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Amplification of the *amoA* Gene from Diverse Species of Ammonium-Oxidizing Bacteria and from an Indigenous Bacterial Population from Seawater†

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Because the chemolithotrophic ammonium-oxidizing bacteria are an integral component of nitrogen biogeochemistry, a sensitive and accurate method to detect this ecologically important group of microorganisms is needed. The *amoA* gene of these organisms encodes the active site of ammonia monooxygenase, an enzyme unique to this group of nitrifying bacteria. We report here the use of the PCR technique to detect the *amoA* gene from pure cultures of chemolithotrophic ammonium-oxidizing bacteria, ammonium oxidizers introduced into filtered seawater, and the natural bacterial population of an unfiltered seawater sample. Oligonucleotide primers, based on the published *amoA* sequence from *Nitrosomonas europaea*, were used to amplify DNA from pure cultures of *Nitrosomonas europaea*, *Nitrosomonas cryotolerans*, and *Nitrosococcus oceanus* and from bacteria in seawater collected offshore near the Florida Keys. Partial sequencing of the amplification products verified that they were *amoA*. These primers, used in conjunction with a radiolabeled *amoA* gene probe from *Nitrosomonas europaea*, could detect *Nitrosococcus oceanus* inoculated into filter-sterilized seawater at 10^4 cells liter^{-1}. Native marine bacteria containing *amoA* could also be detected at their naturally occurring titer in oligotrophic seawater. Amplification of the gene for ammonia monooxygenase may provide a method to estimate the distribution and relative abundance of chemolithotrophic ammonium-oxidizing bacteria in the environment.

The chemolithotrophic ammonium-oxidizing bacteria carry out an essential step in the process of nitrification, oxidizing ammonia to nitrite through the intermediate hydroxylamine (38). These microorganisms are an integral component of nitrogen cycling in natural waters, but they are also involved in the cycling of methane and carbon monoxide (9, 11, 12), may contribute to the global cycling of trace gases such as nitrous oxide and nitric oxide (5, 16, 34), degrade numerous organic compounds (8), and are thought to contribute to the subsurface primary nitrite maximum found throughout large regions of the oceans (22, 23). Chemolithotrophic ammonium-oxidizing bacteria grow extremely slowly and may occur in low numbers in natural waters. This makes them difficult to detect in environmental samples by traditional methods such as most-probable-number estimates, viable titer estimates, and direct counting (33). Such traditional methods have indicated concentrations of only a few cells per liter in ocean waters (35). Fluorescent-antibody assays for the direct detection of *Nitrosomonas* and *Nitrosococcus* species have indicated that the numbers of these species are much higher than previously measured, in the range of 10^4 cells liter^{-1} (31, 32). A method of greater sensitivity capable of detecting all chemolithotrophic ammonium-oxidizing bacteria is still needed to estimate ammonia-oxidizing potential in environmental samples.

The PCR technique has been used to detect specific species, as well as groups of microorganisms, in a variety of environmental samples at concentrations previously considered undetectable (1, 24–26). One characteristic unique to the chemolithotrophic ammonium-oxidizing bacteria (3, 18) is the enzyme ammonia monooxygenase (AMO), which oxidizes ammonia to the intermediate hydroxylamine (38). Although AMO can oxidize a variety of substrates, these microorganisms can only acquire energy from the oxidation of ammonia (4, 7). This makes the gene for AMO a promising function-specific target sequence to detect ammonium-oxidizing bacteria in environmental samples by PCR. A polypeptide containing the active site of this unique monooxygenase has been purified from *Nitrosomonas europaea*, and its associated gene sequence (*amoA*; GenBank accession no. L08050) has been determined (18).

