APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1998, p. 3042–3051 Vol. 64, No. 8
0099-2240/98/$04.00+0

Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: *Nitrosococcus mobilis* and *Nitrospira*-Like Bacteria as Dominant Populations

STEFAN JURETSCHKO,[1] GABRIELE TIMMERMANN,[2] MARKUS SCHMID,[1] KARL-HEINZ SCHLEIFER,[1] ANDREAS POMMERENING-RÖSER,[3] HANS-PETER KOOPS,[3] AND MICHAEL WAGNER[1]*

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich,[1] Max-Planck-Institut für Limnologie, D-24302 Plön,[2] and Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, D-22609 Hamburg,[3] Germany

Received 5 February 1998/Accepted 27 May 1998

The ammonia-oxidizing and nitrite-oxidizing bacterial populations occurring in the nitrifying activated sludge of an industrial wastewater treatment plant receiving sewage with high ammonia concentrations were studied by use of a polyphasic approach. In situ hybridization with a set of hierarchical 16S rRNA-targeted probes for ammonia-oxidizing bacteria revealed the dominance of *Nitrosococcus mobilis*-like bacteria. The phylogenetic affiliation suggested by fluorescent in situ hybridization (FISH) was confirmed by isolation of *N. mobilis* as the numerically dominant ammonia oxidizer and subsequent comparative 16S rRNA gene (rDNA) sequence and DNA-DNA hybridization analyses. For molecular fine-scale analysis of the ammonia-oxidizing population, a partial stretch of the gene encoding the active-site polypeptide of ammonia monooxygenase (*amoA*) was amplified from total DNA extracted from ammonia oxidizer isolates and from activated sludge. However, comparative sequence analysis of 13 *amoA* clone sequences from activated sludge demonstrated that these sequences were highly similar to each other and to the corresponding *amoA* gene fragments of *Nitrosomonas europaea* Nm50 and the *N. mobilis* isolate. The unexpected high sequence similarity between the *amoA* gene fragments of the *N. mobilis* isolate and *N. europaea* indicates a possible lateral gene transfer event. Although a *Nitrobacter* strain was isolated, members of the nitrite-oxidizing genus *Nitrobacter* were not detectable in the activated sludge by in situ hybridization. Therefore, we used the rRNA approach to investigate the abundance of other well-known nitrite-oxidizing bacterial genera. Three different methods were used for DNA extraction from the activated sludge. For each DNA preparation, almost full-length genes encoding small-subunit rRNA were separately amplified and used to generate three 16S rDNA libraries. By comparative sequence analysis, 2 of 60 randomly selected clones could be assigned to the nitrite-oxidizing bacteria of the genus *Nitrospira*. Based on these clone sequences, a specific 16S rRNA-targeted probe was developed. FISH of the activated sludge with this probe demonstrated that *Nitrospira*-like bacteria were present in significant numbers (9% of the total bacterial counts) and frequently occurred in coaggregated microcolonies with *N. mobilis*.

