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The Ammonia Monooxygenase Structural Gene *amoA* as a Functional Marker: Molecular Fine-Scale Analysis of Natural Ammonia-Oxidizing Populations

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The naturally occurring genetic heterogeneity of autotrophic ammonia-oxidizing populations belonging to the β subclass of the *Proteobacteria* was studied by using a newly developed PCR-based assay targeting a partial stretch of the gene which encodes the active-site polypeptide of ammonia monooxygenase (*amoA*). The PCR yielded a specific 491-bp fragment with all of the nitrifiers tested, but not with the homologous stretch of the particulate methane monooxygenase, a key enzyme of the methane-oxidizing bacteria. The assay also specifically detected *amoA* in DNA extracted from various aquatic and terrestrial environments. The resulting PCR products retrieved from rice roots, activated sludge, a freshwater sample, and an enrichment culture were used for the generation of *amoA* gene libraries. No false positives were detected in a set of 47 randomly selected clone sequences that were analyzed further. The majority of the environmental sequences retrieved from rice roots and activated sludge grouped within the phylogenetic radiation defined by cultured strains of the genera *Nitrosomonas* and *Nitrosospira*. The comparative analysis identified members of both of these genera in activated sludge; however, only *Nitrosospira*-like sequences with very similar amino acid patterns were found on rice roots. Further differentiation of these molecular isolates was clearly possible on the nucleic acid level due to the accumulation of synonymous mutations, suggesting that several closely related but distinct *Nitrosospira*-like populations are the main colonizers of the rhizosphere of rice. Each of the *amoA* gene libraries obtained from the freshwater sample and the enrichment culture was dominated by a novel lineage that shared a branch with the *Nitrosospira* cluster but could not be assigned to any of the known pure cultures. Our data suggest that *amoA* represents a very powerful molecular tool for analyzing indigenous ammonia-oxidizing communities due to (i) its specificity, (ii) its fine-scale resolution of closely related populations, and (iii) the fact that a functional trait rather than a phylogenetic trait is detected.

The oxidation of ammonia to nitrite by autotrophic nitrifiers is a key process in the global cycling of nitrogen. The first step, the oxidation of ammonia to hydroxylamine, is catalyzed by ammonia monooxygenase (AMO) (20). The rather wide substrate range of this enzyme has led to the assumption that the environmental activity of autotrophic ammonia oxidizers may also contribute to the global cycling of methane and carbon monoxide (3), as well as to the degradation of hydrocarbons and halogenated compounds (22, 47).

The biogeochemical importance of ammonia oxidizers indicates a need for development of reliable methods for identification of these organisms in nature. Due to their chemolithotrophic metabolism ammonia oxidizers are extremely slowly growing bacteria with generation times between 8 h and several days (54). This makes traditional cultivation methods based on most-probable-number techniques (31) or selective plating (14) very time consuming. In addition, such approaches often result in poor counting efficiencies (4) and lead to a collection of ammonia oxidizers that is not representative of their actual diversity and abundance in the habitats under study (19, 45). Immunoassay detection with polyclonal antibodies has been used to study the serological diversity and distribution of nitrifying bacteria in various aquatic and terrestrial environments (5, 42, 52). However, one prerequisite of this technology is the isolation of ecologically relevant pure cultures in order to raise antibodies.

Molecular detection systems which do not rely on traditional cultivation or on serological approaches appear to be promising alternatives. Comparative 16S rRNA gene sequencing analysis revealed that ammonia oxidizers constitute two monophyletic assemblages (18, 46). The first assemblage is characterized by *Nitrosococcus oceanus* and, most probably, *Nitrosococcus halophilus* (26) in the γ subclass of the *Proteobacteria*. The second group belongs to the β subclass of the *Proteobacteria* and comprises the majority of known isolates. This lineage is divided into two subgroups characterized by *Nitrosomonas* spp. (including *Nitrosococcus mobilis*) and *Nitrosospira* spp. (including strains formerly classified as *Nitrosolobus* and *Nitrosovibrio* spp. [18]). The monophyletic nature of the *Nitrosomonas-Nitrosospira* clade and the set of sequence data available favored the use of the 16S rRNA (gene) as a molecular marker for cultivation-independent detection of ammonia oxidizers in environmental samples either by PCR-based methods (19, 45, 49) or by in situ analysis via whole-cell hybridization (35, 50). However, one major drawback is the phylogenetic character of this approach. PCR primers targeting 16S rRNA genes may cross-react with members of other phylogenetic and physiological groups, especially when they are used with environmental samples containing complex microbial gene pools (38, 45; this study).

McTavish et al. (34) reported the complete gene sequence (designated *amoA*) of the membrane-associated active-site polypeptide of the AMO from *Nitrosomonas europaea*. Similar

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substrate and inhibitor profiles suggested that AMO and the particulate, membrane-bound methane monooxygenase are homologous (3). Sequence similarities between *amoA* and the homologous stretches of the *pmoA* gene of methanotrophs provided strong evidence of an evolutionary relationship (21, 43). Comparative sequencing of the major part of the *amoA* gene from *Nitrosospira* sp. strain AHB1 and *Nitrosospira multiformis* C-71 indicated that this functional gene has strong potential for fine-scale differentiation of closely related ammonia oxidizers (41).

The objective of this study was to elucidate the potential of *amoA* for molecular detection and fine-scale characterization of indigenous ammonia-oxidizing populations. One prerequisite for achieving this goal was the development of a highly specific detection system. The PCR-based assay developed amplified a 491-bp stretch of the *amoA* gene from a wide range of ammonia oxidizers, but not the homologous stretch of *pmoA* from methanotrophs. Our investigations focused mainly on the rhizosphere of flooded rice. This habitat is characterized by oxic-anoxic interfaces due to the diffusion of oxygen into the root zone via the aerenchyma of the vascular system of rice (1, 15). That autotrophic ammonia-oxidizing populations were present in this environment could be concluded from increased denitrification rates measured when the soil was amended with urea (2). To evaluate the specificity and the range of diversity of nitrifiers belonging to the β subclass of the *Proteobacteria* that can be detected by this newly developed *amoA*-based assay, the studies were extended to other environments, including activated sludge from a sewage treatment plant, a freshwater lake, and an enrichment culture.

MATERIALS AND METHODS

Strains. Named ammonia-oxidizing bacteria and control strains used for the development of the PCR assay and the examination of assay specificity were obtained from the sources listed in Table 1.

Environmental samples. This study included samples from following sites: rice roots and surface soil layers (depth, 0 to 2 cm) collected from flooded microcosms planted and grown under conditions described previously (15), activated sludge from a sewage treatment plant located near Plön (Schleswig-Holstein, Germany), Lake Plußsee, and an enrichment culture for ammonia oxidizers inoculated with a water sample from Lake Schöhsee (Lake Plußsee and Lake Schöhsee are freshwater lakes located in Schleswig-Holstein, Germany).

