MICROBIAL ECOLOGY

Microb Ecol (1998) 36:293–302


# Functional Gene Hybridization Patterns of Terrestrial Ammonia-Oxidizing Bacteria

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Received: 4 December 1997; Accepted: 31 March 1998

## ABSTRACT

The biochemical pathway and genetics of autotrophic ammonia oxidation have been studied almost exclusively in *Nitrosomonas europaea.* Terrestrial autotrophic ammonia-oxidizing bacteria (AAOs), however, comprise two distinct phylogenetic groups in the β-*Proteobacteria,* the *Nitrosomonas* and *Nitrosospira* groups. Hybridization patterns were used to assess the potential of functional probes in non-PCR-based molecular analysis of natural AAO populations and their activity. The objective of this study was to obtain an overview of functional gene homologies by hybridizing probes derived from *N. europaea* gene sequences ranging in size from 0.45 to 4.5 kb, and labeled with $^{32}P$ to Southern blots containing genomic DNA from four *Nitrosospira* representatives. Probes were specific for genes encoding ammonia monooxygenase (*amoA* and *amoB*), hydroxylamine oxidoreductase (*hao*), and cytochrome *c*-554 (*hcy*). These probes produced hybridization signals, at low stringency (30°C), with DNA from each of the four representatives; signals at higher stringency (42°C) were greatly reduced or absent. The hybridization signals at low stringency ranged from 20 to 76% of the total signal obtained with *N. europaea* DNA. These results indicate that all four functional genes in the ammonia oxidation pathway have diverged between the *Nitrosomonas* and *Nitrosospira* groups. The *hao* probe produced the most consistent hybridization intensities among the *Nitrosospira* representatives, suggesting that *hao* sequences would provide the best probes for non-PCR-based molecular analysis of terrestrial AAOs. Since *N. europaea* can also denitrify, an additional objective was to hybridize genomic DNA from AAOs with probes for *Pseudomonas* genes involved in denitrification. These probes were specific for genes encoding heme-type dissimilatory nitrite reductase (dNir), Cu-type dNir, and nitrous oxide reductase (nosz). No hybridization signals were observed from probes for the heme-type *dNir* or *nosz,* but *Nitrosospira* sp. NpAV and *Nitrosolobus* sp. 24-C hybridized, under low-stringency conditions, with the Cu-type *dNir* probe. These results indicate that AAOs may also differ in their mechanisms and capacities for denitrification.

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## Introduction

Oxidation of ammonia to nitrite by autotrophic bacteria is a key process affecting the fate of nitrogen (N) in wastewater treatment, crop fertilization, and trace gas production (contributing to global warming). Autotrophic ammonia oxidizers (AAOs) obtain their energy by converting $NH_3$ to $NO_2^-$ [15, 47]. They contribute more to global N oxidation than do heterotrophic nitrifiers [17, 20]. Under certain conditions, AAOs can also denitrify [1, 7], thus making their roles in global N cycling more complex [8]. The biochemical pathway and genes involved in autotrophic ammonia oxidation have been studied almost exclusively in cultures of *Nitrosomonas europaea,* a type strain purified from soil enrichments approximately 40 years ago [22] and available as two accessions (19718 and 25978) from the American Type Culture Collection. The difficulty involved in purifying and maintaining AAO cultures [35, 47] has accounted for the extensive use of *N. europaea* in biochemical and kinetic studies of ammonia oxidation [30].

Generalization of *N. europaea's* physiological characteristics to other AAOs may be justifiable, in light of phylogenetic evidence that AAOs appear to be closely related [11, 40]. To date, all AAOs of terrestrial origin fall within the β-subdivision of the class *Proteobacteria* [48]. However, recent studies based on rDNA from cultured AAO isolates and cloned environmental sequences indicate that terrestrial AAOs comprise two distinct groups within the β-subdivision—the *Nitrosomonas* group, which contains *N. europaea,* other *Nitrosomonas* spp., and some *Nitrosococcus* spp [29]; and the *Nitrosospira* group, which contains genera classified as *Nitrosospira, Nitrosolobus,* and *Nitrosovibrio* [11, 40, 47]. Analyses of 16S rDNA sequences from AAO populations in soils by clone libraries and denaturing gradient gel electrophoresis have shown that *Nitrosomonas* representatives are less abundant than *Nitrosospira* representatives [12, 19, 39]. More recent analyses of PCR-amplified *amoA* gene sequences from natural populations showed that *Nitrosospira* representatives were also predominant in rice rhizosphere and freshwater samples [32]. Therefore, experimental data on ammonia oxidation by *N. europaea* may not be applicable to systems in which *Nitrosospira* representatives predominate.

In this study, DNA–DNA hybridizations using *N. europaea* gene sequences were used to assess these sequences as probes for non-PCR-based detection of natural AAO populations. Probe hybridizations, based on bacterial genes encoding enzymes involved in nitrification and denitrification, would be useful in evaluating the relative importance of these processes in the environment [46]. Hybridizations, using probes derived from *N. europaea,* against genomic DNA of four members of the *Nitrosospira* group were compared. To confirm that AAO strains used in the studies were different from each other, REP-PCR band patterns [9] were obtained from their genomic DNA.

Probes were obtained from DNA sequences available for *N. europaea* genes that encode enzymes in the ammonia oxidation pathway. Ammonia monooxygenase (AMO) oxidizes ammonia to hydroxylamine; it is encoded by two genes, *amoA* and *amoB.* The gene *amoA* codes for the protein subunit containing AMO's active site [25], whereas the immediately downstream *amoB* codes for a structural subunit of AMO [5, 26]. Hydroxylamine oxidoreductase (HAO) oxidizes hydroxylamine to nitrite. It is a multimeric protein [16] composed of subunits encoded by the gene *hao* [2, 6, 34]. Cytochrome *c*-554, the immediate electron acceptor for HAO, is encoded by *hcy* [14], also called *cytA* [6]. Each of these genes is present in at least two copies in the genome of *N. europaea* [6, 14, 26].