Nitrification, the bacterially catalyzed oxidation of ammonia to nitrate is a key process in the global cycling of nitrogen (39) and an integral component of modern wastewater treatment plants. Reduction of the ammonia content of sewage is important, as ammonia is toxic to aquatic life (e.g., reference 3) and creates a large oxygen demand in receiving waters. Furthermore, nitrification is a prerequisite for total N removal from sewage via subsequent denitrification. Two distinct, physiologically defined groups of bacteria catalyze the two separate steps involved in nitrification (7, 27). First, chemolithoautotrophic ammonia-oxidizing bacteria convert ammonia to nitrite, which is subsequently transformed to nitrate by nitrite-oxidizing bacteria. Sixteen species of lithoautotrophic ammonia-oxidizing bacteria have been isolated and validly described (21, 23, 25, 26, 60). Based on comparative 16S rRNA gene (rDNA) sequence analysis, cultured ammonia-oxidizing bacteria comprise two monophyletic groups within the *Proteobacteria*. *Nitrosococcus oceanus* and *N. halophilus* belong to the gamma subclass of the class *Proteobacteria* (63), while the members of the genera *Nitrosomonas* and *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* (the latter three being closely related to each other [16]), as well as *Nitrosococcus mobilis* (actually a member of the genus *Nitrosomonas*) constitute a closely related assemblage within the beta subclass of *Proteobacteria* (16, 38, 47, 50, 51, 62). Based on ultrastructural properties, cultivated nitrite-oxidizing bacteria have been assigned to the four recognized genera *Nitrobacter*, *Nitrospina*, *Nitrococcus*, and *Nitrospira*. Comparative 16S rDNA sequence analyses revealed that one of these genera, *Nitrobacter* (61), with its three species (5, 6), is a member of the alpha subclass of *Proteobacteria* (37, 50). The genera *Nitrospina* (58) and *Nitrococcus* (58), with one species each, belong to the delta and gamma subclass of *Proteobacteria*, respectively (50). The remaining genus, *Nitrospira* (59), encompassing the species *Nitrospira moscoviensis* (14) and *N. marina* (59), is a member of the *Nitrospira* phylum of the domain *Bacteria* (14).

Investigation of the diversity and ecology of nitrifying bac-

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Arcisstr. 16, Technische Universität München, D-80290 München, Germany. Phone: 49 89 2892 2373. Fax: 49 89 2892 2360. E-mail: wagner@biol.chemie.tu-muenchen.de.

TABLE 1. Probe sequences, target sites, and formamide concentrations in the hybridization buffer required for specific in situ hybridization

Probe	Sequence (5′–3′)	Target site[a] (16S rRNA positions)	% Formamide
Nso190	CGATCCCCTGCTTTTCTCC	190–208	55
Nso1225	CGCCATTGTATTACGTGTGA	1225–1244	35
Nsv443	CCGTGACCGTTTCGTTCCG	444–462	30
Nsm156	TATTAGCACATCTTTCGAT	156–174	5
NEU	CCCCTCTGCTGCACTCTA	653–670	40
CTE	TTCCATCCCCCTCTGCCG	659–676	—[b]
S-*-Nse-1472-a-A-18	ACCCCAGTCATGACCCCC	1472–1489	50
NmV	TCCTCAGAGACTACGCGG	174–191	35
S-*-Ntspa-1026-a-A-18	AGCACGCTGGTATTGCTA	1026–1043	20
NIT3	CCTGTGCTCCATGCTCCG	1035–1048	40
CNIT3	CCTGTGCTCCAGGCTCCG	1035–1048	—[c]

[a] *E. coli* numbering (10).
[b] Used as unlabeled competitor together with probe NEU.
[c] Used as unlabeled competitor together with probe NIT3.

teria in natural and engineered systems by traditional cultivation techniques has been hampered by their slow growth rates and by the biases inherent in all culture-based studies (e.g., reference 4; 53, 60). Most studies on nitrification were performed with *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, as they represent ammonia- and nitrite-oxidizing bacteria, which are easy to obtain from international bacterial culture collections but might not represent those nitrifying bacteria dominant in the environments analyzed (39, 60). This appears particularly likely in light of recent molecular studies exploiting 16S rDNA sequence information which demonstrated a sequence diversity in the monophyletic line of the beta subclass ammonia oxidizers which significantly exceeded the species diversity recognized in cultured ammonia oxidizers (28, 31, 48). Unfortunately, the limited degree of sequence diversity within some of the environmental ammonia oxidizer sequence clusters makes it difficult to interpret how many of the sequences represent new, as yet uncultured, species (46). In addition, quantitative dot blot (19) and in situ hybridization (56) studies using 16S rRNA-targeted probes specific for the nitrite-oxidizing bacteria of the genus *Nitrobacter* indicated low *Nitrobacter* numbers in a variety of nitrifying environments and consequently highlighted the importance of non-*Nitrobacter* nitrite-oxidizing bacteria for the nitrification process.

