MICROBIAL ECOLOGY

Microb Ecol (1997) 33:87–96

Phylogenetic Diversity of Natural Populations of Ammonia Oxidizers Investigated by Specific PCR Amplification

B.B. Ward,[1] M.A. Voytek,[1] K.-P. Witzel[2]

[1] Institute of Marine Sciences, University of California, Santa Cruz, Santa Cruz, CA 95064, USA
[2] Max Planck Institut für Limnologie, D-24306 Plön, Germany

Received: 25 September 1995; Revised: 15 January 1996; Accepted: 20 February 1996

Abstract

The species composition of ammonia-oxidizing bacteria in aquatic environments was investigated using PCR primers for 16S rRNA genes to amplify specific subsets of the total ammonia-oxidizer population. The specificity of the amplification reactions was determined using total genomic DNA from known nitrifying strains and non-nitrifying strains identified as having similar rDNA sequences. Specificity of amplification was determined both for direct amplification, using the nitrifier specific primers, and with nested amplification, in which the nitrifier primers were used to reamplify a fragment obtained from direct amplification with Eubacterial universal primers. The present level of specificity allows the distinction between *Nitrosomonas europaea, Nitrosomonas* sp. (marine) and the other known ammonia-oxidizers in the beta subclass of the *Proteobacteria.* Using total DNA extracted from natural samples, we used direct amplification to determine presence/absence of different species groups. Species composition was found to differ among depths in vertical profiles of lake samples and among samples and enrichments from various other aquatic environments. Nested PCR yielded several more positive reactions, which implies that nitrifier DNA was present in most samples, but often at very low levels.

Introduction

The oxidation of ammonium to nitrite, as the first step of nitrification, is performed by ammonia-oxidizing bacteria in soils, sediments, freshwater, and marine environments. Evidence from serological studies using immunofluorescence [22] indicated that most autotrophic ammonia-oxidizing isolates from seawater could be assigned to one of two serogroups of marine ammonia-oxidizing bacteria, *Nitrosomonas* sp. (marine) and *Nitrosococcus oceanus.* Bacteria that reacted with these sera could be identified and enumerated in various marine and estuarine environments on the basis of this seroreactivity [19, 21, 22, 25].

Ribosomal RNA sequence data [5, 16, 28, 29] detected two main lines of descent within the ammonia oxidizers. One line is in the beta-subclass of the *Proteobacteria* and includes two clusters: *Nitrosomonas* (including the previously identified groups *Nitrosomonas* and *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosospira, Nitrosovibrio,*

Correspondence to: B.B. Ward. e-mail: bbw@cats.ucsc.edu

Table 1. Results of PCR amplification of known strains using enbacterial universal (EUB) primers and three sets of nitrifer-specific primers (NitAB, NitAF, NitDB)

Strain	Source[a]	Morphology[b]	Environment[c]	Serotype[d]	EUB	NitAB	NitAF	NitDB
Beta-subclass ammonium oxidizers								
Nitrosomonas europaea	a	R	terrestrial	Nm	+	+	+	+
Nitrosomonas sp. (WH-2)	a	R	terrestrial	Nm	+	+	+	+
Nitrosomonas eutropha C-19	b	R	terrestrial	nr	+	+	+	−
Nitrosomonas sp. (marine)	b	R	marine	Nm	+	+	−	−
Nitrosolobus multiformis C-71	b	L	terrestrial	nr	+	+	+	−
Nitrosospira briensis C-128	b	S	terrestrial	nr	+	+	+	−
Nitrosovibrio tenuis NV-1	b	V	terrestrial	nr	+	+	+	−
Nitrosococcus mobilis NC-2	b	C	marine	nr	+	+	+	−
Gamma-subclass ammonium oxidizers								
Nitrosococcus oceanus	b	C	marine	No	+	−	−	−
Nitrosococcus oceanus	c	C	marine	No	+	−	−	−

[a] a, E.L. Schmidt (University of Minnesota, Minneapolis, Minn.) b, S.W. Watson (Woods Hole Oceanographic Institution, Woods Hole, Mass.) c, A.F. Carlucci (Scripps Institution of Oceanography, La Jolla, Calif.).
[b] Morphology: C, coccoid; R, rod; V, vibrio; S, spiral; L, lobular.
[c] Environment: terrestrial, freshwater medium; marine, seawater medium.
[d] Serotype [22]: Nm, *Nitrosomonas* sp. (marine); No, *Nitrosococcus oceanus;* nr, no reaction.

and *Nitrosolobus*). The other line of descent is in the gamma-subclass (*Nitrosococcus oceanus*). Voytek and Ward [17] showed that the *Nitrosomonas* serotype is a subset of the *Nitrosomonas* 16S rRNA group, which is, in turn, a subset of the beta-subclass ammonia-oxidizer group. They found that all of the *Nitrosomonas* serotype organisms, as well as all nine known strains in the beta subclass ammonia-oxidizer group, amplified with a set of PCR primers designed for selective amplification of the beta-subclass nitrifiers. *Nitrosococcus oceanus*-type organisms did not amplify with the beta-subclass primers, and constituted a different serogroup.