An additional objective of this study was to explore functional diversity among AAOs using hybridization tests with probes from denitrification genes of *Pseudomonas* spp., in which the denitrification pathways and genetics have been well characterized [37, 49]. *N. europaea* is known to produce a Cu-type nitrite reductase enzyme [31], and cultures of *N. europaea* produce NO and $N_2O$ [1, 7]. To determine if *Pseudomonas* gene probes could detect similar genes in AAOs, AAO genomic DNA was hybridized with gene probes for a Cu-type dissimilatory nitrite reductase (dNir) [49], a heme-type dNir [37] and nitrous oxide reductase [44]. Our goal was to investigate the genetic diversity affecting nitrogen metabolism in this functionally important group of bacteria.

## Materials and Methods

### Bacterial Strains

Six strains of ammonia-oxidizing bacteria, previously isolated from soils, were used: *N. europaea* ATCC 25978, neotype isolated from soil enrichment [22]; *N. europaea* ATCC 19718; *Nitrosolobus multiformis* ATCC 25196, neotype isolated from soil in Surinam, South America [47]; *Nitrosolobus* sp. strain 24-C; *Nitrosospira* sp. NpAV (Apple Valley); and *Nitrosospira* sp. Np39-19. The latter three cultures were obtained from Dr. Ed Schmidt, University of Minnesota. They had been isolated from agricultural soils in Minnesota [24].

Cultures of three heterotrophic bacteria were grown in tryptic soy broth, to provide DNA for positive and negative controls. *Pseudomonas stutzeri* strain JM300 and *Pseudomonas* sp. strain G-179

were positive controls for the heme-type [37] and Cu-type [49] dissimilatory nitrite reductases, respectively. The type strain of *Arthrobacter globiformis* (ATCC 8010) was also included, because it is a heterotrophic nitrifier found in soils [21, 43], and to see if its DNA would cross-hybridize with functional gene probes from an autotrophic bacterium.

### *AAO Batch Culture Scale-up, Genomic DNA Extraction, and REP-PCR*

Tube cultures of AAOs in filter-sterilized ATCC Medium 929 were grown, to a cell concentration of 1–2 × $10^7$ cells $ml^{-1}$ (indicated by slight turbidity), with pH adjustment, using 0.05 M $K_2CO_3$ to neutralize the cultures, every other day over a 2-week period. One-ml aliquots were transferred to 50 ml fresh medium in 250-ml Erlenmeyer flasks. The pH of these cultures was repeatedly adjusted, with 0.1 M or 0.3 M $K_2CO_3$, for 2–3 weeks. Five-ml aliquots from the small flask cultures were transferred to 500 ml fresh medium in 2-liter Erlenmeyer flasks. These cultures were grown for another 2–4 weeks. All incubations were at 25°C in the dark, without shaking. Typical DNA recoveries from 4-week-old cultures were 100–120 ng DNA $ml^{-1}$. DNA recoveries were lower from older cultures (e.g., 6 weeks), presumably because of extracellular polysaccharides that interfered with extraction. Slightly improved recoveries could be achieved with AAO cultures that had just reached slight turbidity (0.5–1 × $10^7$ cells $ml^{-1}$).

Cells from the large flask cultures were harvested by centrifugation in 250-ml bottles, at 5000 × *g*, for 30 min. Cell pellets from two to four bottles were resuspended in Medium 929, without $(NH_4)_2SO_4$, and transferred to 30-ml Oak Ridge tubes, to concentrate bacterial biomass. A miniprep procedure for extracting bacterial genomic DNA [4] was modified by using tenfold larger volumes of all extraction reagents. It was used to obtain genomic DNAs of all AAO strains and the two *Pseudomonas* strains. Genomic DNA of *A. globiformis,* which was more resistant to lysis, was extracted according to the method of Visuvanathan et al. [45].

REP-PCR was performed on genomic DNA samples from AAOs, using a Perkin-Elmer GeneAmp PCR System 9600 (Perkin-Elmer, Foster City, CA) and the method and primers described by de Bruijn [9]. This procedure enabled us to compare the AAOs' REP fingerprints, which are characteristic DNA band patterns in agarose gels produced after PCR amplification using primers for conserved, repeated DNA elements in bacteria.

### *DNA Probes*

DNA probes used in hybridization tests, and their sources, are listed in Table 1. *N. europaea* gene fragments had been cloned into vectors which had *Eco*RI restriction sites flanking their insertion sites. Thus, probes for *N. europaea* genes were obtained by digestion of these plasmids with *Eco*RI alone, or with *Eco*RI and another restriction enzyme that cut within the gene fragment. Other probe inserts, cloned into vectors described in references listed in Table 1, were isolated by cutting with appropriate enzymes at mapped restriction sites. Probes were obtained by extracting plasmid DNA from cultures of *Escherichia coli* clones, purifying the DNA in cesium chloride gradients, cutting the DNA with restriction enzymes, and isolating probe fragments by agarose gel electrophoresis, according to standard methods [33]. Probe DNA was purified with a Gene-Clean kit (Bio-101, Inc., LaJolla, CA) and labeled with [α-$^{32}$P]dCTP (3000 Ci/mM, DuPont New England Nuclear Research Products, Wilmington, DE), using a random priming kit from Boehringer Mannheim Biochemicals (Indianapolis, IN). Unincorporated nucleotides were removed from the labeled probes using spun columns [33]. Labeled probe was added to hybridization fluid to obtain a final activity of ~$10^6$ cpm $ml^{-1}$.

### *Restriction Digests, Southern Blots, and Probe Hybridizations*

Genomic DNA preparations were quantified by measuring their absorbance spectra in an HP-8452 UV-Vis spectrophotometer (Hewlett-Packard Co., Englewood, CO). DNA was digested with *Eco*RI, loaded into lanes on 1% agarose gels, and subjected to electrophoresis [33]. For each gel lane, 3 μg of digested DNA was added, except for DNA from *Nitrosolobus* sp. strain 24-C, which had 1.5 μg DNA $lane^{-1}$. The latter reached lower cell densities than the other AAOs, making it difficult to obtain comparable amounts of DNA. Southern blots were prepared from replicate gels on Hybond N+ membranes (Amersham Life Sciences, NY). DNA on the blots was UV-cross-linked in a Stratalinker (Stratagene, La Jolla, CA). Blots were prehybridized for 16 h in a standard prehybridization solution [33], then hybridized for 24 h at 30°C (low stringency) or 42°C (moderate stringency). After hybridization, blots were washed once, for 15 min, at either 30°C or 42°C, with 0.1× SSC, pH 7, with 0.1% sodium dodecyl sulfate. Either X-omat (Kodak) or DuPont NEN film was exposed to the probed blots using one Cronex Lightning Plus KE intensifying screen (DuPont), at −70°C, for 7–10 h prior to development. To permit re-probing, blots were stripped of radioactivity by washing twice, for 15 min, in distilled water that had been boiled and amended with 1% SDS. Membranes were kept moist between hybridizations in airtight containers at 4°C.