In this report we describe the use of oligonucleotide primers, targeting a 665-bp region of the published *Nitrosomonas europaea amoA* sequence (18), to amplify by PCR a region of the *amoA* gene from pure cultures of ammonium-oxidizing bacteria. These cultures included two members of the beta subdivision of proteobacteria (27, 37), *Nitrosomonas europaea*, found in soil and freshwater, and *Nitrosomonas cryotolerans*, a psychrotolerant ammonium-oxidizing species isolated from cold marine waters; and one member from the gamma subdivision (27, 36), *Nitrosococcus oceanus*, widely found throughout marine waters. Other researchers have recently reported the use of PCR to amplify a DNA fragment unique to *Nitrosomonas europaea* (21) and to specifically amplify a 16S rRNA gene sequence from ammonium-oxidizing bacteria (29). The work reported here, however, is the first describing the use of PCR primers to specifically amplify the functional gene encoding AMO. We report here the design of primers for the amplification of *amoA*, the identification of *amoA* amplified from these pure cultures by both hybridization and partial sequencing, estimation of the sensitivity of this *amoA* detection for

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control samples, and the use of this method to detect *amoA*-containing genomes in natural marine samples.

MATERIALS AND METHODS

Cultures. The ammonium-oxidizing bacteria *Nitrosomonas europaea* (ATCC 25978), *Nitrosomonas cryotolerans* (13), and *Nitrosococcus oceanus* (ATCC 19707) were grown in the dark in 4-liter chemostat culture units, as previously described (10, 28).

DNA isolation and purification from pure cultures. Cells from 1-liter samples of the continuous cultures described above were harvested by centrifugation, washed twice, and resuspended in 30 ml of sterile 1× STE buffer (17). DNA was isolated from these bacterial suspensions by using lysozyme, proteinase K, phenol-chloroform extraction, and ethanol precipitation (17). DNA extracts were further purified by CsCl ultracentrifugation, with CsCl added to a final refractivity of 1.39, for 48 h at 50,000 rpm in a Beckman L8-55M ultracentrifuge with a Beckman Ti SW65 rotor. After dialysis, extraction, and precipitation, genomic DNA concentration was determined by the A_{260} (17).

DNA isolation and purification from seawater. Seawater was collected in 8-liter polyethylene bottles (Nalgene) from oligotrophic surface waters near Fowey Rocks in the Florida Keys (25°35.400′N 80°06.000′W) and filtered by passage first through Whatman GF/F glass fiber filters and then through Gelman Supor-200 membrane filters (0.2-μm pore size). Cells of *Nitrosococcus oceanus* from continuous culture were harvested by centrifugation, washed, and resuspended in filtered seawater. The titer of the suspension was determined by direct cell count, using a Petroff-Hausser counting chamber with a Zeiss phase-contrast microscope. One-liter filter-sterilized seawater samples were inoculated with *Nitrosococcus oceanus* at specific titers of 10^3, 10^4, 10^5, 10^7, and 10^9 cells liter^{-1}.

To extract total genomic DNA from the control inoculated seawater and from unfiltered natural seawater, bacterial cells from the seawater samples were collected by passage through Millipore HV membrane filters (0.45-μm pore size) which were transferred to sterile 1.5-ml microcentrifuge tubes and stored frozen at −20°C until further processing. DNA was extracted from these filters by using the EnviroAmp Sample Preparation Kit (Perkin-Elmer-Roche) according to the manufacturer's directions, except that the lysis buffer contained 5% Chelex 100 (Bio-Rad). The resulting DNA extracts were passed through sterile membrane filter (0.2-μm pore size) spin columns, further purified with the GeneClean II Kit (Bio 101, La Jolla, Calif.), and ethanol precipitated. DNA pellets were resuspended in 50 μl of sterile distilled water, and 3 μl of 3% sterile bovine serum albumin (heat shock fraction V; Promega) was added to each sample. Ten-microliter portions of these DNA preparations were used in the PCRs.