DNA extraction and purification. For purification of genomic DNA from both pure and enrichment cultures, cells were pelleted, resuspended in 50 μl of TE buffer (1 mM EDTA, 10 mM Tris; pH 8.0) and subsequently lysed by boiling for 10 min. After centrifugation the supernatant was transferred to a new tube, and 1-μl aliquots were directly used for PCR amplification of the target gene. Total DNAs from aquatic samples and from activated sludge were obtained as described by Ward et al. (53). For extraction of total DNA from the soil we used a protocol described by Smalla et al. (44). Total DNA from rice roots was isolated and purified as previously described by Flemming et al. (13). The DNA extracts were finally suspended in 100 μl of TE buffer. One-microliter aliquots were used in PCR assays.

PCR amplification of *amoA*. The forward primer used (*amoA*-1F; 5′-GGGG TTTCTACTGGTGGT) targets a stretch corresponding to positions 332 to 349 and the reverse primer used (*amoA*-2R; 5′-CCCCTCKGSAAAGCCTTCTTC [K = G or T; S = G or C]) targets a stretch corresponding to positions 802 to 822 of the open reading frame published previously for the *amoA* gene sequence of *Nitrosomonas europaea* (34). The primer system generated a specific PCR product 491 bp long. Amplification was performed in a total volume of 100 μl in 0.2-ml Eppendorf tubes by using a DNA thermocycler (model 2400; Perkin-Elmer Cetus, Foster City, Calif.). Reactions were performed in a solution containing 1× PCR buffer (20 mM Tris-HCl, 25 mM KCl, 1.5 mM $MgCl_2$, 0.5% Tween 20, 100 μg of bovine serum albumin per ml), 20 nmol of each deoxynucleoside triphosphate, 30 pmol of each primer, 1 μl of template DNA, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). The enzyme was added after the first denaturation step. The standard thermal profile used for the amplification of the *amoA* target sequence was as follows: 5 min at 94°C; pause at 80°C to add polymerase; then 36 cycles (pure cultures) or 42 cycles (environmental samples) consisting of 90 s at 60°C (annealing), 90 s at 72°C (elongation), and 60 s at 94°C (denaturation); and a final cycle consisting of 90 s at 60°C and 10 min at 72°C. Aliquots (10 μl) of the PCR products were electrophoresed and visualized in 1% agarose gels by using standard electrophoresis procedures.

TABLE 1. Bacterial strains used in this study

Organism	Source or reference[a]	*amoA* amplification
Ammonia oxidizers (autotrophs)		
Beta subclass of the *Proteobacteria*		
Nitrosomonas europaea C-31	ATCC 25978	+
Nitrosomonas eutropha C-91	18	+
Nitrosospira multiformis C-71	ATCC 25196	+
Nitrosospira tenuis Nv1	18	+
Nitrosospira briensis C-128	18	+
Nitrosospira sp. strain C-57	ATCC 25197	+
Nitrosospira sp. strain AHB1	41	+
Gamma subclass of the *Proteobacteria*		
Nitrosococcus oceanus C-107	ATCC 19707	−
Ammonia oxidizers (heterotrophs)		
Alcaligenes faecalis	ATCC 8750	−
Alcaligenes sp. strain 9006/1	Environmental strain	−
Methane oxidizers		
Methylosinus trichosporium QB3b	NCIMB 11131	−
Methylomonas methanica S1	NCIMB 11130	−
Methylococcus capsulatus Bath	NCIMB 11132	−
Methylocystis parvus OBBP	NCIMB 11129	−
Methylomicrobium agile A30	NCIMB 11124	−
Other bacteria		
Escherichia coli JM109	ATCC 53323	−
Azoarcus communis Sbub3	39	−

[a] ATCC, American Type Culture Collection; NCIMB, National Collections of Industrial and Marine Bacteria Ltd.

Hybridization. Aliquots (10 μl) of the *amoA* PCR products were separated in 1% agarose gels. The DNA fragments were transferred to a nylon membrane (Qiagen, Hilden, Germany) by using a Vacugene XL blotting apparatus (Pharmacia, Uppsala, Sweden) and were fixed by UV cross-linking. An *amoA-amoB* gene probe was used to confirm the identity of the PCR products. This probe was generated from a cloned partial *amoA-amoB* fragment of *Nitrosomonas europaea* by PCR with a primer system previously described by Rotthauwe et al. (41). The approximately 1.5-kb probe was labeled during the PCR. For labeling, dTTP and biotin-16-dUTP were mixed at a molar ratio of 3:1 and added to the reaction mixture at a concentration of 200 μM. The labeled probe was purified by using a Prep-A-Gene system according to the instructions of the manufacturer (Bio-Rad, Hercules, Calif.). Hybridization was done at 55°C overnight. The probe-target hybrid was visualized via chemoluminescence by using a Gene Images nonisotopic nucleic acid detection system (U.S. Biochemicals, Cleveland, Ohio). The complete posthybridization treatment, including washing steps, antibody reaction, and exposure to film, was performed by using the recommendations of the manufacturer.

Cloning. The environmental *amoA* PCR products were cloned by using the TA Cloning kit (Invitrogen, Leek, The Netherlands). Clones were randomly selected for further analysis. For preparation of phagemid DNA, colonies grown on agar plates were picked, and the material was resuspended in 100 μl of TE buffer. Cells were lysed by boiling, and the resulting debris was pelleted by centrifugation. The supernatant was transferred to a new tube, and 1-μl aliquots were directly used for PCR. Cloned inserts were amplified by using primers that targeted vector sequences, as described in the manual for the TA Cloning kit.

Sequencing. The PCR-amplified *amoA* products generated either from genomic DNA of pure cultures or from cloned environmental sequences were checked for size and purity on a 1% agarose gel and purified by using the Prep-A-Gene system (Bio-Rad). Sequencing was done nonradioactively by using a PRISM ready reaction dye terminator cycle sequencing kit according to the instructions of the manufacturer (Applied Biosystems, Foster City, Calif.). The reaction mixtures were analyzed with an automatic DNA sequencer (model 373A; Applied Biosystems). The complete sequences of the PCR-amplified *amoA* fragments were determined by using primer *amoA*-1F and universal forward and reverse primer targeting vector sequences.