In initial experiments with DNA extracts of natural samples using the nitrifier primers, Voytek and Ward [17] were able to amplify nitrifier 16S rDNA from the PCR products obtained from amplifications of total DNA with universal eubacterial 16S rDNA primers. The nitrifier target sequences were detected in water samples from a permanently frozen lake in Antarctica, and from as deep as 900 m in the Southern California Bight, a marine basin off the coast of California. Detection of this group in such different environments suggested that sequences amplified from the environment were probably in the same rRNA group as culturable ammonia-oxidizing bacteria from which the primers were developed, and that individual genera of ammonia oxidizers have global distributions.

In order to investigate the diversity of natural populations of ammonia oxidizers at a more specific level, PCR primers with specificity for different species groups (see below) within the beta-subclass of nitrifiers were investigated. These primers were used to determine the distribution of different species of ammonia oxidizers from a variety of aquatic habitats.

Materials and Methods

Strains

Nitrifying bacteria used for characterization of the PCR primers and amplification assays were obtained from the sources listed in Table 1 and grown in media based on either distilled water [15] for the terrestrial strains or seawater [24] for the marine strains, as indicated.

Primers

The sequence and location in the *Escherichia coli rrnb* genome sequence for each of the primers used in this study are shown in Fig. 1. The primer set that amplifies the entire beta-subclass group of ammonia oxidizers has been described [17]; these primers are designated NitA and NitB (Fig. 1). They amplify a 1,080 bp fragment near the 5′ end of the rRNA gene. Two additional primers, NitF (5′-AGCTACGTTACCAGTCCGT) and NitD (5′-TAGTCGGAAAGAAAGAGTTGCAA) were designed for use in combination with NitA and NitB. The NitA-NitF primer pair generates a fragment approximately 700 bp in size, internal to the NitA-NitB fragment. The NitD-NitB primer pair generates on

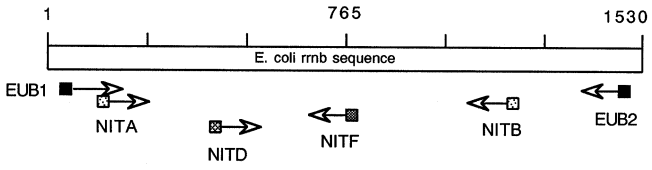

Fig. 1. Map of primer locations and sequences relative to *E. coli* RNA gene.

800 bp fragment, also internal to the NitA-NitB fragment. Eubacterial (EUB) universal primers were those described by Liesack et al. [8]. The EUB primers (5′ positions 9–27 and 3′ positions 1,525–1,542 in the *E. coli* sequence) amplified a region of approximately 1,530 bp.

PCR

Amplifications were performed using either a Perkin-Elmer Cetus DNA Thermocycler (the lake and enrichment samples) or a Barnstead Thermolyn Amplitron-1 (for the pure culture work). Reactions were done in 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1% Triton X-100, 1.25 mM $MgCl_2$), with 200 μM of each deoxynucleotide triphosphate, 0.1 μM each primer, 1 μl template DNA, and 2 U of Taq DNA polymerase (Promega Biological Research Products, Madison, Wis.) in a total volume of 100 μl. The standard amplification reaction for the EUB and NitA-NitB primers used the following cycle: 10 min at 95°C, pause at 82°C to add polymerase, then 35 cycles of 2 min at 57°C, 2.5 min at 72°C, 1 min at 94°C, followed by a final annealing step and a last 5 min elongation at 72°C. The standard reaction for the NitA-NitF and NitD-NitB primer pairs were exactly the same, except annealing occurred at 60°C. One μl of sample DNA extract was routinely amplified (see below). Taq polymerase and dNTPs were obtained from Boehringer Mannheim. PCR products were visualized in 0.7% agarose gels using standard electrophoresis procedures.

The specificity of the primers was tested using genomic DNA from known nitrifying strains in culture (see below for DNA purification). A selection of nonnitrifying strains (see [17]) was chosen from strains showing potential homology with the primer sequences in the GENBANK and EMBL databases and the RNA data base project (RDP; [10]). Sequence comparisons were made using the Experimental GENINFO BLAST Network Service (National Center for Biotechnology Information). These strains were also tested for amplification using the standard reaction conditions. Serial dilutions of genomic DNA from *N. europaea* were used to estimate the sensitivity of the amplification reaction. To detect the presence or absence of different groups, the different Nit primer pairs were used to amplify nitrifier DNA directly from the DNA extract, or to reamplify DNA that had been initially amplified using the eubacterial universal primers or the general beta-subclass primers, NitA-NitB. For reamplification, 1 μl of the first PCR mixture (i.e., the successful EUB amplification) was used as a template in the second amplification reaction with NitA-NitB, NitA-NitF, or NitD-NitB primers. Reaction conditions for the second, or nested, amplification were exactly those used for the direct amplification, except that annealing was always at 60°C.