PCR amplification and detection of *amoA*. Oligonucleotide primers targeting the *amoA* gene were designed from the published *Nitrosomonas europaea* sequence (18) (GenBank accession no. L08050) with the aid of OSP, the Oligonucleotide Selector Program (6), and synthesized with a PCR-Mate 391 DNA synthesizer (Applied Biosystems):

AMO-F: 5′-gggaattcAGAAATCCTGAAAGCGGC-3′
(T_m = 63.6°C; G+C = 50 mol%)

AMO-R: 5′-ggggatccGATACGAACGCAGAGAAG-3′
(T_m = 66.7°C; G+C = 57 mol%)

Lowercase sequences represent linkers with restriction sites, EcoRI for AMO-F and BamHI for AMO-R. AMO-F and AMO-R hybridize to nucleotide positions 269 to 286 and 917 to 934, respectively, of the published *Nitrosomonas europaea* sequence, producing a 665-bp amplification product.

Ten-microliter portions of genomic DNA were amplified by PCR with a model 480 DNA thermal cycler (Perkin-Elmer). PCRs were carried out in 100-μl final volumes with 10 μl of AmpliTaq 10× reaction buffer (Perkin-Elmer), 1 μM (each) AMO-F and AMO-R primers, 1 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, and 2 U of AmpliTaq polymerase (Perkin-Elmer) per reaction. Samples were denatured for 3 min at 95°C and then with 30 cycles of the following: 95°C for 1 min, 50°C for 30 s, and 72°C for 30 s. This was followed by a final extension at 72°C for 10 min. Reamplification followed the procedure described above except 1 μl of the previously amplified product was added for the target DNA.

To verify that amplification signals were not due to contamination of buffers or reagents with target DNA, a variety of negative PCR control samples were tested. These negative controls included filter-sterilized uninoculated seawater samples that were subjected to the same filtration, DNA extraction, and amplification procedures, sterile filters subjected to the same DNA extraction and amplification procedures, and PCR mixtures that received no target DNA.

Amplification products were electrophoresed on 2% agarose gels, and ethidium bromide-stained bands were digitally recorded with an SW-5000 video gel documentation system (UVP, Inc.) and processed with the PhotoFinish software package (ZSoft Corp.). For Southern blots, DNA was blotted from the gels to Nytran nylon membranes by alkaline capillary transfer (17) and cross-linked to the membranes with a Fisher Biotech FB-UVXL-1000 UV cross-linker. An *amoA* gene probe was prepared by radiolabeling the gel-purified 665-bp *amoA* amplification product from *Nitrosomonas europaea* with the Random Primers DNA Labeling Kit (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.). Membranes were hybridized overnight at 65°C in 7% sodium dodecyl sulfate (SDS)–1 mM EDTA–0.263 M Na_2HPO_4–1% bovine serum albumin and then washed twice for 30 min each time at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (17) with 0.5% SDS. Membranes were exposed to Kodak XAR-5 film, and the resulting autoradiograms were digitized and processed with the SW-5000 gel documentation system (UVP, Inc.).