Data analysis. Partial *amoA* gene sequences and deduced amino acid sequences were manually aligned with each other and with the *pmoA* sequences. No insertions or deletions were observed in the two data sets used for the reconstruction of gene genealogies. The first data set included a set of publicly

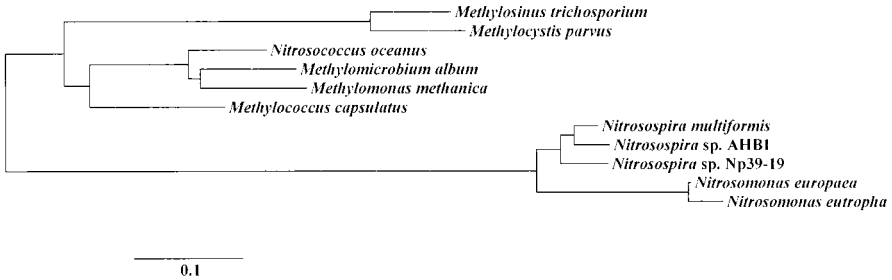

FIG. 1. Unrooted Fitch-Margoliash tree based on 165 deduced amino acid sites, showing the phylogenetic positions of partial *amoA* gene stretches from autotrophic β-proteobacterial ammonia oxidizers (*Nitrosomonas-Nitrosospira* cluster) in relation to homologous *pmoA* sequences from the α-proteobacterial methanotrophs (*Methylosinus trichosporium* and *Methylocystis parvus*) and the γ-proteobacterial methanotrophs (*Methylomicrobium album*, *Methylomonas methanica*, and *Methylococcus capsulatus*) plus the *amoA* sequence from *Nitrosococcus oceanus*. Scale bar = 0.1 substitution per amino acid site.

available partial *amoA* and *pmoA* gene sequences (for accession numbers see below) and covered a continuous stretch of 165 amino acid sites corresponding to the nucleotide sequence from position 169 through position 663 of the *amoA* gene from *Nitrosomonas europaea* (34). This data set was used to infer the evolutionary relationship of *amoA* and *pmoA* gene sequences (Fig. 1). The second data set comprised the partial *amoA* gene sequences obtained in this study and covered a continuous stretch of 450 nucleotides and 150 amino acid sites that corresponded to positions 352 through 801 (numbering based on the *amoA* gene sequence of *Nitrosomonas europaea*). This set of data was used to construct the *amoA* trees (Fig. 2 and 3). The phylogenetic trees were constructed by using computer algorithms supplied by the software package PHYLIP (10). On the amino acid level, evolutionary distances between pairs of sequences were calculated by using the PAM matrix-based distance correction implemented in the protdist program of PHYLIP. On the nucleic acid level, evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor equation (23) implemented in the dnadist program of PHYLIP. Distance dendrograms estimating the phylogenetic relationships were derived by using the method developed by Fitch and Margoliash (12).

Nucleotide sequence accession numbers. The environmental *amoA* gene sequences have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Z97833 through Z97851, and the partial *amoA* sequence stretches obtained for *Nitrosospira* sp. strain C-57, *Nitrosospira briensis* C-128, *Nitrosospira tenuis* Nv1, and *Nitrosomonas eutropha* C-91 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Z97858 through Z97861. The accession numbers for *amoA* and *pmoA* sequences used as references for the reconstruction of gene genealogies are as follows: *Methylococcus capsulatus*, L40804; *Methylomicrobium album*, U31654; *Methylomonas methanica*, U31653; *Methylocystis parvus*, U31651; *Methylosinus trichosporium*, U31650; *Nitrosomonas europaea*, L08050; *Nitrosospira* sp. strain AHB1, X90821; *Nitrosospira* sp. strain Np39-19, AF006692; *Nitrosospira* sp. strain NpAV, U38250 (*amoA1*), U20644 (*amoA2*), and U38251 (*amoA3*); *Nitrosospira multiformis*, U91603 (*amoA1*), U15733 (*amoA2*), and U89833 (*amoA3*); and *Nitrosococcus oceanus*, U31652.

RESULTS

Specificity of the *amoA* assay. Based on an alignment of publicly available *amoA* sequences (25, 34, 41), several primers were designed specifically to amplify a partial stretch of the *amoA* gene from a wide range of autotrophic ammonia oxidizers belonging to the β subclass of the *Proteobacteria*. The primers were tested in different combinations by using both template DNA from cultured strains and template DNA from environmental samples. The primer combination consisting of *amoA*-1F and *amoA*-2R provided the most reliable performance in these studies and, consequently, was used in all further studies. The assay resulted in a specific 491-bp PCR product for all of the ammonia oxidizers tested except *Nitrosococcus oceanus*, an ammonia-oxidizing member of the γ subclass of the *Proteobacteria*. The assay clearly discriminated between the *amoA* gene and the *pmoA* gene of the particulate methane monooxygenase in that neither nonspecific nor specific products were generated from methanotrophs. In addi-

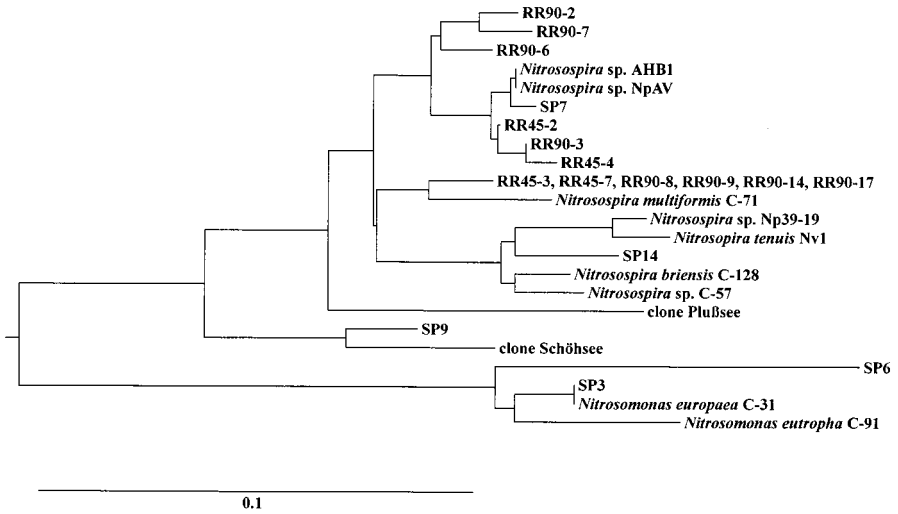

FIG. 2. Fitch-Margoliash tree constructed for partial *amoA* gene sequences based on 150 deduced amino acid sites. The tree shows the relationship between cultured ammonia oxidizers and environmental sequences retrieved from rice roots at two different vegetation stages (after 45 days [RR45-2, RR45-4, RR45-3, and RR45-7] and after 90 days [RR90-2, RR90-7, RR90-6, RR90-3, RR90-8, RR90-9, RR90-14, and RR90-17]), from activated sludge from a sewage treatment plant (SP3, SP6, SP9, SP14, and SP7), from Lake Plußsee, and from the Lake Schöhsee enrichment culture. The homologous *pmoA* stretch of *Methylococcus capsulatus* Bath was used to root the tree. Scale bar = 0.1 substitution per amino acid site.