Recently, the battery of molecular tools used to infer the presence of ammonia-oxidizing bacteria in the environment in a cultivation-independent way has been supplemented by sets of specific or semispecific PCR primers for amplification of 16S rDNA (12, 18, 38, 52, 57) or the ammonia monooxygenase structural gene *amoA* (15, 40, 44). While such PCR-based methods have provided exciting new insights into the sequence diversity and environmental distribution of ammonia oxidizers, they do not permit accurate quantification of cell numbers. For direct enumeration and simultaneous in situ analysis of the spatial distribution of environmental populations of nitrifying bacteria, we and others have used in situ hybridization with fluorescent oligonucleotide probes (33, 43, 55, 56). Since the presence of ammonia oxidizers can be correlated with their characteristic activity, in situ probe counts can be compared with total nitrification rates to calculate the specific in situ activity per cell (55). However, isolation of dominant ammonia- and nitrite-oxidizing bacteria identified by molecular methods is still required to obtain a more comprehensive picture of their physiology. The inevitable bias induced by standard cultivation can be partly compensated for by the monitoring of enrichment and isolation using hybridization with oligonucleotide probes designed from environmentally derived 16S rRNA sequences (22).

The present study was undertaken to identify the most important species of the nitrifying bacterial population present in activated sludge with a high nitrifying capacity that originated at an industrial wastewater treatment plant. The diversity of ammonia-oxidizing bacteria was studied by (i) fluorescent in situ hybridization techniques on activated sludge samples using previously published phylogenetic probes (33, 38, 55), (ii) comparative sequence analysis of environmentally derived *amoA* gene sequences, and (iii) isolation and subsequent characterization (using fluorescent in situ hybridization, 16S rDNA sequencing, and DNA-DNA hybridization) of the numerically dominant ammonia oxidizer population. Since we failed in a previous study to detect nitrite-oxidizing bacteria of the genus *Nitrobacter* in the activated sludge we analyzed (56), we used comparative analysis of 16S rDNA sequences to test for the presence of other nitrite-oxidizing genera. Confocal laser scanning microscopy and fluorescent in situ hybridization using probes designed from environmentally derived sequences affiliated with the genus *Nitrospira* were used to monitor their abundance and spatial distribution in activated sludge.

(A preliminary part of this work has been presented at the Second International Conference on Microorganisms in Activated Sludge and Biofilm Processes, 21 to 23 July 1997, Berkeley, Calif.)

MATERIALS AND METHODS

Organisms, culture conditions, and cell fixation. The nitrifying bacteria investigated in this study were cultured as described previously (6, 26). For in situ hybridization, cells were fixed with paraformaldehyde (2) from cultures which had oxidized 70 to 80% of the ammonia and nitrite, respectively, originally present in the cultivation medium.

Sampling. Grab samples were collected in October 1996 and September 1997 from the intermittently aerated nitrification-denitrification basin of an industrial wastewater treatment plant receiving sewage from an animal waste processing facility (Tierkörperbeseitigungsanstalt Kraftisried, Kraftisried, Germany; 6,000 population equivalents [PE] [1 PE = 60 g of biological oxygen demand day^{-1}). For DNA isolation, aliquots of the samples were pelleted by centrifugation ($10{,}000 \times g$ for 2 min), immediately frozen on dry ice, and stored at −80°C after their arrival at our laboratory. For in situ hybridization, a subsample of activated sludge was fixed for 3 h with 4% paraformaldehyde as described by Amann (2).