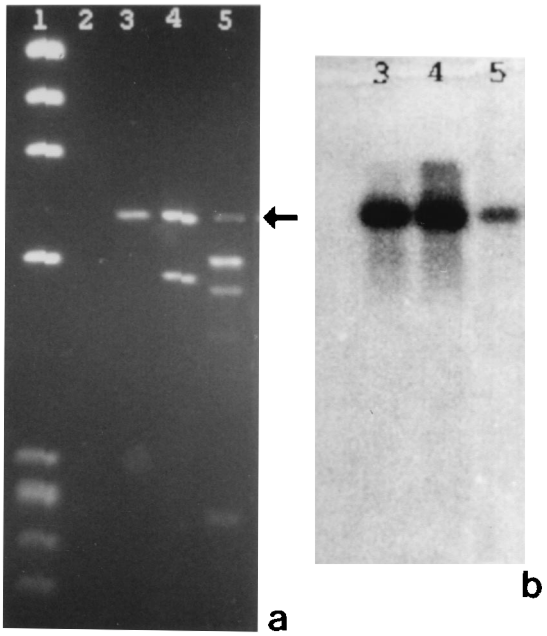

FIG. 1. Amplification with the AMO-F and AMO-R primers from pure cultures of ammonium-oxidizing bacteria (a) and *amoA* hybridization with the PCR products of these primers (b). (a) A 2% agarose gel stained with ethidium bromide and digitally photographed under UV light with a charge-coupled device (CCD) video camera, using a 0.8-s integration. Lane 1 contains phage ϕX174 DNA digested with *Hae*III as a standard size marker. Lane 2 was loaded with the amplification product from a negative PCR control mixture that received no target DNA. Lanes 3, 4, and 5 were loaded with the amplification products from *Nitrosomonas europaea*, *Nitrosomonas cryotolerans*, and *Nitrosococcus oceanus*, respectively. The arrow indicates the expected size of the *amoA* amplification product based on the published *amoA* sequence for *N. europaea* (18). (b) Autoradiogram from a Southern blot (24-h exposure) of the 2% agarose gel shown in panel a. DNA was transferred to a nylon membrane by alkaline capillary transfer and probed with the gel-purified and ^{32}P-labeled 665-bp *amoA* amplification product from *N. europaea.*

Partial sequencing of amplification products. PCR products were electrophoresed on 3% low-melting-temperature agarose gels, and the 665-bp bands were excised and purified with the Magic PCR-Preps kit (Promega, Madison, Wis.). The AMO-F and AMO-R primers were end labeled with [γ-^{32}P]dATP and used to partially sequence the gel-purified 665-bp PCR products with the Amplitaq Cycle-Sequencing Kit (Perkin-Elmer).

RESULTS

Design of primers and amplification of *amoA*. Primers were chosen to target the central portion of the *amoA* gene, encoding the active site of the AMO complex. These primers were chosen on the basis of the target product they amplified, physical characteristics such as G+C content (moles percent), similar melting temperatures, and lack of internal or primer-primer complementarity. Since chemolithotrophic ammonium oxidizers are a diverse group of microorganisms, species isolated from varied habitats representing both the beta and the gamma subdivisions of the proteobacteria were tested. We were uncertain of the ability of these primers to amplify *amoA* from such different organisms, but Fig. 1a shows amplification of CsCl-purified DNA from *Nitrosomonas europaea*, *Nitrosococcus oceanus*, and *Nitrosomonas cryotolerans*.

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LINEUP of: AMOA  from: 1  to: 660   October 28, 1993  14:53  ..

                270
 Fowey Rock                          .......... .nn....... ..........
   AMOa Amp  GAAATCCTGA AAGCGGCCAA GATGCCGCCG GAAGCGGTTC ATATGTCACG

                320
 Fowey Rock  .......... .......... .......... .......... ..........
   AMOa Amp  TCTGATTGAT GCAGTTTATT TTCCAATTCT GATTATTTTG CTGGTGGGTA

                370
 Fowey Rock  .......... .......... ...
  N oceanus                      .. .......... .......... ..........
  N cryotol        .... .......... .......... .......... ..........
   AMOa Amp  CCTACCACAT GCACTTTATG CTGCTGGCAG GTGACTGGGA TTTCTGGATG

                420
  N oceanus  .......... .......... .......... .......... ........
  N cryotol  .......... .......... .......... .......... ...
   AMOa Amp  GACTGGAAAG ATCGTCAATG GTGGCCGGTT GTAACGCCAA TCGTGGGGAT

                                   //

                718
  N oceanus       ...... .......... .......... .......... ..........
N europaea      ........ .......... .......... .......... ..........
  N cryotol              . .......... .......... .......... ..........
   AMOa Amp  TCGGTCTGCT GTTCTATCCG GGTAACTGGC CGATTTTTGG ACCAACCCAT