DNA purification

Genomic DNA was purified from pure cultures of bacteria by standard methods as previously described [17]. Total DNA was purified from aquatic samples using the protocol described by Ward et al. [26] with the following modifications. Particulate material from 4 liters of water was concentrated using Amicon tangential flow fiber filters (Type H1 P100 fiber cartridge). The final concentrate, resuspended in TE (1 mM EDTA, 10 mM Tris, pH 8), was centrifuged in a microcentrifuge to produce a pellet which was resuspended in 0.8 ml of 0.5 M EDTA. The pellet was vortexed vigorously to resuspend, and could be stored frozen until extraction. The Aufwuchs sample from Belauer See, which consisted of the scum scraped off the stems of aquatic plants and collected by centrifugation, was washed in TE and then resuspended in 0.5 M EDTA and frozen. Aliquots (1–3 ml) from cultures were collected by centrifugation, washed in TE, and resuspended in 0.5 M EDTA and frozen. The 0.5 M EDTA suspensions were thawed and extracted as previously described [26]. Aliquots of this extract (1 μl) were amplified directly, unless otherwise noted.

For enrichment cultures, and other smaller volume samples, a simple chelex extraction method [18] was also used to obtain amplifiable DNA. Small volumes (20 μl to 1.5 ml) were centrifuged and the pellet washed in buffer (0.05 mM EDTA, 100 mM Tris, pH 8). Chelating resin (iminodiacetic acid, Sigma, St. Louis, Mo.; 200–500 μl; 10–15% suspended in the same buffer) was added, mixed well, and the sample heated at 80°C for 25 min, then boiled for 10 min. The sample was centrifuged and the supernatant used directly for amplification. In the case of the sewage treatment plant sample, the initial chelex purification was extracted once with phenol, the DNA was precipitated with propanol and dissolved in TE prior to amplification.

Samples

The study sites included Schöhsee, Plußsee, and Belauer See (three lakes in Schleswig Holstein, Germany), Hohwachter Bucht on the Baltic coast of Germany, and the sewage treatment plant near the town of Plön, Germany. Lake samples were obtained using a 2-liter Ruttner sampler closed repeatedly at each depth in order to obtain 4 liters of water. The same sampler was used to obtain water for N_2O determinations. Gas samples were collected in 50-ml serum vials without headspace and preserved with 2% formalin (see below).

Enrichment cultures from various sources were obtained by inoculating medium for ammonia oxidizers [13] with 0.1 vol of sample and repeated subculturing of actively nitrifying cultures.

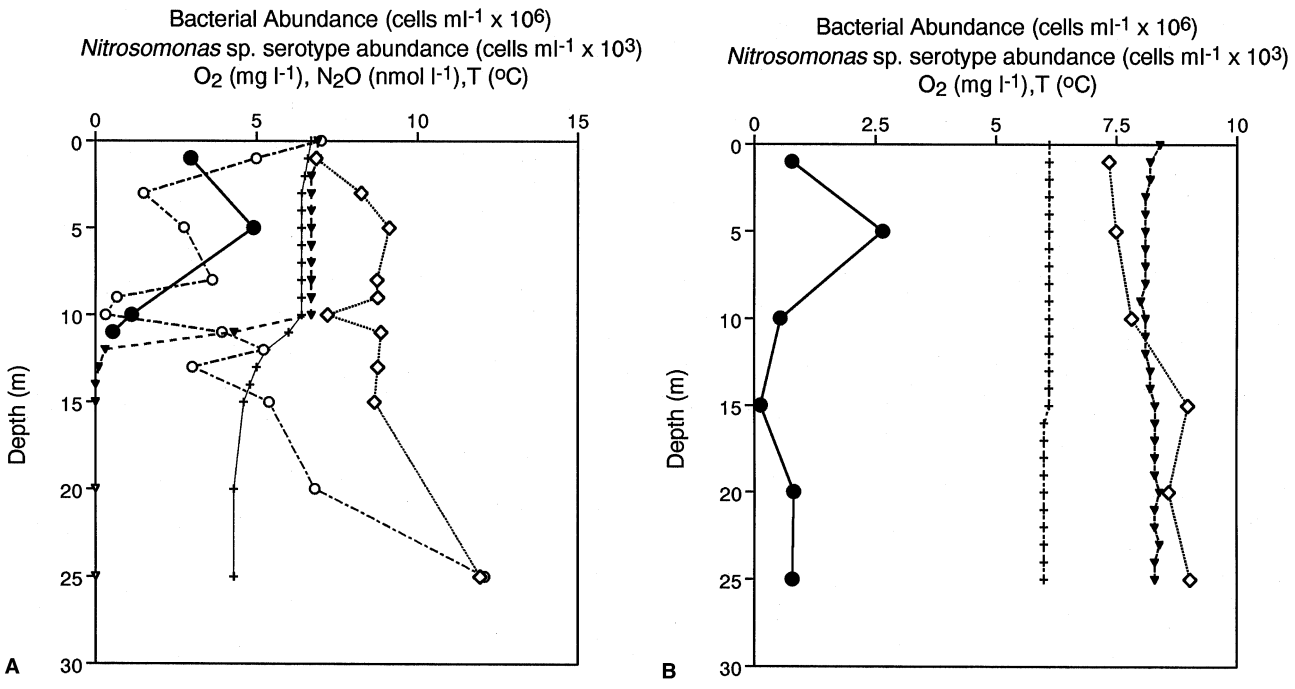

Fig. 2. Hydrographic data from **(A)** Plußsee, 2 November 1993 and **(B)** Belauer See, 16 November 1993. Total bacterial abundance, (◇); *Nitrosomonas* serotype cells, (●); oxygen, (▼); temperature, (+); nitrous oxide, (○). (N_2O not shown for Belauer See.)