### *Densitometric Measurements from Autoradiograms*

Digitized images of autoradiogram films were obtained with the CCD camera of a Gel Print 2000i system (BioPhotonics Corp., Ann Arbor, MI). Hybridization band intensities were determined by densitometric measurements of the digitized images with Spectrum IP Lab image processing software (Signal Analytics Corp., Vienna, VA). Band intensities measured for *Nitrosolobus* and *Nitrosospira* strains were compared to those of *N. europaea,* to estimate percent sequence similarities between the probes and homologous genes. Densitometric measurements of bands from *Nitrosolobus* sp. 24-C DNA were doubled to compensate for the lower amount of target DNA in these lanes.

## Results

### *REP-PCR Patterns*

REP-PCR patterns were obtained from purified genomic DNA from the six AAO stock strains (Fig. 1). The REP-PCR

**Table 1.** DNA probes used in this study

| Probe name | Organism (source of probe sequence) | Plasmid and restriction enzymes used to obtain probe | Protein encoded by gene containing probe sequence | Approximate length of gene | Number of *Eco*RI sites within gene | Length of probe and region of gene corresponding to probe | Laboratory sources and references |
|---|---|---|---|---|---|---|---|
| amoA | *N. europaea* ATCC 19718 | pNH901 *Kpn*I-*Eco*RI | Ammonia monooxygenase (AMO) acetylene-binding polypeptide | 0.8 kb | 0 | 0.7 kb of 3′ end of gene (probe starts at internal *Kpn*I site) | N. Hommes and D. Arp [25] |
| amo B | *N. europaea* ATCC 19718 | pNH902 *Eco*RI-*Eco*RI | Polypeptide involved in AMO activity | 1.4 kb | 0 | 0.7 kb of 5′ end of gene (probe ends at internal *Kpn*I site) | N. Hommes and D. Arp [26] |
| hao | *N. europaea* ATCC 19718 | pNH110 *Kpn*I-*Eco*RI | Hydroxylamine oxidoreductase | 1.7 kb | 1 | 0.7 kb (probe starts at *Kpn*I site 43 nt from N terminus and ends at *Eco*RI site) | N. Hommes and D. Arp [34] |
| hcy | *N. europaea* ATCC 19718 | pNHc55p1 *Bam*HI-*Eco*RI | Cytochrome *c*-554, believed to accept electrons from HAO | 0.7 kb | 1 | 0.45 kb (probe starts at *Bam*HI site 48 nt from N terminus and ends at *Eco*RI site) | N. Hommes and D. Arp [34] |
| TN45 probe | *N. europaea* ATCC 25978 | pTN45 *Eco*RI-*Eco*RI | Polypeptide possibly involved in HAO activity | unknown | 0 | 4.5 kb (probe consists of entire plasmid insert) | T. Tokuyama [41] |
| Cu-dNir | *Pseudomona* sp. strain G-179 | pRTc1.9 *Eco*RI-*Bam*HI | Copper-containing nitrite reductase | 1.2 kb | 0 | 1.9 kb (probe consists of entire gene plus 660 nt upstream and 80 nt downstream) | J. Tiedje [49] |
| Heme-dNir | *P. stutzeri* strain JM300 | pBsGTH2.4 *Hind*III-*Cla*I | Heme-containing ($c,d_1$-) nitrite reductase | 1.6 kb | 0 | 2.4 kb (probe consists of entire gene plus flanking regions) | J. Tiedje [37] |
| nosz | *P. stutzeri* strain ZoBell | pAV21 *Pst*I-*Pst*I | Nitrous oxide reductase | 1.9 kb | 0 | 1.2 kb (probe consists of gene region between $2^{nd}$ and $3^{rd}$ *Pst*I sites from 5′ end) | J. Tiedje [44] |

patterns for *N. europaea* ATCC 25978 and *N. europaea* ATCC 19718 were identical, suggesting that the strains were the same [9]. This observation was supported by the observation that the source of the strains had been a culture purified from a soil enrichment obtained from D.L. Jensen in Denmark (D. Pramer, personal communication). REP-PCR patterns were unique for all other AAO strains (Fig. 1).

### *Hybridizations with Probes for Ammonia Oxidation Genes*

*N. europaea* DNA digested with *Eco*RI exhibited two hybridization bands with each of the *amoA* and *amoB* probes, three bands with the *hao* probe, and one band with the *hcy* probe (Figs. 2 and 3). Similar results with *N. europaea* DNA have been obtained in other laboratories for *amo* [25, 26], *hao* [26, 34], and *hcy* [6, 14]. All *N. europaea* probes hybridized with DNA of other AAOs. Hybridization signals at 42°C, however, were greatly reduced or absent, relative to those obtained at 30°C (Figs. 2 and 3). Other AAO strains also appeared to have multiple copies of at least some of the homologous genes (Figs. 2 and 3).