                768
  N oceanus  .......... .......... .......... ..
N Europaea   .......... .......... .......... .......... ..........
  N cryotol  .......... .......... .......... ....nn...n n.........
   AMOa Amp  TTGCCAATCG TTGTAGAAGG CACATTGCTG TCGATGGCTG ATTACATGGG

                818
N Europaea   .......... .......... .......... .......... ..........
  N cryotol  .......... .......... .......... .......... ..........
   AMOa Amp  ACATCTGTAT GTTCGTACAG GTACACCCGA GTATGTTCGT CATATTGAGC

                868
N Europaea   .......... .......... .......... ....
  N cryotol  .......... .......... ......
   AMOa Amp  AAGGTTCACT GCGTACCTTT GGTGGTCATA CCACGGTTAT TGCAGCATTC
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FIG. 2. Partial sequencing of *amoA* amplification products from the AMO-F and AMO-R primers. Multiple sequence alignments were produced with the LINEUP and PRETTY programs (Genetics Computer Group software package, provided via the Interdisciplinary Center for Biological Research Computing Facility). AMOa Amp, expected sequence of the amplification product from these primers based on the published *Nitrosomonas europaea* sequence (18); Fowey Rock, partial sequence data determined for the 665-bp product amplified from DNA isolated from the native bacterial population in 1 liter of seawater collected near Fowey Rocks, Fla. Dots indicate matches with AMOa Amp, mismatches are indicated by the letter of the base mismatch, and the letter n represents an undetermined nucleotide at that position.

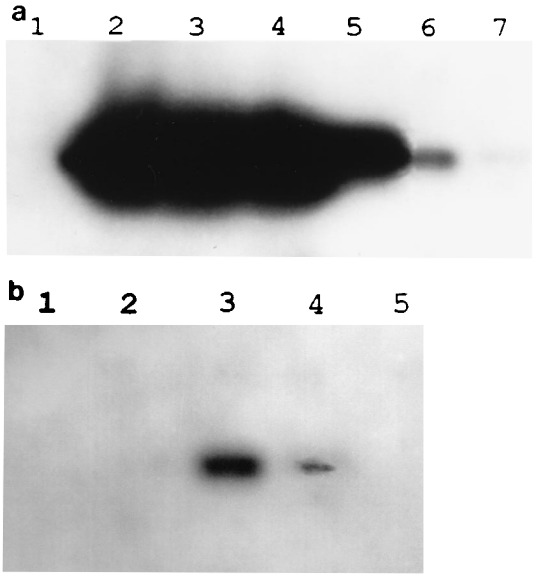

FIG. 3. Autoradiograms of a Southern blot (48-h exposure) showing the *amoA* amplification and hybridization signal for a range of *Nitrosomonas europaea* target DNA concentrations (a) and for DNA isolated from seawater samples with a range of known ammonium oxidizer titers (b). (a) PCR products were run on 2% agarose, blotted to nylon, and then probed and washed as described in the legend to Fig. 1b. Lane 1 contains phage ϕX174 DNA digested with *Hae*III as a standard size marker (not radioactively labeled). Lanes 2 to 7 show the *amoA* hybridization signal from PCR mixtures receiving 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg of CsCl-purified *N. europaea* DNA, respectively. This represents the theoretical DNA yield from approximately 2×10^7 (lane 2), 2×10^6 (lane 3), 2×10^5 (lane 4), 2×10^4 (lane 5), 2×10^3 (lane 6), and 2×10^2 (lane 7) cells. The PCR products in lanes 2 and 3 were diluted 1:100 before loading on the gel, and those in lanes 4 and 5 were diluted 1:10. Controls (not shown) consisting of PCR mixtures receiving no target DNA show no amplification or hybridization. (b) Lanes 1 to 5 were loaded with the PCR products amplified from 20% of the genomic DNA isolated from 1-liter samples of filtered seawater inoculated with *Nitrosococcus oceanus* at titers of 10^9, 10^7, 10^5, 10^4, and 10^3 cells liter^{-1}, respectively. This therefore represents the signal from 2×10^8 (lane 1), 2×10^6 (lane 2), 2×10^4 (lane 3), 2×10^3 (lane 4), and 2×10^2 (lane 5) cells. Amplification controls (not shown) of DNA extractions from filtered uninoculated seawater, a sterile filter, and a PCR mix with no target DNA added were run on a separate gel. No amplification or hybridization was seen for any negative control.