The salt content of the medium was adjusted to match that of the environment of origin.

Bacterial Abundance

Total bacteria were enumerated using DAPI staining and epifluorescence microscopy [12]. *Nitrosomonas* serotype ammonia oxidizers were enumerated in selected lake samples using an antiserum produced against *Nitrosomonas* sp. (marine) [19]. This antiserum reacts with several terrestrial and aquatic ammonia-oxidizing isolates, and has a reaction with *N. europaea* equivalent to that of the immunizing strain. Up to 20 ml of preserved lake water (2% v/v formalin) was filtered and cells stained by the procedures previously described [22]. The serotype designation of the beta-subclass ammonia oxidizers was reported previously by Ward and Carlucci [22] and Voytek and Ward [17].

Ancillary Measurements

Temperature and oxygen concentration with depth were measured using a handheld WTW OXI 191 Oximeter. N_2O was measured in formalin-preserved samples after equilibration with a headspace of helium, using a Siemens Dichromat 1-4 gas chromatograph (Seimens AG, Karlsruhe, Germany) with N_2 as a carrier gas, a Poraplot Q column and a ^{63}Ni ECD detector [4]. Dissolved N_2O was calculated using the solubility coefficients of Weiss and Price [27]. Dissolved nutrients were determined with a Technicon (Technicon Instrument Corporations, Tarrytown, NY) continuous flow system according to Albrecht [1].

Results

Hydrographic Data

The two lakes from which the depth profiles were obtained were sampled at different stages of fall overturn. The seasonal cycle of nutrients [7] and the general microbial ecology [11] for the Plußsee has been described. Plußsee, sampled 2 November, was still stratified with surface waters at 8°C and deep waters at about 5°C (Fig. 2A). The weakening thermocline between 9 and 11 m coincided with the oxic/anoxic interface. Phosphate and ammonium had accumulated to high levels below 11 m, where concentrations of nitrite and nitrate were low (not shown). Nitrous oxide concentration exceeded atmospheric saturation both above and below the interface. The total bacterial abundance approached 10^7 cells ml^{-1} throughout the water column, with a minimum in the surface layer and slightly higher numbers in the deepest sample. The minimum at the oxic/anoxic interface appears to be statistically significant (ave = 6.84×10^6, SD = 0.33×10^6, $N = 3$).

The Belauer See was sampled two weeks later (16 November) and was no longer stratified (Fig. 2B). Temperature was constant at 6°C, oxygen was uniform throughout at slightly more than 8 mg ml^{-1}, and the major nutrients showed little variation with depth, being uniformly high.

Total bacterial abundance increased slightly from the surface to the deepest sample, and was on the order of 7–10 × 10^6 cells ml^{-1}.

Primer Specificity

The specificity of the NitA-NitB primer set has been described [17]: the NitA-NitB primers amplify all nine sequenced strains of beta-subclass ammonia oxidizers [5]. As reported previously, an extensive suite of nonnitrifying strains, identified by sequence comparisons ([10]; DNASTAR, Inc., Computer Systems for Molecular Biology and Genetics, London) as being similar to the target sequences was tested for amplification with the NitA-NitB primers. The only known strain that amplifies with the NitA-NitB primers under the standard reaction conditions is *Spirillum volutans* [17] and this is also the only strain in which reamplification with the NitA-NitB primers of the EUB product yields a product that is approximately the correct size.

For the NitD-NitB and NitA-NitF primer sets, specificity was initially evaluated by comparison of sequences in GENBANK and EMBL databases ([10]; DNASTAR, Inc, Computer Systems for Molecular Biology and Genetics). Amplification experiments with known strains verified the predicted specificity and fragment size (Table 1). Under the standard conditions described above, the NitA-NitF primer pair amplified all members of the beta-subclass ammonia-oxidizer group except *Nitrosomonas* sp. (marine). The sequence of NitF differs by 2 bp from the NitC oligonucleotide probe used previously [17] to verify the identity of NitA-NitB amplification fragments. This difference is enough that NitC amplifies *Nitrosomonas* sp. (marine) and NitF does not. Thus, an unknown nitrifier target can be tentatively identified as *Nitrosomonas* sp. (marine) if it amplifies with the NitA-NitB pair, but with neither of the NitD-NitB nor NitA-NitF primer pairs. The NitD-NitB primer set was designed to be specific for *N. europaea* (100% homology). NitD has less than 80% similarity with all other species in the group (and in the database) except *N. eutropha* (83% homology). The NitD-NitB primer set was indeed specific, amplifying only *N. europaea* and one other strain, *Nitrosomonas* WH-2, which, on the basis of sequence information (Voytek, unpublished), is indistinguishable from *N. europaea* (Table 1). None of these primers amplified strains designated as *Nitrosococcus oceanus* in the gamma-subclass of the *Proteobacteria*. No amplification (either direct or nested within the EUB amplification fragment) with any nonnitrifying strains was observed for the NitA-NitF and NitD-NitB primer sets (data not shown; strains tested are listed in Table 1 of ref [17]).