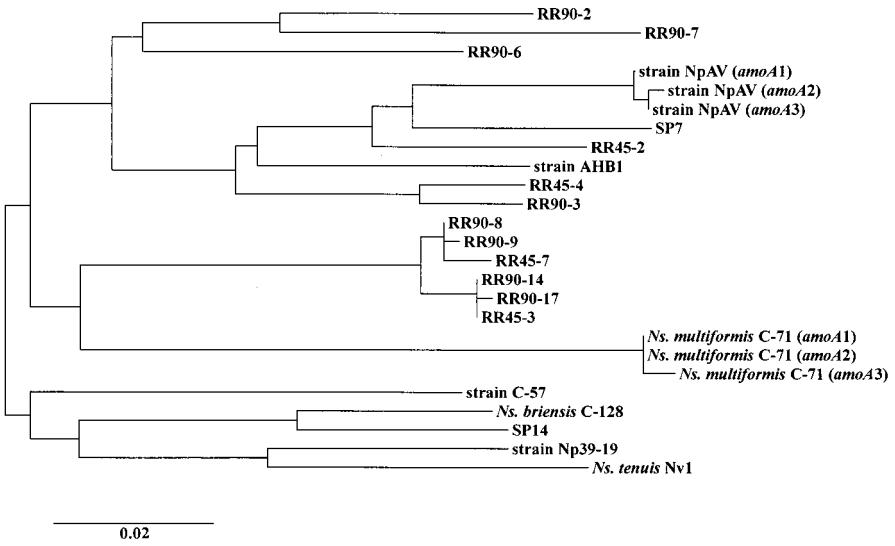

FIG. 3. Unrooted Fitch-Margoliash tree constructed for partial *amoA* gene stretches belonging to the traditional *Nitrosospira* cluster based on 450 nucleotide sequence positions. The *amoA* sequences were obtained from rice roots at two different vegetation stages (after 45 days [RR45-2, RR45-4, RR45-7, and RR45-3] and after 90 days [RR90-2, RR90-7, RR90-6, RR90-3, RR90-8, RR90-9, RR90-14, and RR90-17]), from activated sludge from a sewage treatment plant (SP7 and SP14), and from cultured strains of the traditional *Nitrosospira* cluster. The partial *amoA* gene sequences designated strain NpAV (*amoA*1) through strain NpAV (*amoA*3) and *Ns. multiformis* C-71 (*amoA*1) through *Ns. multiformis* C-71 (*amoA*3) were the three different *amoA* gene copies present in the genomes of strain NpAV and *Nitrosospira multiformis*, respectively (24, 25, 36, 37). Scale bar = 0.02 substitution per nucleotide sequence position. *Ns.*, *Nitrosospira.*

tion, genomic DNAs from control organisms, including *Alcaligenes faecalis* (heterotrophically ammonia-oxidizing strains), *Azoarcus communis* (Fig. 4), and *Escherichia coli* (Fig. 5), reacted negatively as well (Table 1). Southern blot hybridization with a nonradioactively labeled *amoA* gene probe confirmed the identity of the PCR products (Fig. 4B). Applied to total DNA extracted from different natural environments, the PCR assay showed the same specificity that it showed for pure cultures (Fig. 5).

Molecular characterization of indigenous ammonia-oxidizing populations. The *amoA* PCR products retrieved from environmental samples (i.e., from rice roots, activated sludge, Lake Plußsee, and an enrichment culture) were used for the generation of *amoA* gene libraries. A total of 47 clones were randomly selected and further analyzed by comparative sequencing. No false positives were detected in this set of molecular isolates. The identity values for the partial *amoA* sequences obtained either from pure cultures or from environmental samples varied between 74.0 and 100% on the amino acid level. In contrast, the levels of identity between these *amoA* sequences and the homologous stretch of the *pmoA* gene from *Methylococcus capsulatus* Bath (43) were significantly lower; these values ranged between 45.2 and 48.9%. The latter *Methylococcus capsulatus* Bath sequence was used as a representative of the *pmoA* database, because it is the only publicly available *pmoA* sequence that almost completely overlaps the homologous *amoA* stretch analyzed in this study (135 amino acid sites). On the nucleic acid level, the values were not as significant as the values on the amino acid level, but did not overlap (52.3 to 63.5% identity between the *amoA* sequences and the corresponding stretch of *pmoA* versus 66.7 to 100% identity between each pair of sequences in the *amoA* sequence data set).

Evolutionary relationship of *amoA* and *pmoA* sequences of known species. For phylogenetic inference of the main lines of descent, the reconstruction of gene genealogies focused on the amino acid level. Amino acid-based trees probably are less biased by multiple substitutions at particular positions than trees derived from the corresponding nucleotide sequences and therefore are more accurate phylogenetically (9, 48, 55). However, this conclusion might be different when very closely related organisms are compared (55; this study). The treeing

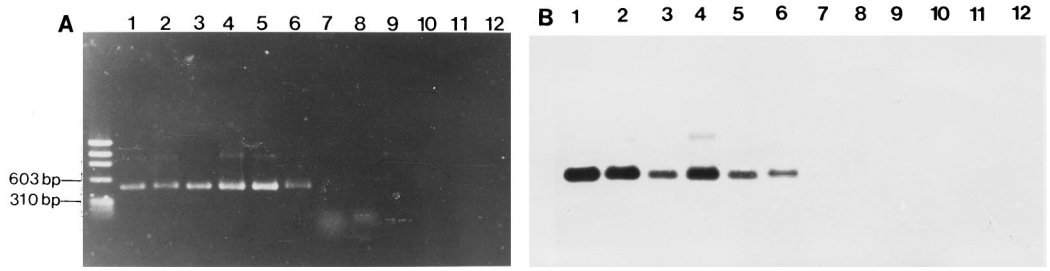

FIG. 4. (A) PCR amplification of the specific 491-bp fragment of the *amoA* gene from ammonia-oxidizing pure cultures and negative controls. (B) Southern hybridization of the corresponding blot with a biotin-labeled *Nitrosomonas europaea amoA* gene probe. Lane 1, *Nitrosomonas europaea*; lane 2, *Nitrosomonas eutropha*; lane 3, *Nitrosospira multiformis*; lane 4, *Nitrosospira tenuis*; lane 5, *Nitrosospira briensis*; lane 6, *Nitrosospira* sp. strain AHB1; lane 7, *Nitrosococcus oceanus*; lane 8, *Alcaligenes faecalis*; lane 9, *Methylosinus trichosporium*; lane 10, *Methylomonas methanica*; lane 11, *Methylococcus capsulatus*; lane 12, *Azoarcus communis*. The size marker was an *Hae*III digest of phage ϕX174 DNA.