Nitrosomonas europaea, the organism from which the primers were designed, showed only a single amplification product of the expected size, 665 bp. *Nitrosococcus oceanus* and *Nitrosomonas cryotolerans* also amplified DNA of 665 bp as well as smaller products, which were reproducibly amplified independently of the purification method of the DNA. Variations in the banding patterns from these primers may prove to be diagnostic for these specific microorganisms.

Identifying *amoA* by hybridization. Because multiple amplified products were produced from these primers, we attempted to identify the sequences by hybridization with *amoA*. Only the bands of the expected size hybridized with the *amoA* probe (Fig. 1b), suggesting that the smaller amplified products were from other regions of the genome.

Partial sequencing of *amoA*. Because we wanted to use the *amoA* gene to identify and enumerate ammonium-oxidizing bacteria and to corroborate the identification of our amplification products as *amoA*, we sequenced portions of the 665-bp amplified product after gel purification (Fig. 2). The sequences from *Nitrosomonas europaea*, *Nitrosococcus oceanus*, and *Nitrosomonas cryotolerans* for the regions so far examined were identical to the reported *Nitrosomonas europaea amoA* sequence. Thus, species-specific *amoA* primers cannot be designed from our current sequence data.

Sensitivity of *amoA* detection from purified DNA. To determine how much *amoA*-containing DNA was required to give a detectable signal by amplification and hybridization, CsCl-purified genomic DNA from *Nitrosomonas europaea* was tested over a range of target concentrations (Fig. 3a). Some hybridization signal was detected from as little as 1 pg of target DNA (lane 7). Assuming an average bacterial chromosome size of about 4.7×10^6 bp, this would represent the DNA yield from approximately 200 cells.

Sensitivity of *amoA* detection from cells inoculated into seawater. Because a future goal of this project is to utilize *amoA* amplification and hybridization to detect and enumerate ammonium oxidizers, the minimum titer of ammonium oxidizers necessary for a detectable signal was investigated (Fig. 3b). Known numbers of *Nitrosococcus oceanus* were added to samples of cell-free seawater, and DNA was extracted and amplified as described above. A hybridization signal could be detected from the amplification products resulting from 20% of the DNA extracted from filtered seawater inoculated with 10^4 cells liter^{-1}. This method could thus detect as few as 2,000 cells from a seawater sample (lane 4). When 20% of the DNA extracted from higher titers (10^7 and 10^9 cells liter^{-1}) was tested, an amplification signal was not detected (Fig. 3b, lanes