Sensitivity of PCR Assay for Nitrifiers

The minimum concentration of nitrifier DNA that could be amplified directly by the Nit primer pairs was estimated by amplification of serial dilutions of DNA from pure cultures of *N. europaea.* The NitA-NitF and NitD-NitB primers reproducibly amplified target sequence from less than 1 pg of purified DNA, as did the NitA-NitB primers (Voytek and Ward, unpublished). The detection limit for amplification of known target sequence in DNA in extracts from complex natural populations was not determined.

Distribution of Ammonia-Oxidizer DNA in Lake Samples

Depth profile samples from two of the lakes, and individual samples from other sources, were tested for amplification with the Nit and EUB primers. All lake DNA samples amplified directly with the eubacterial universal primers (Table 2). Samples from most of the depths in the Plußsee amplified directly with the NitA-NitB primer pair, but only one of the Belauer See samples did so. Surprisingly, direct amplification with the NitA-NitF and NitD-NitB primer sets was successful in Belauer See samples that did not amplify directly with the NitA-NitB primers. Although the NitA-NitB set is comprehensive in terms of its ability to amplify the beta-subclass ammonia oxidizers (experiments on DNA extracted from pure cultures, Table 1), we have observed differences in the efficiency and apparent sensitivity of the different primer pairs; this may explain their different behavior in natural samples (see discussion).

NitA-NitB target DNA was detected in nearly every sample when amplified from a fragment that resulted from initial amplification with eubacterial primers (two-stage amplification; Table 2). In fact, the NitA-NitF and NitD-NitB pairs also amplified many more samples in the second stage amplification than they did directly from the DNA extract (Table 2). The Nit primers amplify the eubacterial amplification fragment with greater sensitivity because the eubacterial amplification product is highly enriched in target sequences relative to the complex whole DNA extract.

Enrichment Cultures

Results of direct and two-stage amplification of three enrichment cultures of ammonia oxidizers are shown in Table

Table 2. Amplification of environmental samples with EUB and Nit primers

Site	Depth (m)	Direct amplification of total DNA extract				Reamplification from product of amplification with EUB primers		
		EUB	NitAB	NitAF	NitDB	NitAB	NitAF	NitDB
Plussee	1	+	+	−	+	+	+	−
(2 Nov 1993)	3	+	+	−	−	+	+	+
	5	+	−	−	−	+	+	−
	8	+	+	−	−	+	+	−
	9	+	−	−	+	−	+	−
	10	+	+	−	+	+	+	+
	11	+	+	−	−	+	+	+
	12	+	+	+	−	+	+	−
	13	+	+	+	+	+	+	+
	15	+	−	−	−	+	−	+
	20	+	+	+	−	+	+	+
	25	+	+	+	−	+	+	+
Belauer See	1	+	−	−	+	+	+	−
(16 Nov 1993)	5	+	+	+	+	+	+	+
	15	+	−	−	−	+	+	+
	20	+	−	−	−	+	+	+
	25	+	−	+	−	+	+	+
	Inlet	+	−	+	−	+	+	+
	Littoral	+	−	+	−	+	+	+
	Aufwuchs	+	−	+	−	+	+	+
Schohsee (6 Dec 1993)	0	+	+	+	+			
Baltic coast (6 Dec 1993)	0	+	+	+	−			
Sewage treatment plant (6 Dec 1993)		+	+	−	+			

3. The MTP enrichment amplified directly with all Nit primer pairs, while the M1 enrichment could be amplified only with the Nit primers after amplification with the EUB primers. The Puget Sound enrichment amplified directly with NitA-NitB and NitA-NitF, but not NitD-NitB.

Immunofluorescence

The distribution of cells detected by immunofluorescence staining with the *Nitrosomonas* antiserum is shown for the Plußsee and Belauer See depth profiles in Fig. 2. In both lakes, maximum *Nitrosomonas* sp. (marine) serotype abundances were observed at 5 m (4,898 and 2,650 cells ml^{-1} for the Plußsee and Belauer See, respectively). Immunofluorescence counts were not obtained from samples in the anoxic bottom layer of the Plußsee, because nitrifiers were not expected to occur in the anoxic layer; *Nitrosomonas* sp. (marine) serotype abundance had decreased to 536 cells ml^{-1} at the oxic/anoxic interface. *Nitrosomonas* sp. (marine) serotype cells were detected at all depths in the Belauer See at levels from 122 cells ml^{-1} (15 m) up to the maximum of 2,650 cells ml^{-1} observed at 5 m. Due to limitation on the amount of antiserum available, only single counts, instead of replicates, were made. These counts exceed the limit of detection for reproducible immunofluorescence counts [23], which was estimated to be 20 cells ml^{-1} for 20-ml sample volumes. Previous use of this technique [22, 23] would suggest a coefficient of variance for such counts of about 30%.