Oligonucleotide probes. The following 16S rRNA-targeted oligonucleotide probes were used: (i) NEU, complementary to a signature region of most halophilic and halotolerant ammonia oxidizers (55); (ii) Nso190, specific for the ammonia oxidizers in the beta subclass of *Proteobacteria* (33); (iii) Nso1225, specific for the ammonia oxidizers in the beta subclass of *Proteobacteria* (33); (iv) Nsm156, specific for the *Nitrosomonas* cluster (33); (v) Nsv443, specific for the *Nitrosospira* cluster (33); (vi) NmV, specific for the *N. mobilis* lineage (38); (vii) S-*-Nse-1472-a-A-18, targeted against ammonia oxidizer isolate Nm103 and all

other members of the *N. europaea* lineage; (viii) NIT3, complementary to a region of all previously sequenced *Nitrobacter* species (56); and (ix) S-*-Ntspa-1026-a-A-18, specific for *N. moscoviensis* and activated-sludge-derived clone sequences A-4 and A-11. The sequences and target sites of all of the probes are listed in Table 1. The probes developed in this study were named in accordance with the standard proposed by Alm et al. (1). The names of previously published probes were left unchanged to avoid confusion. Oligonucleotides were synthesized with a C6-trifluoracetylamino amino linker at the 5′ end (Interactiva, Ulm, Germany). Labeling with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) and with the monofunctional, hydrophilic sulfoindocyanine dyes Cy3 and Cy5 (Amersham, Buckinghamshire, United Kingdom) and purification of the oligonucleotide-dye conjugates were performed as described by Amann (2). For labeling with the Cy dyes, the fluorochrome was suspended in a 1:1 mixture of 200 mM sodium carbonate buffer (pH 9.0) and dimethyl formamide.

In situ hybridization and probe-specific cell counts. Optimal hybridization conditions were determined for probes NmV, S-*-Nse-1472-a-A-18, and S-*-Ntspa-1026-a-A-18 by using the hybridization and wash buffers described by Manz et al. (30). Optimal hybridization stringency required the addition of formamide to final concentrations of 20% for probe S-*-Ntspa-1026-a-A-18, 35% for probe NmV, and 50% for probe S-*-Nse-1472-a-A-18. All hybridizations were performed at a temperature of 46°C. Subsequently, a stringent wash step was performed for 10 min at 48°C. Simultaneous hybridization with probes requiring different stringency was realized by a successive-hybridization procedure (54). Probes NEU and NIT3 were applied together with the competitor oligonucleotides CTE and CNIT3, respectively (55, 56; Table 1). Dual staining of cells with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent oligonucleotides was modified from the method of Hicks et al. (17) so that cells were stained after in situ hybridization with DAPI (0.5 $\mu g\ ml^{-1}$) for 10 min on ice. Probe-specific cell counts were determined by enumerating at least 5,000 cells stained with DAPI.

Microscopy. A Zeiss LSM 510 scanning confocal microscope equipped with a UV laser (351 and 364 nm), an Ar ion laser (450 to 514 nm), and two HeNe lasers (543 and 633 nm) was used to record optical sections. Image processing was performed with the standard software package delivered with the instrument (version 1.5). Reconstructed and processed images were printed by using the software package Microsoft Power Point (version 7.0) in combination with a Kodak 8650 PS printer.

Enrichment and isolation of ammonia oxidizers. Enrichments were performed on a mineral salt medium containing 10 or 100 mM NH_4Cl, 0.4 mM KH_2PO_4, 1 mM KCl, 0.2 mM $MgSO_4$, 10 or 200 mM NaCl, 5-g $liter^{-1}$ $CaCO_3$, 1-m $liter^{-1}$ 0.05% (wt/vol) cresol red solution, and 1-ml $liter^{-1}$ trace element solution [0.2 mM $MnSO_4$, 0.8 mM H_3BO_3, 0.15 mM $ZnSO_4$, 0.03 mM $(NH_4)_6Mo_7O_{24}$, 2.5 mM $FeSO_4$, and 0.1 mM $CuSO_4$ in 0.01 N HCl]. Isolations were carried out by plating the enrichments on mineral medium solidified by addition of 10 g of agar per liter and picking single colonies after 2 months of incubation at 30°C. Purity of the cultures was checked by microscopic examinations and by inoculation of an organic medium containing 0.5-g $liter^{-1}$ (each) yeast extract, beef extract, and peptone (pH 7.4).