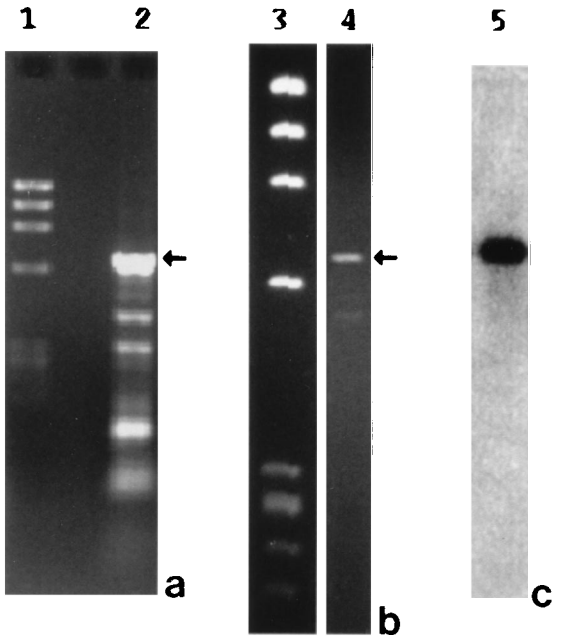

FIG. 4. (a) Initial amplification with the AMO-F and AMO-R primers from bacteria filtered from 1 liter of seawater collected near Fowey Rocks, Fla. Shown is a 2% agarose gel stained with ethidium bromide and digitally photographed under UV light with a CCD video camera, using a 3.6-s integration. Lane 1 contains phage ϕX174 DNA digested with *Hae*III as a standard size marker. Lane 2 was loaded with the PCR products amplified from 20% of the DNA isolated from 1 liter of Fowey Rocks seawater. The arrow indicates the expected size of the *amoA* amplification product based on the published *amoA* sequence for *Nitrosomonas europaea* (18). Amplification controls (not shown) of DNA extractions from filtered uninoculated seawater, a sterile filter, and a PCR mix with no target DNA added were run on a separate gel. No amplification or hybridization was seen for any negative control. (b) Reamplification of the gel-purified 665-bp product from the initial amplification of DNA isolated from the native bacterial population of Fowey Rocks seawater. Shown is a 2% agarose gel stained with ethidium bromide and digitally photographed under UV light with a CCD video camera, using a 0.8-s integration. Lane 3 contains phage ϕX174 DNA digested with *Hae*III as a standard size marker. Lane 4 was loaded with the PCR products reamplified from the sample shown in panel a, lane 2. No amplification was seen for a negative control sample receiving no target DNA (not shown). (c) *amoA* hybridization with the PCR products of the gel-purified and reamplified 665-bp product from Fowey Rocks seawater bacteria. Lane 5 is the autoradiogram from a Southern blot prepared as described in the legend to Fig. 1b of the gel shown in panel b, lane 4.

1 and 2). Amplification was seen when 0.2% of the DNA extracted from these titers was added to the PCR mixture (data not shown).

Detection of *amoA* in natural seawater samples. To verify that this method could detect naturally occurring bacteria containing *amoA*, we tested the *amoA* amplification and hybridization signal from genomic DNA extracted from the native bacterial population of 1 liter of oligotrophic seawater from Fowey Rocks, Fla. (Fig. 4), which was expected to have a relatively low titer (10^2 to 10^4 cells liter^{-1}) of chemolithotrophic ammonium oxidizers. PCR products seen in Fig. 4a (lane 2) were amplified from 20% of the total DNA isolated from 1 liter of seawater. A product of 665 bp, the expected size of the *amoA* product, was seen, as well as smaller products. To prepare enough of the 665-bp product for sequencing, the product was gel purified and reamplified (Fig. 4b, lane 4). The reamplified product hybridized to the *amoA* probe from *Nitrosomonas europaea* (Fig. 4c, lane 5), and the partial sequence determined for the reamplified product was identical to the published *amoA* sequence (Fig. 2).