Discussion

All the sample DNAs amplified directly with EUB primers. It appears, therefore, that failure of the nitrifier primers to amplify some samples was not due to inhibition of the polymerase by some artifact of the extraction method. It is still not straightforward, however, to deduce the relative abundance of each target in the DNA sample. Such quantification would require the use of internal standard fragments that amplified with each of the different primer sets [2], and this was not done in the present study. Therefore, the dis-

Table 3. Amplification of enrichment cultures with EUB and Nit primers

Culture	Source	Direct amplification of total DNA extract				Reamplification from product of amplification with EUB primers		
		EUB	NitAB	NitAF	NitDB	NitAB	NitAF	NitDB
MTP	Belauer See	+	+	+	+	+	+	+
M1	Hypersaline pond	+	−	+	+	+	−	+
PS	Puget Sound	+	+	+	−	+	+	−

cussion is restricted to interpretation of amplification results in terms of presence/absence of different target sequences. Even presence/absence determinations depend on the relative efficiency of different primer sets. We observed that the NitA-NitB primers, which were designed to have the broadest specificity, exhibit the lowest sensitivity. The more specific primer sets, e.g., NitD-NitB, are more sensitive: they amplify the *N. europaea* target even in samples in which the NitA-NitB primers failed to amplify (Table 2).

Immunofluorescence vs. DNA

The data on cross reactivity with immunofluorescence and amplification with different primer sets, presented here and previously [17], allows us to assess the relative specificity and usefulness of the two assays. The *Nitrosomonas* antiserum is much more specific than the NitA-NitB primer set, in that it recognizes only some of the *Nitrosomonas* strains and does not react with any of the other beta-subclass ammonia oxidizers. It was previously reported [22] that the *Nitrosomonas* antiserum appeared to have relatively broad specificity in that it reacted with many otherwise unidentified ammonia oxidizers isolated from various aquatic environments. Those results can now be interpreted to suggest that enrichment and isolation methods used to obtain those strains had selected for *Nitrosomonas* types.

Immunofluorescence based on cell-surface antigens has both advantages and disadvantages in ecological studies. Its primary advantage is that individual strains can be quantified in natural samples. Its disadvantages are that the strain must first be cultured in order to produce the antibody (which is a serious constraint because of the low culturability of natural populations), and that the detection is based on antigenic characteristics that may be quite variable and not particularly linked to the genetic identify of the strain.

Both of these disadvantages are overcome in large part by PCR. Although the primer sequences in this case were derived from cultured strains, they are capable of detecting target in samples where organisms cannot be cultured. rDNA primers target, specifically, the one molecule containing the most phylogenetic information, so that primers with different specificities can be designed explicitly to include or exclude certain types. Thus, PCR analysis of natural samples may, in the future, allow us to determine the extent to which isolates represent the natural population diversity.

Structure and Diversity of Natural Populations

The use of different primer sets which selectively amplify different members of the ammonia-oxidizer population makes it possible to investigate the structure and diversity of in situ populations without enrichment, culturing, or microscopy. Based on the results of amplification with the different primer sets when tested on pure cultures of known strains of ammonia-oxidizing bacteria, the three Nit primer sets can be used to define and detect subgroups within the group of ammonia oxidizers that have been previously characterized as members of the beta subclass of the *Proteobacteria.* Samples that amplify with the NitA-NitB and NitA-NitF primers contain at least some cells from this group. Samples that amplify with the NitD-NitB primers are specifically identified as containing *N. europaea. Nitrosomonas* sp. (marine) is the only strain in the database that amplifies with NitA-NitB but not NitA-NitF; samples that show this pattern must contain *Nitrosomonas* sp. (marine) or another similar type not presently in the culture collection. In the case of enrichments or unidentified isolates, differential amplification can be used to identify the organisms present. In the case of natural samples, differential amplification can be used to gain insight into population composition of different ammonia oxidizers in the population.

While one attraction of PCR is that isolation is no longer necessary for identification and detection of target strains, isolation and culture methods remain important for biochemical and physiological studies and the development of probes for genes other than ribosomal. Thus, PCR with

primers of known specificity is an additional way to characterize enrichment cultures and isolates [9]. On the basis of differential amplification, it can be concluded that different species were present in the enrichment cultures obtained from different sources. MTP, the enrichment from Belauer See, was probably *N. europaea;* it amplified with the specific *N. europaea* primer set, NitD-NitB. The M1 enrichment, obtained from the top centimeter of a microbial mat of an experimental, hypersaline (80°/°°) pond at the Steinitz Marine Laboratory in Eilat, Israel, also probably contained *N. europaea*–type cells (NitD-NitB) even though it did not amplify directly with NitA-NitB. The Puget Sound enrichment culture amplified with NitA-NitF but not with NitD-NitB. It appears, therefore, that this culture contains neither *N. europaea* nor *Nitrosomonas* sp. (marine), but another member(s) of the beta-subclass group. The cells in this enrichment stained brightly with the *N. marina* antiserum and were rod-shaped. They thus appear to be another marine strain of *Nitrosomonas.*

