–2382.
8. **Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt.** 1974. Autoecological study of the chemoautotroph *Nitrobacter* by immunofluorescence. Appl. Microbiol. **27:**124–149.
9. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:** 60–62.
10. **Head, I. M., W. D. Hiorns, T. Martin, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **139:**1147–1153.
11. **Horrigan, S. G., A. F. Carlucci, and P. M. Williams.** 1981. Light inhibition of nitrification in sea surface films. J. Mar. Res. **39:**557–565.
12. **Jinks-Robertson, S., R. L. Gourse, and M. Nomura.** 1983. Expression of rRNA and tRNA genes in *Escherichia coli*: evidence for feedback regulation by products of rRNA operons. Cell **33:**865–876.
13. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living bacteria. Can. J. Microbiol. **25:**415–420.
14. **Koops, H.-P., and U. C. Möller.** 1992. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2637. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer. (ed.), The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag, New York.
15. **Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J. M. López-Pila.** 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol. **59:**1213–1219.
16. **Liesack, W., H. Weyland, and E. Stackebrandt.** 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. **21:**191–198.

16a. **Priscu, J.** Unpublished data.

17. **Priscu, J. C., B. B. Ward, and M. T. Downes.** 1993. Water column transformations of nitrogen in Lake Bonney, a perennially ice-covered Antarctic lake. Antarct. J. U.S. **28:**237–239.
18. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
19. **Schmidt, E. L.** 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. **118:**141–149.
20. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:**4371–4378.
21. **Seal, S. E., L. A. Jackson, and M. J. Daniels.** 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3751–3758.
22. **Smorczewski, W. T., and E. L. Schmidt.** 1991. Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment. Can. J. Microbiol. **37:**828–833.
23. **Soriano, S., and N. Walker.** 1968. Isolation of ammonia oxidizing autotrophic bacteria. J. Appl. Bacteriol. **31:**493–497.
24. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. **49:**1379–1384.
25. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54:**2185–2191.
26. **Steffan, R. J., J. Goksoyr, A. K. Bej, and R. M. Atlas.** 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54:**2908–2915.
27. **Sykes, P. J., S. H. Neoh, M. J. Brisco, E. Highes, J. Condon, and A. A. Morley.** 1992. Quantitation of targets for PCR by use of limiting dilution. BioTechniques **13:**444–449.
28. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
29. **Tsai, Y. L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
30. **Völsch, A., W. F. Nader, H. K. Geiss, G. Nebe, and C. Birr.** 1990. Detection and analysis of two serotypes of ammonia-oxidizing bacteria in sewage plants by flow cytometry. Appl. Environ. Microbiol. **56:**2430–2435.
31. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:**506–513.

31a. **Ward, B.** Unpublished data.

32. **Ward, B. B.** 1982. Oceanic distribution of ammonium-oxidizing bacteria determined by immunofluorescence. J. Mar. Res. **40:**1155–1172.
33. **Ward, B. B.** 1987. Nitrogen transformations in the Southern California Bight. Deep-Sea Res. **34:**785–805.
34. **Ward, B. B., and A. F. Carlucci.** 1985. Marine ammonia- and nitrite-oxidizing bacteria: serological diversity determined by immunofluorescence in culture and in the environment. Appl. Environ. Microbiol. **50:**194–201.
35. **Ward, B. B., and K. A. Kilpatrick.** 1993. Methane oxidation associated with mid-depth methane maxima in the Southern California Bight. Cont. Shelf Res. **13:**1111–1122.
36. **Ward, B. B., and M. J. Perry.** 1980. Immunofluorescent assay for the marine ammonium-oxidizing bacterium *Nitrosococcus oceanus*. Appl. Environ. Microbiol. **39:**913–918.
37. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
38. **Watson, S. W., E. Bock, E. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Nitrifying bacteria, p. 1808–1834. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
39. **Wegmüller, B., J. Lüthy, and U. Candrian.** 1993. Direct polymerase chain reaction detection of *Campylobacter jejuni* and *Campylobacter coli* in raw milk and dairy products. Appl. Environ. Microbiol. **59:**2161–2165.
40. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of the purple bacteria: the gamma subdivision. Syst. Appl. Microbiol. **6:**25–33.
41. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of the purple bacteria: the beta subdivision. Syst. Appl. Microbiol. **5:**327–336.
42. **Yoshioka, T., H. Hisayoshi, and Y. Saijo.** 1982. Growth kinetic studies of nitrifying bacteria by the immunofluorescent counting method. J. Gen. Appl. Microbiol. **28:**169–180.