Enrichment and isolation of nitrite oxidizers. A nitrite-oxidizing bacterium was enriched and isolated from the activated sludge as described by Bock et al. (6). Purity of the nitrite oxidizer isolate was checked by (i) inoculation of an organic culture medium (containing 0.5 g of yeast extract, 0.5 g of peptone, 0.5 g of beef extract, and 0.584 g of NaCl per liter; pH 7.4) and (ii) fluorescent in situ hybridization with probe NIT3 (56).

PCR amplification of 16S rDNA. DNA was extracted from a 0.25-g (wet weight) activated sludge pellet by using three different methods (11, 45, 64). For PCR amplification of the 16S rDNA of the ammonia and nitrite oxidizer isolates, high-molecular-weight DNA was isolated by the method of Chan and Goodwin (11). Oligonucleotide primers targeting the 16S rDNAs of all bacteria were used for PCR with a thermal capillary cycler (Idaho Technology, Idaho Falls) to obtain almost-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the primers were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*Escherichia coli* 16S rDNA positions 8 to 27 [10]) and 5′-CAKAAAGGAGGTGATCC-3′ (*E. coli* 16S rDNA positions 1529 to 1545). Reaction mixtures were prepared in accordance with the manufacturer's recommendations in a total volume of 50 μl by using 20 mM $MgCl_2$ reaction buffer. Thermal cycling was carried out with an initial denaturation step of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 51°C for 20 s, and elongation at 72°C for 30 s; cycling was completed by a final elongation step of 72°C for 1 min. Positive controls containing purified DNA from *E. coli* were included in all sets of amplifications along with negative controls (no DNA added). The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of the reaction product.

PCR amplification of the *amoA* gene fragment. For PCR amplification of the *amoA* gene fragment of the ammonia oxidizer isolates, high-molecular-weight DNA was isolated by the method of Chan and Goodwin (11). A 665-bp fragment of the *amoA* gene was amplified from 100 ng of DNA by primers AMO-F and AMO-R (44) for PCR with a thermal capillary cycler (Idaho Technology). Reaction mixtures with each primer at 15 pM were prepared in accordance with the manufacturer's recommendations in a total volume of 50 μl by using 20 mM $MgCl_2$ reaction buffer and 1.5 U of *Taq* polymerase (Promega, Madison, Wis.). Thermal cycling was carried out by an initial denaturation step of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 48°C for 40 s, and elongation at 72°C for 50 s; cycling was completed by a final elongation step of 72°C for 1 min. For *amoA* gene fragment amplification from the bacterial population present in the activated sludge, total genomic DNA was extracted by the following protocol. A 0.25-g activated sludge pellet was resuspended in a 2-ml polypropylene tube with a screw-on plastic cap with 675 μl of DNA extraction buffer (100 mM Tris-HCl [pH 8.0]; 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% cetyltrimethylammonium bromide) and treated for 30 s with a blender (Ultraturrax; Janke and Kunkel, Freiburg, Germany). After addition of 50 μl of enzyme mixture I (lysozyme [Fluka, Buchs, Switzerland], lipase Typ7 [Sigma, Deisenhofen, Germany], pectinase [Roth, Karlsruhe, Germany], and β-glucuronidase [Sigma], each at 10 mg $liter^{-1}$), the mixture was incubated for 30 min at 37°C. Subsequently, 50 μl of enzyme mixture II (proteinase K [Boehringer Mannheim], protease Typ9 [Sigma], and pronase P [Serva, Heidelberg, Germany], each at 10 mg $liter^{-1}$) was added and the mixture was incubated for another 30 min at 37°C. After addition of 75 μl of 20% sodium dodecyl sulfate and incubation at 65°C for 2 h, cell lysis was completed by addition of 600 μl of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and 20 min of incubation at 65°C. After vortexing, the mixture was centrifuged for 10 min at 10,000 × *g* at room temperature. The aqueous phase was carefully transferred to a fresh tube, mixed with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged again for 10 min at 10,000 × *g*. The aqueous phase was transferred to a fresh tube, and nucleic acids were precipitated by incubation with 0.6 volume of isopropanol for 1 h at room temperature and subsequent centrifugation for 20 min at 10,000 × *g*. Pellets were washed with 1 ml of 70% ethanol, dried, and finally resuspended in 50 μl of double-distilled H_2O. The amount and quality of DNA were determined by spectrophotometric analysis of the ratio of optical densities at 260 and 280 nm (41). Amplification of *amoA* gene fragments was initially performed with 25, 50, and 100 ng of DNA and primers AMO-F and AMO-R as described above. To increase the specificity of *amoA* amplification from activated sludge, a nested PCR protocol was developed. For this purpose, two additional primers, AMO-F2 (5′-AAGATGCCGC CGGAAGC-3′) and AMO-R2 (5′-GCTGCAATAACTGTGGTA-3′), comprising the inner primer set, were designed from the *amoA* sequences of *N. europaea* Nm50, isolate Nm93, and isolate Nm103. AMO-F2 and AMO-R2 hybridize to nucleotide positions 288 to 305 and 895 to 913, respectively, of the published *N. europaea* sequence (32). For nested PCR, 25, 50, and 100 ng of genomic DNA isolated from activated sludge was amplified by using primers AMO-F and AMO-R and the conditions described above. A 1-μl volume of the reaction product was subjected to a further round of PCR amplification with 15 pM (each) AMO-F2 and AMO-R2 as a second primer set. Reaction mixtures were prepared as described above. Thermal cycling was carried out by an initial denaturation step of 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 40 s, and elongation at 72°C for 50 s; cycling was completed by a final elongation step of 72°C for 1 min. Positive controls containing purified DNA from *N. europaea* Nm50 were included in all of the amplification sets along with negative controls (no DNA added). The presence and size of the amplification products were determined by agarose (0.8%) gel electrophoresis of the reaction product. Ethidium bromide-stained bands were digitally recorded with a Cybertech video documentation system (Cybertech, Hamburg, Germany).