Based on results of direct amplification only, the population composition in terms of the Nit primer specificities varied with depth in the Plußsee and Belauer See (Table 2), but members of the beta-subclass group of ammonia oxidizers were present in most samples we analyzed. Using two-stage amplification, nitrifier sequences were detected in every sample. It can be seen (Table 2) that targets amplified by the two selective primer sets, NitD-NitB and NitA-NitF, have different depth distributions in the Plußsee. In the two-stage amplification, the NitD-NitB target was not detected in some of the upper layer samples, while the NitA-NitF target was found throughout the profile. In the Plußsee, only one depth below theoxic/anoxic interface amplified directly with the NitD-NitB primers, while NitA-NitF amplified at four of the five depths sampled at greater than 11 m (Table 2). Although absolute abundances cannot be determined, detection by direct amplification discerns which types were present at levels detectable in complex samples without preselection (by amplification with eubacterial primers). These results may imply that different strains of ammonia oxidizers were present at different relative abundances at different depths, but quantitative methods will be required to test this hypothesis.

In the Belauer See, all three Nit primer sets detected target throughout the water column. The greater differences in target distribution with depth in the Plußsee may correlate with the fact that the Plußsee samples were collected while the water column was stratified, and the Belauer See samples were collected after destratification. In three samples (Plußsee 1 m, 9 m, and Belauer See 1 m), NitD-NitB detected target in the whole DNA extract but not in the eubacterial amplification fragment. Because small differences in efficiency can drastically affect the outcome of the PCR, loss of the NitD-NitB reaction may be due to selection by the eubacterial primers, such that different nitrifier sequences were differentially represented in the eubacterial fragment.

The metabolism of ammonia oxidizers is narrowly restricted to chemoautotrophy, and would seem to leave little room for adaptive diversification. This is consistent with the tight phylogenetic clustering observed among known ammonia oxidizers in the two subclasses of the *Proteobacteria* where they occur. However, the presence of multiple strains in one environment, and their differential distribution as detected by selective primer amplification, implies that environmental variables are capable of inducing selection and therefore imposing structure within the ammonia-oxidizer population of an environment. Oxygen concentration, temperature, and inorganic nutrient concentration are all variables across the oxic/anoxic interface of the Plußsee that might cause the observed distributions of ammonia oxidizer types. Population structure in the nitrifiers was also detected in the Belauer See, even though chemical distributions indicated that overturn was complete and physical/chemical stratification had been destroyed. Both *Nitrosomonas* sp (marine) serotype distribution and amplification results suggest that some other factor might be exerting selective influence in the Belauer See at this time.

Similarly, variations in salinity and organic content could be important variables in determining the composition of nitrifier populations in the other three samples shown in Table 2. The Schöhsee is a low-productivity freshwater lake; the Baltic is a moderately saline, moderately eutrophic coastal marine environment and the sewage treatment plant sample had a very high organic load and low salinity. *N. europaea* was not detected in the Baltic sample, but was present in the other two, implying that salinity could be a determining factor in its distribution. The failure of NitA-NitF to amplify the sewage treatment sample, however, suggests the presence of *Nitrosomonas* sp. (marine), the marine analog of *N. europaea,* in this environment.

Results from the three enrichments (Table 3) imply the importance of other factors as well. *N. europaea* was detected in both the Belauer See (low salinity) and the hypersaline pond enrichments, but not in the Puget Sound enrichment. Sequencing or restriction analysis could reveal just how similar the Belauer See and mat enrichment strains are. Both were identified as *N. europaea* by PCR, but the mat enrich-

ment, for example, might be a new strain with high salt tolerance. *N. europaea* is reported to have limited salt tolerance [6]. Enrichment cultures are not entirely reliable indicators of selective factors in the environment, however, due to the selective nature of enrichment itself. Identification of possible selective factors, whether in enrichment cultures or within environmental heterogeneity, should help identify environmental conditions that might harbor as-yet uncultured strains of ammonia oxidizers, and guide the development of enrichment techniques to culture these organisms.

For example, conditions (anoxic, sulfidic) in the Plußsee at 25 m were unlikely to be conducive to the support of typical aerobic chemoautotrophic ammonia oxidizers. Yet, both NitD-NitB and NitA-NitF primers detected target sequence there. Unfortunately, we did not attempt to enumerate *Nitrosomonas* by immunofluorescence below the chemocline in the Plußsee. Smorczewski and Schmidt [14] detected and isolated ammonia oxidizers from anoxic lake sediments, which implies that the cells can retain viability under these conditions. Detection of a target sequence by PCR does not prove that the target was present in liver organisms. Other means such as fluorescence in situ hybridization might be used to investigate whether whole cells were present. But methods to determine viability of individual cells in situ are almost non-existent; fluorescence in situ hybridization in combination with autoradiography, as has been done with immunofluroescence [3, 20], is a possibility.