The intensities of all hybridization bands recovered at 30°C were summed to obtain an estimate of total probe

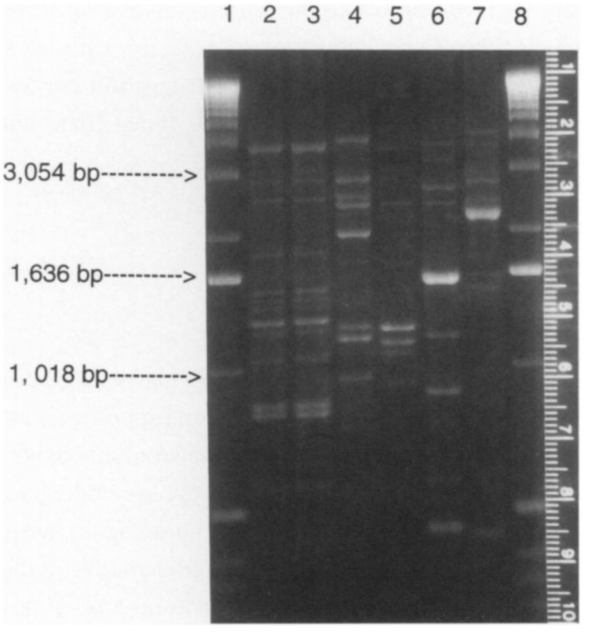


Fig. 1. REP-PCR patterns of AAO DNA observed in agarose gels stained with ethidium bromide and illuminated under UV light: Lane 1, DNA size marker; Lane 2, *N. europaea* ATCC 25978; Lane 3, *N. europaea* ATCC 19718; Lane 4, *N. multiformis* ATCC 25196; Lane 5, *Nitrosolobus* sp. 24-C; Lane 6, *Nitrosospira* sp. NpAV; Lane 7, *Nitrosospira* sp. Np39-19; Lane 8, DNA size marker.

signal from each AAO under low-stringency conditions. The total probe signal depends on both gene copy number and percent similarity between the DNA probe and corresponding genes in different strains. Since all probes were derived from *N. europaea* DNA, the total probe signal of *N. europaea* was designated as 100%. Total probe signals from the other four AAO strains were divided by the total probe signal from *N. europaea,* to obtain percentage values (Fig. 4). These percentages reflect the relative signal that could be obtained from one genome of an AAO strain when it is hybridized with the corresponding *N. europaea*-derived probe. In general, *N. multiformis* exhibited the lowest percentages (20–48%) of the four strains tested. The *hao* probe gave the highest overall percentages (48–68%) among the strains (Fig. 4).

In replicate Southern blots of each AAO strain run in parallel, locations of hybridization bands from the *amoA* probe were identical to those for the *amoB* probe (Fig. 2). Since *amoB* is located immediately downstream of *amoA* in *N. europaea* [25], these results indicate that the *amoA* and *amoB* genes are also linked in the four *Nitrosospira* representatives.

No hybridization was observed at low stringency between any of the *N. europaea* probes and DNA from *A. globiformis, P. stutzeri* strain JM300, or *Pseudomonas* sp. strain G-179 (data not shown for latter two strains). Another *N. europaea* DNA probe obtained from a 4.5 kb insert in pTN45 was also tested (Table 1). This construct had been reported to carry a putative *hao* gene that conferred a low level of hydroxylamine oxidation activity when transformed into *Pseudomonas* sp. [41]. The pTN45 probe hybridized only with genomic DNA from *N. europaea* (data not shown). DNA comple-

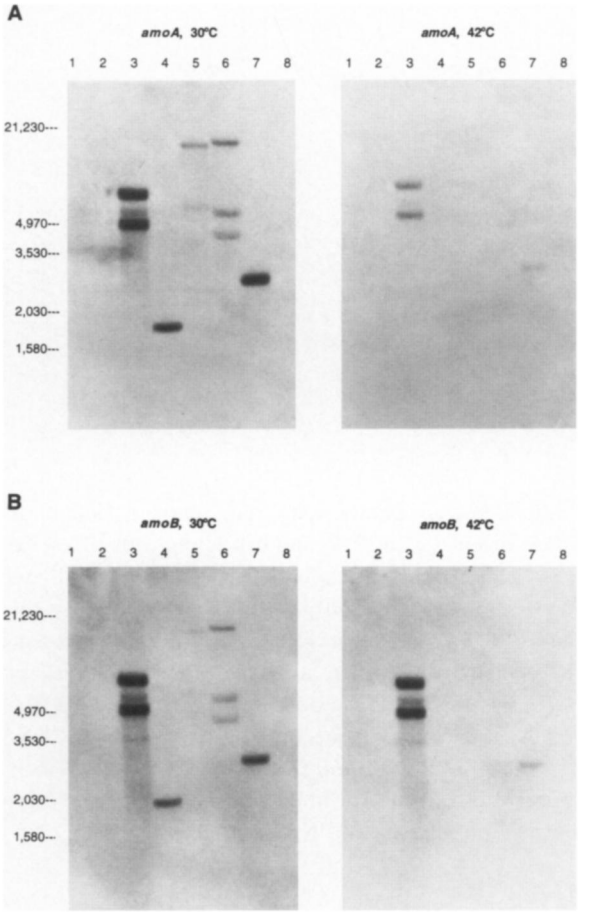


Fig. 2. Autoradiogram photographs from Southern blots of genomic DNAs digested with *Eco*RI and hybridized with $^{32}$P-labeled *N. europaea* gene probes. A: *amoA* probe. B: *amoB* probe. Left-hand photographs show hybridization patterns at lower stringency (hybridization at 30°C, washing at 42°C in 0.1× SSC). Right-hand photographs show hybridization at higher stringency (hybridization at 42°C, washing at 42°C in 0.1× SSC). Blots contained DNA from the following cultures: Lane 1, *A. globiformis*; Lane 2, DNA size marker; Lane 3, *N. europaea*; Lane 4, *N. multiformis*; Lane 5, *Nitrosolobus* sp. 24-C; Lane 6, *Nitrosospira* sp. NpAV; Lane 7, *Nitrosospira* sp. Np39-19; Lane 8, DNA size marker.