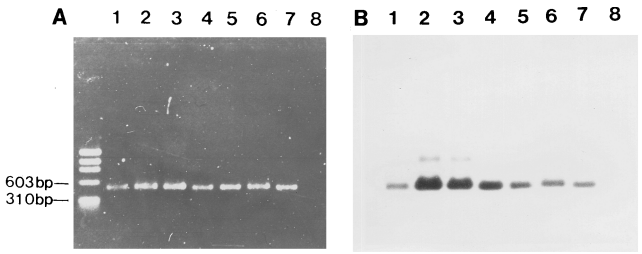

FIG. 5. (A) PCR amplification of the specific 491-bp *amoA* fragment from environmental DNAs extracted from various aquatic and terrestrial habitats. (B) Southern hybridization of the corresponding blot with a biotin-labeled *Nitrosomonas europaea amoA* gene probe. Lane 1, surface soil layer (depth, 0 to 2 cm) of a flooded rice microcosm; lane 2, rice roots (45 days); lane 3, rice roots (90 days); lane 4, aquatic sample (Lake Plußsee); lane 5, enrichment culture (Lake Schöhsee); lane 6, activated sludge (sewage plant in Plön); lane 7, *Nitrosospira* sp. strain AHB1 (positive control); lane 8, *E. coli* (negative control). The size marker was an *Hae*III digest of phage ϕX174 DNA.

analysis of the evolutionary relatedness between partial *amoA* and *pmoA* sequences (Fig. 1) resulted in a branching order which largely mirrors that of the corresponding 16S ribosomal DNA (rDNA) tree; i.e., the representatives of the α-proteobacterial methanotrophs (*Methylosinus trichosporium* and *Methylocystis parvus*) form one group, the four members of the γ subclass of the *Proteobacteria*, including *Nitrosococcus oceanus*, form a second group, and the five autotrophic ammonia oxidizers belonging to the β subclass form a third closely related cluster (17, 18, 46).

According to the 16S rDNA results (18, 41, 46), the tree constructed for the available *amoA* sequence data separates the β-subclass ammonia oxidizers into two main lines of descent characterized by (i) *Nitrosomonas europaea* and *Nitrosomonas eutropha* and (ii) *Nitrosospira* spp. (Fig. 2). The *Nitrosospira* spp. fall into three different branches characterized by (i) strains AHB1 and NpAV, (ii) *Nitrosospira multiformis*, and (iii) *Nitrosospira tenuis*, *Nitrosospira briensis*, and strains Np39-19 and C-57.

Phylogenetic affiliation of environmental *amoA* gene sequences. (i) Rice roots. All partial *amoA* sequences retrieved from roots of flooded rice grouped within the phylogenetic radiation of known *Nitrosospira* strains. *Nitrosomonas*-like sequences were not detected. The *amoA* sequences formed three distinct lineages. The first group (clone sequences RR45-2, RR45-4, and RR90-3) formed a common branch with strains AHB1 and NpAV. The members of the second cluster were molecular isolates RR90-2, RR90-7, and RR90-6. The third group was characterized by a set of partial *amoA* sequences that could not be distinguished on the amino acid level but could be distinguished on the nucleic acid level (clone sequences RR45-3, RR45-7, RR90-8, RR90-9, RR90-14, and RR90-17) (Fig. 2 and 3). The last two groups showed no significant affiliation with any of the *Nitrosospira* strains included in this study. No environmental sequences that could be assigned to the *Nitrosospira tenuis-Nitrosospira briensis* branch were retrieved from rice roots.

(ii) Activated sludge. The majority of environmental partial *amoA* sequences retrieved from activated sludge from a sewage treatment plant belonged to the *Nitrosomonas* branch. Nine of 14 clones randomly selected for comparative sequence analysis were completely identical on the amino acid level (represented by clone SP6 in Fig. 2). Interestingly, one environmental *amoA* sequence (clone SP3) was absolutely identical, even on the nucleic acid level, to the sequence of *Nitrosomonas europaea*. In addition, a few *amoA* sequences which belonged to the *Nitrosospira* branch, including *amoA* sequence types represented by clones SP7, SP9, and SP14 (Fig. 2), were identified.

(iii) Samples from freshwater lakes. Two *amoA* gene libraries were generated, the first from an *amoA* PCR product obtained from Lake Plußsee and the second from an enrichment culture inoculated with material collected from Lake Schöhsee. Comparative analysis of seven randomly selected clones resulted in the detection of only one distinct *amoA* sequence type for each of the two gene libraries. These sequence types could be assigned to the *Nitrosospira* branch, but formed two individual lines of descent (branch Plußsee and branch Schöhsee) (Fig. 2) that were clearly separated from the lineage characterized by cultured strains of the genus *Nitrosospira* (for clarity, this lineage is referred to below as the "traditional *Nitrosospira*" cluster). Interestingly, one environmental sequence retrieved from activated sludge (clone SP9) clustered on branch Schöhsee.

DISCUSSION

Specificity of the *amoA* PCR assay. The autotrophic ammonia-oxidizing bacteria do not represent a monophyletic clade but are members of at least two phylogenetically different groups. The first group is characterized by members of the *Nitrosomonas-Nitrosospira* clade in the β subclass of the *Proteobacteria*, and the second group is characterized by strains of the species *Nitrosococcus oceanus*, and, probably, *Nitrosococcus halophilus* (26) in the γ subclass of the *Proteobacteria*. Holmes et al. (21) demonstrated that the *amoA* gene from *Nitrosococcus oceanus* is evolutionarily more closely related to the *pmoA* gene from *Methylomicrobium album*, *Methylomonas methanica*, and *Methylococcus capsulatus* Bath, which are also members of the γ subclass of the *Proteobacteria*, than to the homologous stretch of the *amoA* gene from ammonia oxidizers belonging to the β subclass of the *Proteobacteria* (Fig. 1). This implies that the development of a universal *amoA* assay which targets the complete range of all known ammonia oxidizers but not other homologous sequence types (i.e., the *pmoA* gene of methanotrophs) will probably not be possible. However, the obvious need to restrict the target specificity of *amoA*-based assays to defined subgroups of known ammonia oxidizers also applies to the available 16S rDNA-based approaches due to their phylogenetic nature.