In the future, the PCR products themselves can yield more information about species composition by making use of restriction fragment length polymorphisms, characteristic of known strains (Voytek and Ward, unpublished), or complete sequence analysis to investigate which species are represented in the amplified fragment. This will provide another layer of information on population diversity and structure, beyond what is now possible with selective primers.

Acknowledgments

C. Burghardt and K. Eckert provided technical assistance and performed the microscopy for bacterial enumeration and DNA concentration measurements. W. Liesack helped identify a sequence region for the NitD primer. M. Gerdes measured the gas samples, and D. Albrecht provided the nutrient data. R. Herwig provided a sample of the Puget Sound enrichment. This work was supported by the National Science Foundation (OCE-9115040 and DPP-9117907) and a visiting scientist grant from the Max-Planck-Gesellschaft.

References

1. Albrecht D (1973) Verbesserte Methoden zur automatischen Wasseranalyse in der Limnologie. Vom Wasser 41:129–135
2. Atlas RM, Bej AK (1994) Polymerase Chain Reaction. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. ASM, Washington D.C., pp 418–435
3. Fliermans CB, Schmidt EL (1975) Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. Appl Environ Microbiol 30:676–684
4. Hall KC (1980) Gas chromatographic measurements of nitrous oxide dissolved in water using a headspace analysis technique. J Chrom Sci 18:22–24
5. Head IM, Hiorns WD, Martin T, McCarthy AJ, Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J Gen Microbiol 139:1147–1153
6. Hunik JH, Meijer HJG, Tramper J (1992) Kinetics of *Nitrosomonas europaea* at extreme substrate, produce, and salt concentrations. Appl Microbiol Biotechnol 37:802–807
7. Krambeck H-J, Albrecht D, Hickel B, Hofmann W, Arzbach HH (1993) Limnology of the Plußsee. In: Overbeck J, Chrost RJ (eds) Microbial ecology of Lake Plußsee. Springer-Verlag, New York, pp 1–23
8. Liesack W, Weyland H, Stackebrandt E (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
9. McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol Lett 120:363–367
10. Olsen GJ, Larsen N, Woese CR (1991) The ribosomal RNA database project (RDP). Nucl Acid Res 19(Suppl):2017–2021
11. Overbeck J, Chrost RJ (1993) (eds) Microbial ecology of Lake Plußsee. Springer-Verlag, New York
12. Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948
13. Rheinheimer G (1959) Mikrobiologische Untersuchungen über den Shtickstoffhaushalt der Elbe. Arch Mikrobiol 34: 358–373
14. Smorczewski WT, Schmidt EL (1991) Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment. Can J Microbiol 37:828–833
15. Soriano S, Walker N (1968) Isolation of ammonia-oxidizing autotrophic bacteria. J Appl Bacteriol 31:493–497
16. Teske A, Alm E, Regan JM, Toze S, Rittmann BE, Stahl DA (1995) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J Bacteriol 176:6623–6630
17. Voytek MA, Ward BB (1995) Detection of ammonia-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl Environ Microbiol 61: 1444–1450

18. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–513
19. Ward BB (1982) Oceanic distribution of ammonia-oxidizing bacteria determined by immunofluorescent assay. J Mar Res 40:1155–1172
20. Ward BB (1984) Combined autoradiography and immunofluorescence for estimation of single cell activity by ammonia-oxidizing bacteria. Limnol Oceanogr 29:402–410
21. Ward BB (1987) Nitrogen transformations in the Southern California Bight. Deep-Sea Res 34:785–805
22. Ward BB, Carlucci AF (1985) Marine ammonia- and nitrite-oxidizing bacteria: serological diversity determined by immunofluorescence in culture and in the environment. Appl Environ Microbiol 50:194–201
23. Ward BB, Cockcroft AR (1993) Immunofluorescence detection of the denitrifying bacterium *Pseudomonas stutzeri* (ATCC 14405) in seawater and intertidal sediment environments. Microb Ecol 25:233–246
24. Ward BB, MJ Perry MJ (1980) Immunofluorescent assay for the marine ammonia-oxidizing bacterium *Nitrosococcus oceanus*. Appl Environ Microbiol 39:913–918
25. Ward BB, Glover HE, Lipschultz F (1989) Chemoautotrophic activity and nitrification in the oxygen minimum zone off Peru. Deep-Sea Res 36:1031–1051
26. Ward BB, Cockcroft AR, Kilpatrick KA (1993) Antibody and DNA probes for detection of nitrite reductase in seawater. J Gen Microbiol 139:2285–2293
27. Weiss RF, Price BA (1980) Nitrous oxide solubility in water and seawater. Mar Chem 8:347–359
28. Woese CR, Weisburg WG, Paster BJ, Hahn CM, Tanner RS, Krieg NR, Koops H-P, Harms H, Stackebrandt E (1984) The phylogeny of the purple bacteria: the beta subdivision. System Appl Microbiol 5:327–336
29. Woese CR, Weisburg WG, Hahn CM, Paster BJ, Zablen LB, Lewis BJ, Macke TJ, Ludwig W, Stackebrandt E (1985) The phylogeny of the purple bacteria: the gamma subdivision. System Appl Microbiol 6:25–33