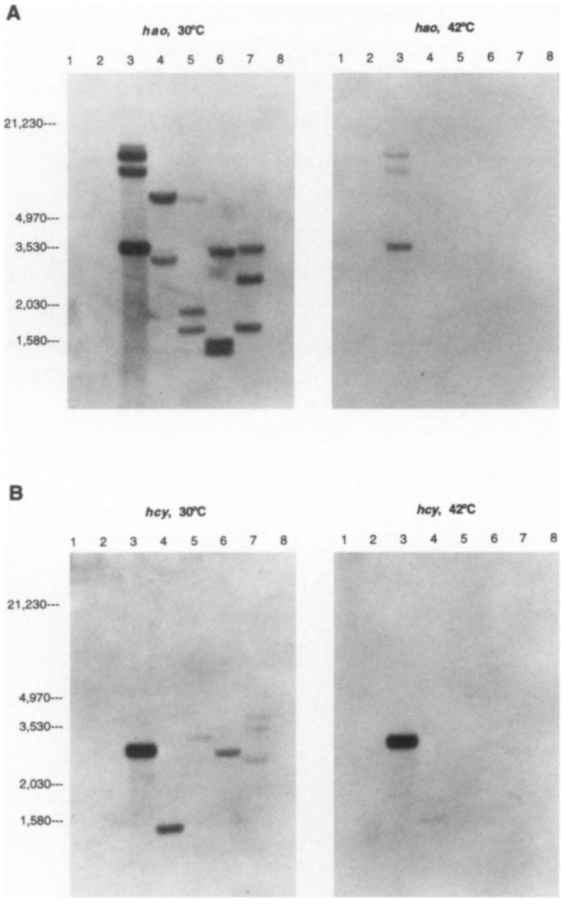


Fig. 3. Autoradiogram photographs from Southern blots of genomic DNAs digested with *Eco*RI and hybridized with $^{32}$P-labeled *N. europaea* gene probes: A: *hao* probe. B: *hcy* probe. Left-hand photographs show hybridization patterns at low stringency (hybridization at 30°C, washing at 42°C in 0.1× SSC). Right-hand photographs show hybridization at higher stringency (hybridization at 42°C, washing at 42°C in 0.1× SSC). Blots contained DNA from the following cultures: Lane 1, *A. globiformis*; Lane 2, DNA size marker; Lane 3, *N. europaea*; Lane 4, *N. multiformis*; Lane 5, *Nitrosolobus* sp. 24-C; Lane 6, *Nitrosospira* sp. NpAV; Lane 7, *Nitrosospira* sp. Np39-19; Lane 8, DNA size marker.

mentary to this fragment was thus not observed in genomes of the other AAO strains.

### *Hybridizations with Probes for Denitrification Genes*

DNA from *Nitrosolobus* sp. strain 24-C and *Nitrosospira* sp. NpAV each produced hybridization bands at 30°C, with the probe for the Cu-type *dNir* gene from *Pseudomonas* sp. strain G-179. DNA from *N. europaea* on the same blots did not cross-react with this probe (Fig. 5). These results indicate that the copper nitrite reductase enzyme observed in *N. europaea* [31] is not similar to this Cu-type dNir. Probes for heme-type *dNir* and *nosz* hybridized, as expected, with DNA from the control *Pseudomonas* strains, but they did not hybridize with DNA from any of the AAOs tested (data not shown).

## Discussion

Terrestrial AAOs comprise two distinct phylogenetic groups in the β-*Proteobacteria.* These hybridization experiments indicate that all four functional genes in the ammonia oxidation pathway of *Nitrosospira* representatives have undergone varied degrees of divergence, relative to homologous genes in the classic type strain *N. europaea* (a representative of the *Nitrosomonas* group). Recent analyses of natural AAO populations have demonstrated that *Nitrosospira* representatives were more abundant than *Nitrosomonas* in soils [12, 19, 39, 42], rice rhizosphere, and freshwater samples [32]. Stephen et al. [39] have also described the predominance of "specialist" AAO populations (e.g., *Nitrosospira* Cluster 2 in acid soils) that may undergo selection in particular environments. Therefore, fundamental understanding of the biochemistry and kinetics of ammonia oxidation, which is based primarily on experiments done with *N. europaea* [15], should not be extrapolated or generalized to *Nitrosospira* representatives without more comprehensive studies.

The extent of divergence between *N. europaea* and *Nitrosospira* representatives, however, was not sufficient to preclude cross-hybridization at low stringency. Since the *N. europaea* probes were based on nearly full-length gene sequences (Table 1), shorter functional probes (fewer than 100 nucleotides), developed from highly conserved regions, should exhibit more consistent hybridization signals among terrestrial AAOs. Short, conserved regions can also serve as PCR primer sites for functional gene amplification. Rotthauwe et al. [32] used primers to amplify portions of the *amoA* gene from cultured *Nitrosomonas* and *Nitrosospira* strains, as well as from enriched and nonenriched environmental samples. The 491-bp PCR products corresponded to the *amoA* gene sequence of *N. europaea* between positions 332 and 822 [32]. In their data set, consisting of at least 50 partial *amoA* sequences, identity values between each pair of *Nitrosomonas* and *Nitrosospira* sequences were all higher than 67%. Our similarity values, based on relative intensities of hybridization signals from the 703-bp *amoA* probe on Southern blots of *Nitrosospira* DNA, ranged from 36 to 76%