DISCUSSION

The primers designed for this study amplified *amoA* from two widely divergent genera of ammonium-oxidizing bacteria, *Nitrosococcus* and *Nitrosomonas*. It was surprising to find that the partial sequence of the amplified product from *Nitrosococcus oceanus* was identical to the published sequence from *Nitrosomonas europaea*. Recently, two sequences for *amoA* from *Nitrosolobus multiformis* (accession no. U15733) and *Nitrosospira* sp. strain NpAV (accession no. U20644) have been directly submitted to the GenBank database (13, 14). These two sequences show a significant difference at the DNA level from the published *Nitrosomonas europaea* sequence; however, the *amoA* genes from these two organisms were identical. Interestingly, the regions that our primers were designed to target show five mismatches of 18 bases for the forward primer compared with the *Nitrosolobus* and *Nitrosospira* sequence and three mismatches of 18 bases for the reverse primer. It is believed that there are two copies of *amoA* in ammonium oxidizers (3, 18). It may be that if there are differences between these copies in some species, our primers may have only amplified one of the copies and that the sequence reported for *Nitrosolobus* and *Nitrosospira* species might represent the other copy. Comparison of these *amoA* sequences against the GenBank and EMBL databases with the BLAST program (2) indicated that they were unique, having no significant homology to other currently reported sequences, including other monooxygenases, and confirming previous observations (3, 18, 19). Thus, while *amoA* might not be appropriate for examining phylogenetic relationships, it may be a good target sequence for specifically detecting chemolithotrophic ammonium-oxidizing bacteria from environmental samples. We are continuing to sequence the *amoA* genes from *Nitrosomonas europaea*, *Nitrosomonas cryotolerans*, and *Nitrosococcus oceanus*, and we have designed additional PCR primers to test for the presence of the *Nitrosolobus*-type *amoA* sequence in these cultures.

With our current protocol we could detect down to 1 pg of CsCl-purified DNA, which represents the theoretical yield from 200 cells. Perhaps continued optimization would increase this sensitivity, but our primary interest is in the detection of *amoA*-containing bacteria in seawater. With our current methodology we could detect an *amoA* signal from 20% of the DNA extracted from 1 liter of seawater containing 10^4 ammonium oxidizers liter^{-1} (this represents the signal from approximately 2,000 cells). The step most limiting sensitivity is probably DNA isolation, particularly the quality of the DNA isolated from seawater. The higher titers did not amplify, unless diluted. This suggests that some PCR inhibitor may be copurifying with the DNA. Preliminary evidence from other experiments (not shown) suggests that these inhibitors may be from our artificial seawater, not natural seawater. Optimizing DNA isolation for *Nitrosococcus oceanus*, however, may not give optimal results for amplification of the bacterial population of seawater. Further optimization should involve increasing *amoA* detection sensitivity for DNA isolated from natural waters. Still, this procedure is at least as sensitive as the methods currently employed, being able to detect *amoA*-containing cells at the titers expected for marine waters. In addition, our assay should be able to detect more widely divergent ammonium oxidizers than the current immunological methods. It is unclear why higher titers of *Nitrosococcus oceanus* did not amplify as well as expected, but we are primarily interested in the lower limits. It is unlikely that the high titers tested would be observed in natural waters.

With our current protocol we could detect a strong amplification signal of the proper size from the native bacterial

population of 1 liter of oligotrophic seawater, in which the titers of ammonium oxidizers are expected to be relatively low. When the 665-bp product was gel purified and reamplified, hybridization and partial sequencing studies verified that this product from the native bacterial population was indeed *amoA*. We have thus shown that this method could detect *amoA* from the population of ammonium-oxidizing bacteria at naturally occurring titers in seawater. It is also interesting that among the smaller amplification products from the seawater sample, there appeared to be a combination of the banding patterns seen for the pure cultures, perhaps reflecting *amoA* amplification of a population of different ammonium oxidizers.

Further work on this project will involve improving detection sensitivity (modifying extraction procedures to improve the quality of DNA isolated from seawater), continued sequencing of *amoA* from a variety of cultures, and testing the specificity of several *amoA* primers and probes for both of the known *amoA* sequences against a wide variety of microorganisms. In addition, coamplifiable internal controls (20, 30) will be developed for the quantitation of *amoA* amplification from environmental samples.

The work reported here indicates that PCR primers and hybridization probes targeting the *amoA* gene should be sensitive enough and general enough to detect a broad range of bacteria containing AMO in environmental samples. Furthermore, this approach can be used to detect unidentified *amoA*-containing species in natural waters without the need for isolating or culturing these microorganisms.

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