In addition to the products obtained from all of the *Nitrosomonas* and *Nitrosospira* strains tested, specific *amoA* PCR products were obtained from various terrestrial and aquatic samples, as indicated by the specific hybridization signals when an *amoA* gene probe was used (Fig. 5). Additional evidence for the specificity of the *amoA* assay is the fact that no false positives were detected in a set of 47 randomly selected clone sequences that were analyzed further. The lowest level of identity between the environmental *amoA* sequences obtained was 74.0% on the amino acid level. This value is in the same range as the values reported previously for *amoA* sequences of cultured autotrophic ammonia oxidizers (41) and sharply contrasts with the overall levels of identity obtained for the *amoA* sequence cluster when it was compared to the homologous stretch of the *pmoA* gene of *Methylococcus capsulatus* Bath (45.2 to 48.9%). The same is true for the 165-amino-acid stretch that was used to construct the Fitch-Margoliash tree for partial *amoA* and *pmoA* sequences, as shown in Fig. 1. The levels of identity between the *pmoA* sequences of the α- and γ-proteobacterial methanotrophs, including the *amoA* gene from *Nitrosococcus oceanus* on the one hand and the homologous *amoA* stretch from the β-proteobacterial clade of am-

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                     1                                                      50                                                          100                                                         150
strain AHB1          HYPINFVLPS TMIPGALIMD TVMLLTRNWM ITALVGGGAF GLLFYPGNWP IFGPTHLPLV AEGVLLSLAD YTGFLYVRTG TPEYVRLIEQ GSLRTFGGHT TVIAAFFSAF VSMLMFCVWW YFGKLYCTAF YYVKGPRGRV TMKNDVTAYG
strain NpAV          .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...G...... .......... .......... .......... ..........
clone SP7            .......... .......V.. .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
clone RR45-2         .......... .......... ..L....... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
clone RR90-3         .......F.. .......... ..L....... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
clone RR45-4         .......F.. .......... ..L....... .......... .......... .......... .......... .......... .......... .......... .........S .......... .......... .......... ..........
clone RR90-7         .......... .......... ..L....... .......... .......... .......... .......V.. .......... .......... .......... .......... .......... ....I..... F........I S.........
clone RR90-2         .......F.. .......... ..L....... ....I..... .......... .......... .......V.. .......... .......... .......... .......... .......... ....I..... F......... S.........
clone RR90-6         .......F.. .......V.. .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ....I..... F......... S.........
Ns. multiformis C-71 ...M...F.. .......V.. ..L....... .......... .........T .......... .......V.. .......... .......... .......... ....S..... ......T... ....V..... .....A.... S........F
clone RR45-7         .......F.. .......V.. ..L....... V...I..... .......... .......... .......V.. .......... .......... .......... .......... .......... ....V..... .....A.... S........F
strain C-57          ....S..F.. .......V.. .......... .......... .........T .......... T......V.. .......... .......... .......... ....S..A.. .......... .......... F....A.... .........F
Ns. briensis C-128   ....S..F.. .......V.. .......... .......... .......... .......... .......V.. .......... ......N... .......... ....S..A.. .......L.. .......... F....A.... .........F
Ns. tenuis Nv1       ...MS..F.. .......V.. .......... .......... .......... .......... V......V.. .......... ......N... .......... .......A.. I.....TI.. .......... F....H.... .........F
clone SP14           ....A..F.. .......V.. .......... .......... .......... .......... V......V.. .......... ......N... .......... ....S..A.. ......S... .L........ F......... .........F

clone Plußsee        .......... .......V.. .CL....S.L .......... .......... .......... .......V.. .......... .......... .......... .......A.. ......L... .....F..S. .......... AE.Y.....F

clone Schöhsee       .......... .......ML. .IL...G..L V...L...FW ..F....... .......... V......V.. .......... .......... .......... .......... .......... .......... .....E...I S..H.....F
clone SP9            .......... .......M.. .I....G..L ....L...F. ..F....... ........V. V......V.. .......... .......... .......... .......A.. .......... .......... .....E...I S........F

clone SP6            .......T.G I.L....ML. LTLY.....L V......AF. .......... ........I. V..H...M.. .M.HM.I... ....T....K .......... .......A.. .......... .L..V..... F....K...I VHRE.....F
Nm. eutropha C-91    ...L...T.G I.L....ML. FT.Y.....L V.......F. ..M....... ........I. V..T...M.. .M.H...... ......H... .......... .......A.. ......A... .L..V..... F....K.R.I VQR......F
Nm. europaea C-31    .......T.G I.L....ML. FTLY.....L V.......F. .......... ........I. V..T...M.. .M.H...... ......H... .......... .......... ......T... .L..V..... F....K...I VHR......F
clone SP3            .......T.G I.L....ML. FTLY.....L V.......F. .......... ........I. V..T...M.. .M.H...... ......H... .......... .......... ......T... .L..V..... F....K...I VHR......F

M.capsulatus Bath    YF.....F.A SLV...I.L. ..LM.SGSYL F..I..AMGW ..I....... .IA.L.V.VE NN.M.M.I.. IQ.YN..... ....I.MV.K .T.....KDV APVS...... M.I.IYFM.H FI.RWFSNER FLQST----- ----------
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FIG. 6. Alignment of the deduced amino acid sequences for the partial 450-bp *amoA* gene stretch examined in this study. Sequence data were obtained from cultured ammonia oxidizers and from environmental samples including rice roots taken from 45- and 90-day-old flooded microcosms (clones RR45-2, RR90-3, RR45-4, RR90-7, RR90-2, RR90-6, and RR45-7), activated sludge (clones SP7, SP14, SP9, SP6, and SP3), Lake Plußsee, and the Lake Schöhsee enrichment culture. The homologous stretch of the *pmoA* gene of *Methylococcus capsulatus* Bath is included as an outgroup reference sequence. Only one representative is shown for each individual partial *amoA* sequence type obtained in this study. Identical amino acid positions are indicated by dots. *Ns.*, *Nitrosospira*; *Nm.*, *Nitrosomonas*; *M.*, *Methylococcus.*

monia oxidizers on the other hand, ranged from 41 to 54% for this stretch, whereas the levels of identity between the *amoA* sequences of members of the *Nitrosomonas-Nitrosospira* group were not less than 80%. These findings resulted in the conclusion that environmental sequences with identity values above a threshold level of about 70% compared to the *amoA* gene of known β-proteobacterial ammonia oxidizers represent *amoA* gene stretches that have been retrieved from the latter group of organisms.

In contrast to the specificity observed for the *amoA* PCR assay, a 16S rDNA-based approach used by Stephen et al. (45) to assess the molecular diversity of β-subclass ammonia oxidizers in soil and marine environments resulted to a large extent in the coamplification of environmental sequences closely related to sequences of nonnitrifying members of the β subclass of the *Proteobacteria*, including *Comamonas testosteroni*, *Sphaerotilus natans*, and *Rubrivivax gelatinosus*. Similarly, we used a 16S rDNA-based assay that has been described as being highly specific for the detection of autotrophic β-subclass ammonia oxidizers (49). This study used one of the total DNA extracts which has been successfully used for the retrieval of environmental *amoA* sequence types (RR45) (Fig. 2 and 3). PCR amplification resulted in a product of the correct size which was subsequently used for the generation of a 16S rRNA gene library. However, comparative sequence analysis of 13 randomly selected 16S rDNA clones did not reveal any clone that could be assigned to the *Nitrosomonas-Nitrosospira* group. Instead, the majority of the sequences belonged to a novel lineage in the β subclass of the *Proteobacteria* that grouped next to *Telluria mixta* and *Telluria chitinolytica* (data not shown). This discrepancy in targeting false positives indicates that the *amoA*-based approach has greater specificity than approaches which use 16S rDNA as a marker. The reason for this might be the ubiquitous presence of 16S rRNA-like genes in all prokaryotes, which may lead to reduced specificity if PCR assays are applied to environmental samples that contain a large variety of as-yet-unknown 16S rDNA sequence types. This might be especially true for a 16S rDNA-based assay that is developed specifically to target a microbial group with a relatively low population size (like ammonia oxidizers) or does not perfectly match all sequence types of its target group (38, 49).