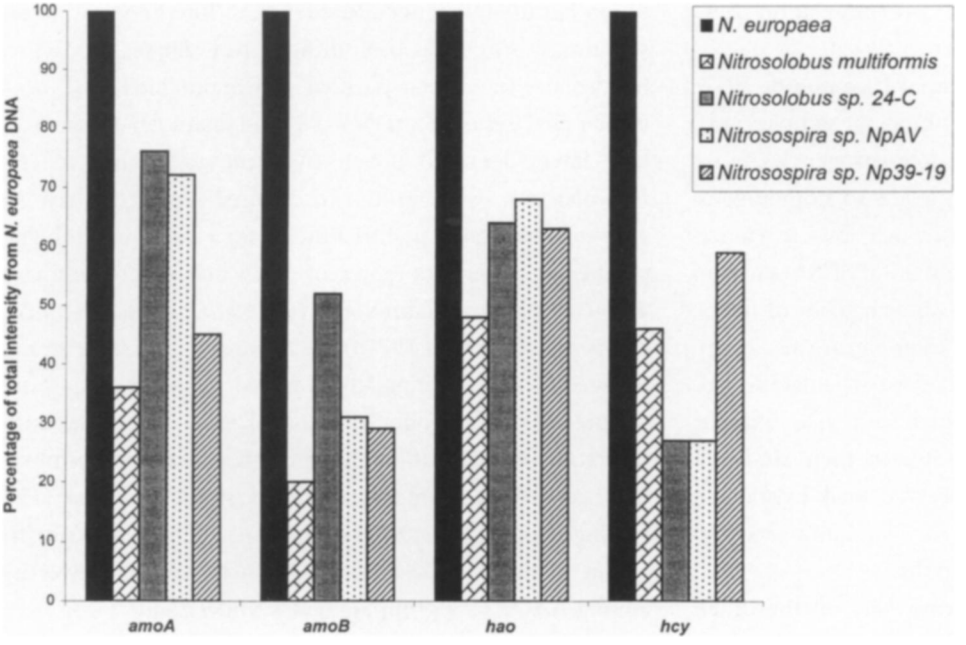


Fig. 4. Histograms showing summed intensities of hybridization bands, expressed as percentages of total signal from *N. europaea* DNA (100%). Band intensities were measured densitometrically, using digitized images of autoradiogram films that had been exposed to Southern blots hybridized with $^{32}$P-labeled probes (Figs. 2 and 3). Band intensities for the five strains obtained with a given probe were all measured from the same blot. AAO strains tested in each set were in the following order: *N. europaea; N. multiformis; Nitrosolobus* sp. strain 24-C; *Nitrosospira* sp. NpAV; and *Nitrosospira* Np39-19.

(Fig. 4). Southern blot hybridizations are, of course, less precise than primary sequence determinations. The low similarities observed with *N. multiformis* (36%) and *Nitrosospira* sp. NpAV (45%) could be due to the *amoA* probe covering 25% more of the gene (from positions 118 to 822). It may also have contained a less conserved region.

The functional probes in this study produced low or no hybridization signals from DNAs of *Nitrosospira* representatives at high stringency (Figs. 2 and 3). This observation has important implications for non-PCR-based methods [13, 27] for assessing the presence and activity of natural AAO populations. These findings indicate that hybridization signals from any single nitrification gene probe of moderate length (400–700 nucleotides) will depend on the species composition of the AAO populations under study. Shorter probes will be required to produce more consistent signals from both *Nitrosomonas* and *Nitrosospira* groups. However, differences in gene copy number would still affect hybridization signals from DNAs of different AAO groups. *N. europaea* possesses two copies of the *amoA* and *amoB* genes [25], three copies of the *hao* gene [26], and three copies of the *hcy* gene [34]. Multiple copies of these genes also appeared evident in at least some of the *Nitrosospira* representatives (Figs. 2 and 3), although we did not perform the additional restriction digests needed to confirm copy numbers. Norton et al. [28] have verified three copies of *amoA* [18] in *Nitrosospira* sp. NpAV. Variation in functional gene copy number among AAO strains, therefore, would confound establishment of a quantitative relationships between

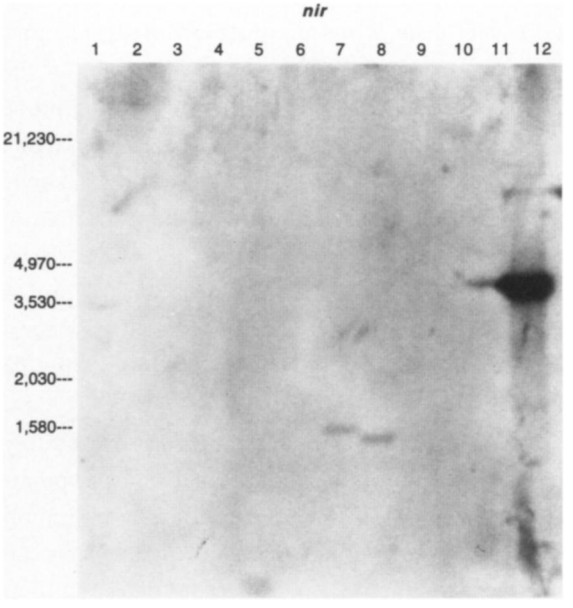


Fig. 5. Autoradiogram photograph of Southern blot hybridized at 30°C with *nir* probe, for a copper-type nitrate reductase gene from *Pseudomonas* sp. G-179. Photograph shows hybridization patterns at low stringency (hybridization at 30°C, washing at 42°C in 0.1× SSC). The blot contained *Eco*RI-digested DNA from the following cultures: Lane 1, *Pseudomonas stutzeri* JM300; Lane 2, blank; Lane 3, *A. globiformis*; Lane 4, DNA size marker; Lane 5, *N. europaea*; Lane 6, *N. multiformis*; Lane 7, *Nitrosolobus* sp. 24-C; Lane 8, *Nitrosospira* sp. NpAV; Lane 9, *Nitrosospira* sp. Np39-19; lane 10, DNA size marker; Lane 11, blank; Lane 12, *Pseudomonas* sp. G-179 (positive control). Lane 11, a blank lane intended to contain the spillover of radioactivity typically observed with *Pseudomonas* sp. G-179 DNA, contains some signal from Lane 12.

DNA–DNA hybridization signals and AAO population sizes, when mixed populations of AAOs are analyzed.

These findings indicate that bacterial community DNA extracted from soils and other environmental samples [13] would have to be hybridized, under low-stringency conditions, with probes from *N. europaea* to detect homologous genes of *Nitrosospira* representatives. Such low-stringency hybridizations could result in overestimation of AAO population size, due to probe binding to similar genes of unrelated organisms. Methanotrophs, for example, are also common in soils [10]. They contain genes for particulate methane monooxygenase (pMMO), which can also oxidize ammonia and is similar to AMO [36]. In their study of pMMO genes, Semrau et al. [36] observed weak hybridization at 42°C between a 693-nt probe for *amoA* and genomic DNA from four different methanotrophs.

Structural gene probes derived from *hao,* on the other hand, would probably be more specific for AAOs, because hydroxylamine is not a known intermediate in the energy-generating pathways of methanotrophs. These results suggest that the *hao* gene is more conserved than the other ammonia oxidation genes, among the AAOs tested. Of the four *N. europaea* probes tested in this study, the *hao* probe produced the least variable intensities in hybridization signal across all five AAO strains (Table 2). However, *hao* may contain sequences similar to those coding for heme-containing peptides in unrelated bacteria [36], and it shares similarity to the *nirT* gene of *E. coli* [6]. Shorter probes (<100 nt), derived from HAO coding regions that are critical to specific enzyme function [3], might prove most specific for AAOs.

DNA probe hybridizations against microbial community DNA could also produce false-negative results if AAO populations are too small to produce detectable hybridization signals. Most-probable-number counts of AAOs in soils can be $10^4$ or fewer per gram [35], and the lower detection limit for high-activity radioactive probes with single-copy targets of 1 kb is $10^4$ organisms per gram [13]. Thus, some AAO populations may be too small to be tracked with functional gene probes, even if the genes are present in two or three copies. It may be more feasible to use probe hybridization analysis with community DNA from soils containing enriched AAO populations (e.g., soils that have been amended with ammonia-based fertilizers, animal manures, or high-nitrogen-loaded sludges).

An additional problem in using *N. europaea* gene probe hybridizations for estimating AAO population size *in situ* stems from the uncertainty regarding genetic similarity between cultured and uncultured AAOs. Physiological diversity among AAOs has been difficult to evaluate, because so few isolates have been purified and maintained [30]. Molecular analyses of 16S rDNA [38] and *amoA* gene sequences [32] have identified novel AAO lineages within the β-*Proteobacteria,* with no known cultured representatives. *N. europaea,* cultivated in enrichments for years prior to being purified [22], may not represent AAOs that are predominant and widespread in nature. The two ATCC accessions of *N., europaea* (25978 and 19718) are apparently the same strain, because they were originally obtained from the same pure culture and they produced identical REP-PCR patterns in this study (Fig. 1). Although ammonia oxidation physiology has been studied almost exclusively in *N. europaea* [15], findings from these studies cannot be generalized to nitrification in the environment until functional gene diversity among AAOs, as a group, is better understood.

Stephen et al. [38, 39] conducted intensive studies of the diversity of natural AAO populations, by using PCR amplification and specific primers [23, 19] to retrieve 16S rDNA sequences of AAOs from microbial community DNA. Seven phylogenetically distinct clusters, four clusters within the *Nitrosospira* clade and three within the *Nitrosomonas* clade, were identified based on partial 16S rDNA sequences corresponding to *E. coli* positions 230 to 530 [38]. Despite the monophyletic status of terrestrial AAOs within the β-subdivision of *Proteobacteria,* no single oligonucleotide within the 16S rDNA database of Stephen et al. could be identified as a primer or probe for detecting all AAOs. Although Rotthauwe et al. [32] developed PCR primers that could amplify *Nitrosomonas* and *Nitrosospira amoA* genes, non-PCR-based molecular analysis methods may require multiple probes to comprehensively assess the presence and activity of AAOs.

The linkage between the *amoA* and *amoB* genes observed in *N. europaea* [25] was also apparent for all four *Nitrosospira* representatives, as indicated by identical hybridization patterns for the *amoA* and *amoB* gene probes (Fig. 2). However, physiological differences among the AAOs in this study were suggested by hybridization bands obtained between the Cu-type *dNir* probe and DNAs from *Nitrosospira* sp. NpAV and *Nitrosolobus* sp. strain 24-C, but not DNA from other AAOs (Fig. 5). Furthermore, the lack of cross-hybridization between the TN45 probe from *N. europaea* and DNA from *Nitrosospira* representatives alludes to physiological attributes in *N. europaea* that are absent in the other AAO strains.

In conclusion, a suite of hybridization tests provided an

overview of functional gene homologies between the two groups of AAOs in the β-*Proteobacteria.* Hybridization patterns indicated that all four functional genes involved in ammonia oxidation have diverged between the *Nitrosomonas* and *Nitrosospira* groups. These two groups may also differ in their denitrification genes. The *N. europaea hao* probe produced the most consistent hybridization intensities among the *Nitrosospira* representatives. This suggests that shorter probes derived from *hao* coding regions important in enzyme activity are the best probes for non-PCR-based molecular analysis of autotrophic ammonia oxidation in terrestrial ecosystems.

## Acknowledgments

We thank Dan Arp and Norman Hommes, of Oregon State University, for the clones containing *N. europaea* probe inserts; E.L. Schmidt, of the University of Minnesota, for ammonia oxidizer cultures; and R. Tokuyama, Nihon University of Japan, for the TN45 clone. The valuable advice of Yuichi Suwa and comments by Roberta Fulthorpe, Jessica Hanson, and two anonymous reviewers were also greatly appreciated. This work was supported by National Science Foundation grant BIR-9120006 to the Center for Microbial Ecology.