Gene genealogies. The topology of the *amoA-pmoA* tree (Fig. 1) is in good agreement with the topology of the corresponding 16S rDNA tree, with only slight deviations in the branching order of the four γ-subclass sequences (17). At first glance, the congruities suggest that the common ancestral gene type of the present-day *amoA* and *pmoA* sequences was already present in a proteobacterial line of descent prior to the separation into the α, β, and γ subclasses. However, more detailed studies must be performed to clarify if horizontal gene transfer can be excluded as one of the underlying processes which resulted in this tree topology. The monophyletic character of the *amoA* gene of the β-subclass ammonia oxidizers is suggested not only by the long common branch found in the *Nitrosomonas-Nitrosospira* clade (Fig. 1), but also by a set of 38 absolutely conserved amino acid residues in all partial *amoA* sequences that are not present in the *pmoA* sequence of *Methylococcus capsulatus* Bath (Fig. 6). In addition, the separation of the ammonia oxidizers into two main lines of descent characterized by *Nitrosomonas europaea* and *Nitrosomonas eutropha* on the one hand and by the traditional *Nitrosospira* species on the other hand also corresponds to the 16S rDNA results (18, 41, 46). It is noteworthy that all partial *amoA* gene sequences belonging to the traditional *Nitrosospira* cluster exhibited nucleotide sequence identities between 59.3 and 63.5% with the

TABLE 2. Amino acid residues indicating the phylogenetic coherence of the traditional *Nitrosospira*, Plußsee, and Schöhsee lineages and amino acid sequence motifs shared by the Schöhsee and *Nitrosomonas*-like lineages

Lineages compared	Position	Amino acid in:				
		Traditional *Nitrosospira* lineage	Plußsee lineage	Schöhsee lineage	*Nitrosomonas*-like lineage	*Methylococcus capsulatus* Bath
Traditional *Nitrosospira*, Plußsee, and Schöhsee	8	Leu/Phe	Leu	Leu	Thr	Phe
	10	Ser	Ser	Ser	Gly	Ala
	11	Thr	Thr	Thr	Ile	Ser
	13	Ile	Ile	Ile	Leu	Val
	21	Thr	Thr	Thr	Leu/Phe	Thr
	24	Leu	Leu	Leu	Tyr	Met
	64	Val	Val	Val	His/Thr	Met
	68	Leu/Val	Val	Val	Met	Ile
	72	Thr	Thr	Thr	Met	Gln
	74	Phe	Phe	Phe	His	Tyr
	143	Lys	Lys	Lys	Arg	
Schöhsee and *Nitrosomonas*-like	18	Ile/Val	Val	Met	Met	Ile
	30	Met	Leu	Leu	Leu	Leu
	39	Ala	Ala	Phe	Phe	Gly

[a] The homologous amino acid residues of the *pmoA* gene from *Methylococcus capsulatus* Bath are shown for reference purposes. The positions correspond to the numbering in Fig. 6.

pmoA sequence of *Methylococcus capsulatus* Bath, whereas the corresponding values for the three *Nitrosomonas*-like sequences (*Nitrosomonas europaea*, *Nitrosomonas eutropha*, and clone SP6) ranged between 52.3 and 56.8%. This observation might indicate that the evolutionary rates for the *amoA* genes in the different lineages of the autotrophic β-subclass ammonia oxidizers were slightly different.

Two novel lineages were detected that could not be assigned to any of the pure-culture sequences (branch Plußsee and branch Schöhsee). However, the root of the *amoA* tree determined by using the homologous *pmoA* sequence of *Methylococcus capsulatus* Bath grouped these lineages and the traditional *Nitrosospira* cluster on a common branch (Fig. 2). A search for amino acid residues that were on the one hand invariant in the Plußsee-, Schöhsee-, and traditional *Nitrosospira*-like sequences but were on the other hand different in the *Nitrosomonas*-like sequences identified 11 such amino acids (Table 2). Eight of these amino acids represented nonconservative substitutions leading to amino acids with different biochemical characteristics. In contrast, only one and three amino acid residues were shared with the residues of the *Nitrosomonas* branch by the Plußsee sequence and the two Schöhsee-like sequences, respectively, and these amino acid residues were not found in the *amoA* sequences of the traditional *Nitrosospira* branch. These findings provide further evidence that these two novel lineages are affiliated with the traditional *Nitrosospira* branch. Recently, Stephen et al. (45) reported the presence of four novel as-yet-unknown phylogenetic clusters of β-proteobacterial ammonia oxidizers in soil and marine environments on the basis of 16S rRNA gene libraries. Three of these novel lineages were identified as *Nitrosospira*-like groups and showed in a 16S rDNA tree based on 1,099 nucleotide sequence positions a phylogenetic affiliation to the traditional *Nitrosospira* cluster similar to the phylogenetic affiliation that branches Plußsee and Schöhsee show in the *amoA* tree. This raises the interesting question of whether Stephen et al. (45) and we detected the same novel ammonia-oxidizing subgroups by using different gene markers. Hopefully, this question will be answered in the near future, because cross-linking of the two data sets would allow additional conclusions about the phylogenetic complexity of *Nitrosospira*-like populations actually existing in natural environments.

Formation of chimeric structures. Analogous to environmental 16S rDNA-based studies (27–29, 51), there might be a risk of formation of chimeric structures during mixed amplification of *amoA* gene sequences. The inclusion of such sequences in a phylogenetic analysis would blur the gene genealogies and indicate lines of descent that do not exist in nature. Because of the unusual positions of branches Plußsee and Schöhsee in the *amoA* tree (Fig. 2), we reanalyzed these environmental sequence types to examine this problem. However, the fact that at least the Schöhsee branch was confirmed with two independently analyzed samples not only indicates that this lineage occurs naturally but also suggests that its members are present in different habitats. In addition, a set of amino acid signature residues that characterize each of these two novel lineages is distributed over the complete *amoA* stretch analyzed (at positions 22, 28, 117, 126, 129, 141, 142, and 144 in branch Plußsee and at positions 22, 27, 35, 43, and 136 in branch Schöhsee) (Fig. 6), and furthermore, these sequence types were the dominant sequence types in the gene libraries generated from the Lake Plußsee sample and the enrichment culture, respectively. However, one chimeric sequence was detected (clone RR90-13) that was shuffled together during PCR from two different *amoA* sequence types. Clone RR90-13 differed in the first 300 bp by only 3 substitutions from clone RR90-7, but in the remaining 150 nucleotides it differed by 24 substitutions. In contrast, these 150 bp had the same nucleotide sequence as the clone RR90-14 sequence, but differed from the latter *amoA* sequence at 33 positions in the first 300 nucleotides. The chimeric nature of clone RR90-13 was recognized in a separate phylogenetic analysis of the 5′ and 3′ regions of the *amoA* nucleotide sequences belonging to the traditional *Nitrosospira* cluster. The characterization of clone RR90-13 as a PCR artifact presupposed that this amplicon does not represent a naturally occurring *amoA* gene type that