## References

1. Anderson IC, Poth M, Homstead J, Burdige D (1993) A comparison of NO and $N_2O$ production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrophic nitrifier *Alcaligenes faecalis.* Appl Environ Microbiol 59:3525–3533
2. Arciero DM, Hooper AB (1993) Hydroxylamine oxidoreductase from *Nitrosomonas europaea* is a multimer of an octaheme subunit. J Biol Chem 268:14645–14654
3. Arciero DM, Lipscomb JD, Hooper AB (1994) The active site of hydroxylamine oxidoreductase from *Nitrosomonas:* Evidence for a new metal cluster in enzymes. J Amer Chem Soc 116:11961–11968
4. Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Sideman JG, Struhl K (eds) (1987) Current protocols in molecular biology. John Wiley & Sons, Inc, New York, NY, 2.4.1
5. Bergmann DJ, Hooper AB (1994) Sequence of the gene, *amoB,* for the 43-kDa polypeptide of ammonia monooxygenase of *Nitrosomonas europaea.* Biochem Biophys Res Comm 204: 759–762
6. Bergmann DJ, Arciero DM, Hooper AB (1994) Organization of the *hao* gene cluster of *Nitrosomonas europaea*: genes for two tetraheme *c* cytochromes. J Bacteriol 176:3148–3153
7. Bock E, Schmidt L, Stuven R, Zwart D (1995) Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donor and nitrite as electron acceptor. Arch Microbiol 163:16–20
8. Conrad R (1995) Soil microbial processes involved in production and consumption of atmospheric trace gases. Adv Microb Ecol 14:207–250
9. deBruijn FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensuses) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Env Microbiol 58:2180–2187
10. Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–471
11. Head IM, Hiorns WD, Embley TM, 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
12. Hiorns WD, Hastings RC, Head IM, McCarthy AJ, Saunders JR, Pickup RW, Hall GH (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidising bacteria. J Gen Microbiol 141:2793–2800
13. Holben WE, Jansson JK, Chelm BK, Tiedje JM (1988) DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl Environ Microbiol 54:703–711
14. Hommes NG, Sayavedra-Soto LA, Arp DJ (1994) Sequence of *hcy,* a gene encoding cytochrome *c*-554 from *Nitrosomonas europaea.* Gene 146:87–89
15. Hooper AB, Vanelli T, Bergmann DJ, Arciero DM (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. Antonie van Leeuwenhoek 71:59–67
16. Hoppert M, Mahony TJ, Mayer F, Miller DJ (1995) Quaternary structure of the hydroxylamine oxidoreductase from *Nitrosomonas europaea.* Arch Microbiol 163:300–306
17. Killham K (1986) Heterotrophic nitrification. In Prosser JI (ed), Nitrification. IRL Press, Oxford, UK, pp 117–126
18. Klotz MG, Norton JM (1995) Sequence of an ammonia monooxygenase subunit A-encoding gene from *Nitrosospira* sp. NpAV. Gene 163:159–160
19. Kowalchuk GA, Stephen JR, de Boer W, Prosser JI, Embley TM, Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol 63:1489–1497
20. Kuenen JG, Robertson JG (1988) Ecology of nitrification and denitrification. In: Cole JA, Ferguson S (eds), The Nitrogen and Sulfur Cycles. Cambridge University Press, Cambridge, UK, pp 161–218
21. Kurokawa M, Fukumori Y, Yamanaka T (1985) A hydroxylamine-cytochrome *c* reductase occurs in the heterotrophic nitrifier *Arthrobacter globiformis.* Plant Cell Physiol 26:1439–1442

22. Lewis RF, Pramer D (1958) Isolation of *Nitrosomonas* in pure culture. J Bacteriol 76:524–528
23. McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol Lett 120:363–368
24. McCarty GW, Bremner JM, Schmidt EL (1991) Effects of phenolic acids on ammonia oxidation by terrestrial autotrophic nitrifying microorganisms. FEMS Microb Ecol 85:345–349
25. McTavish H, Fuchs JA, Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea.* J Bacteriol 175:2436–2444
26. McTavish H, LaQuier F, Arciero D, Logan M, Mundform G, Fuchs JA, Hooper AB (1993) Multiple copies of genes coding for electron transport proteins in the bacterium *Nitrosomonas europaea.* J Bacteriol 175:2445–2447
27. Mobarry BK, Wagner M, Urbain V, Rittman BE, Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol 62:2156–2162
28. Norton JM, Low JM, Klotz MG (1996) The gene encoding ammonia monooxygenase subunit-A exists in nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol Lett 139: 181–188
29. Pomerening-Roser A, Rath G, Koops H-P (1996) Phylogenetic diversity within the genus *Nitrosomonas.* System Appl Microbiol 19:344–351
30. Prosser JI (1989) Autotrophic nitrification in bacteria. Adv Microbiol Physiol 30:125–181
31. Ritchie GAF, Nicholas DJD (1974) The partial characterization of purified nitrite reductase and hydroxylamine oxidase from *Nitrosomonas europaea.* Biochem J 138:471–480
32. Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712
33. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Sayavedra-Soto LA, Hommes NG, Arp DJ (1994) Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea.* J Bacteriol 176:504–510
35. Schmidt EL, Belser LW (1982) Nitrifying bacteria. In: Page AL, Miller RH, Keeney DR (eds) Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties. American Society of Agronomy, Madison, WI, pp 1027–1041
36. Semrau JD, Chistoserdov A, Lebron J, Costello A, Davagnino J, Kenna E, Holmes AJ, Finch, Murrell JC, Lidstrom ME (1995) Particulate methane monooxygenase genes in methanotrophs. J Bacteriol 177:3071–3079
37. Smith GB, Tiedje JM (1992) Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl Environ Microbiol 58:376–384
38. Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62:4147–4154
39. Stephen JR, Kowalchuk GA, Bruns MA, McCaig AE, Phillips CJ, Embley TM, Prosser JI (1998) Analysis of β-subgroup ammonia oxidiser populations in soil by DGGE analysis and hierarchical phylogenetic probing. Appl Environ Microbial 64:2958–2965
40. Teske A, Alm E, Regan JM, Toze S, Rittmann BE, Stahl DA (1994) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J Bacteriol 176:6623–6630
41. Tokuyama T, Tomita Y, Takahashi R (1988) Cloning and expression of the hydroxylamine oxidase gene of *Nitrosomonas europaea* in *Pseudomonas putida.* Hakkokogaku 66:103–107 (translated from the Japanese)
42. Utaker JB, Bakken L, Jiang QQ, Nes IF (1995) Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. System Appl Microbiol 18:549–559
43. Verstraete W, Alexander M (1972) Heterotrophic nitrification by *Arthrobacter* sp. J Bacteriol 110:955–961
44. Viebrock A, Zumft WG (1988) Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri.* J Bacteriol 170:4658–4668
45. Visuvanathan S, Moss MT, Stanford JL, Hermon-Taylor J, McFadden JJ (1989) Simple enzymic method for isolation of DNA from diverse bacteria. J Microbiol Meth 10:59–64
46. Ward BB (1995) Nitrification and denitrification: probing the nitrogen cycle in aquatic environments. Microb Ecol 32:247–261
47. Watson SW, Bock E, Harms H, Koops H-P, Hooper AB (1989) Nitrifying bacteria. In: Staley JT (ed), Bergey's Manual of Systematic Bacteriology, Vol. 3, William & Wilkins Co., Baltimore, MD, pp 1808–1834
48. 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
49. Ye RW, Fries MR, Bezborodnikov SG, Averill BA, Tiedje JM (1993) Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. Appl Environ Microbiol 59:250–254