could come from intragenic recombination of *amoA* gene sequences between different ammonia-oxidizing populations similar to that which has been shown, for example, for some genomic loci of naturally occurring populations of *E. coli* (8, 16). However, considering the relatively high frequency of artificial recombination occurring between similar sequence types during mixed amplification in PCR, as reported for 16S rRNA genes (27, 51), the alternative interpretation appears to be rather unlikely.

Ecological significance. Since only a few strains of autotrophic β-subclass ammonia oxidizers are available from public culture collections, we investigated various natural samples to evaluate the range of diversity that can be detected by the newly developed *amoA* assay in environmental studies; these samples included rice roots, activated sludge, lake water, and an enrichment culture. Using the same primer system, thermal PCR profile, and cloning strategy for all samples, we identified rather diverse ammonia-oxidizing populations in activated sludge. Two *amoA* clone sequences (SP7 and SP14) belonged to two different subgroups of the traditional *Nitrosospira* cluster (Fig. 2), and one (SP9) was affiliated with branch Schöhsee. However, *Nitrosomonas*-like *amoA* sequences were the dominant type in the clone library generated from activated sludge. In contrast, the molecular approach applied to the rice root samples detected members of the traditional *Nitrosospira* cluster almost exclusively. These results, however, do not per se allow the conclusions that *Nitrosomonas* spp. are the most abundant ammonia-oxidizing organisms in activated sludge and *Nitrosospira* spp. are the most abundant ammonia-oxidizing organisms in the rhizosphere of rice. The reason for this is that some method-inherent bias may have occurred during the molecular retrieval of the partial *amoA* gene sequences, which may have led to a shift in the relative proportions of the different *amoA* sequence types present. The conclusion that the data could be biased can be reached from the results of molecular retrieval studies in which the 16S rRNA gene was used as a marker (28). In addition, the assay is not able to detect members of the genus *Nitrosococcus*, and some as-yet-unknown ammonia oxidizers belonging to the *Nitrosomonas-Nitrosospira* clade may escape detection due to a lack of target specificity. Although the assay has been proven to detect a wide range of β-proteobacterial nitrifiers, the oligonucleotide primers used may have to be refined when new *amoA* sequence data become available. Nevertheless, the results obtained in this study are in fair agreement with some data obtained from similar habitats by using other approaches. Wagner et al. (50) identified *Nitrosomonas*-like bacteria as the abundant type of nitrifiers in activated sludge by using in situ hybridization with fluorescently labeled 16S rRNA-targeted oligonucleotide probes. Both culture-based techniques (5, 30, 32, 33) and molecular 16S rDNA-based approaches (19, 45) suggest that *Nitrosospira* spp. are more common in terrestrial environments than *Nitrosomonas* spp.

In order to evaluate if the *amoA* gene allows fine-scale resolution of closely related ammonia-oxidizing populations, as previously suggested by Rotthauwe et al. (41), we constructed a nucleic acid tree based on *amoA* sequences for the traditional *Nitrosospira* group. The topology of this dendrogram largely reflected the branching pattern of the corresponding amino acid tree (Fig. 2 and 3). However, the two trees differed in the branch lengths separating individual sequences. The reason for this is the accumulation of synonymous (neutral) mutations that do not lead to changes of amino acid residues but clearly facilitate further differentiation of the *amoA* cluster. The comparative sequence analysis of the three *amoA* gene copies present in the genomes of *Nitrosolobus multiformis* and strain NpAV detected only two and three nucleotide differences, respectively, in the 450-bp *amoA* stretch which was analyzed by us (Fig. 3) (24, 25, 36, 37). From these data, it is reasonable to conclude that environmental *amoA* gene sequences with significantly more nucleotide differences originate from closely related but distinct ammonia-oxidizing strains, even if the deduced amino acid sequences are almost identical. This is most obvious for the *amoA* sequences of strains AHB1 and NpAV. On the amino acid level, these sequences differ at no position and one position, respectively, depending on which of the three *amoA* gene copies shown to be present in the genome of strain NpAV is considered. In contrast, both sequences differ at 38 nucleotide sequence positions, leading to a different branching order in the nucleotide tree. A second example is molecular isolates RR90-2, RR90-7, and RR90-6, which exhibit only one to three substitutions on the amino acid level, but differ at 37 to 53 positions on the nucleotide sequence level. These data confirm the superiority of nucleotide sequences derived from protein-encoding genes for the analysis of phylogenetic relationships between closely related bacteria, as previously reported by Yamamoto and Harayama (55). However, analysis on the amino acid level may give a more accurate phylogenetic tree when synonymous substitutions may have occurred at the most possible sites between the sequence stretches compared.

Comparative sequence analysis of 16S rDNA and other genes has shown that bacterial groups fall into distinct sequence clusters of closely related strains, which often correspond to ecologically distinct populations (7, 11, 40). The term ecological population is used for groups with individual evolutionary adaptations to defined ecological niches that give them higher fitness in their respective niches than all closely related populations (7). With respect to the genetic diversity of the ammonia oxidizers detected on rice roots, the partial *amoA* sequences exhibit highly similar amino acid patterns but can be clearly further differentiated into distinct lineages on the nucleic acid level. The presence of up to 63 nucleotide substitutions in the molecular isolates belonging to the traditional *Nitrosospira* cluster suggests that these isolates have a common evolutionary history but have been distinct for some time. Bodelier et al. (6) described specific adaptations to low-oxygen or anoxic situations in the indigenous ammonia-oxidizing populations colonizing the root zone of the oxygen-releasing, aerenchymatous, emergent macrophyte *Glyceria maxima*, and the nitrifying capacities were maintained under these conditions compared to nitrifying populations that inhabit permanently oxic habitats. Oxic-anoxic fluctuations are also a characteristic of the rhizosphere of flooded rice and of lake habitats, and, consequently, we assume that similar specific adaptations occur in the indigenous ammonia oxidizers in these environments. One of the very interesting aspects of our future studies will be which level of sequence divergence corresponds to ecologically distinct populations inhabiting different niches in habitats that are characterized by different or fluctuating physicochemical conditions.

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