Cloning, sequencing, and phylogeny inference. 16S rDNA and *amoA* PCR products were excised from the agarose gel, purified with an agarose gel extraction kit (Boehringer Mannheim), and subsequently ligated, in accordance with the manufacturer's recommendations, into the cloning vector (pCRII) supplied with the Original TA cloning kit (Invitrogen Corp., San Diego, Calif.). One *amoA* gene library and three 16S rDNA clone libraries, reflecting the three methods used for DNA extraction, were generated. Nucleotide sequences were determined by the dideoxynucleotide method (42) by cycle sequencing of purified plasmid preparations (Qiagen, Hilden, Germany) with a Thermo Sequenase Cycle sequencing kit (Amersham) and an infrared automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. Dye-labeled sequencing primers (Li-Cor) were used. The 16S rDNA sequences were added to the 16S rRNA sequence database of the Technische Universität München by use of the ARB program package (49). The ARB_EDIT tool was used for sequence alignment. Alignments were refined by visual inspection. Deduced amino acid sequences for *amoA* were aligned manually by pooling the amino acids into six groups with the GDE 2.2 sequence editor implemented in the ARB software package. Nucleic acid sequences of the *amoA* gene fragments were then aligned in accordance with the amino acid alignment. Nucleic acid similarities were computed by using the appropriate tool in the ARB program package. Phylogenetic analyses based on 16S rDNA were performed by applying the ARB parsimony tool and maximum-likelihood analysis (fast DNAml; 29) to different data sets. Checks for chimeric sequences were conducted by independently subjecting the first 513 5′ base positions, the middle 513 base positions, or the last 513 3′ base positions of the insert sequence to phylogenetic analysis. Phylogenetic trees based on comparative analysis of the *amoA* gene fragments were computed by performing maximum-likelihood analysis on *amoA* nucleic acid alignments with the appropriate tool in the ARB software package.

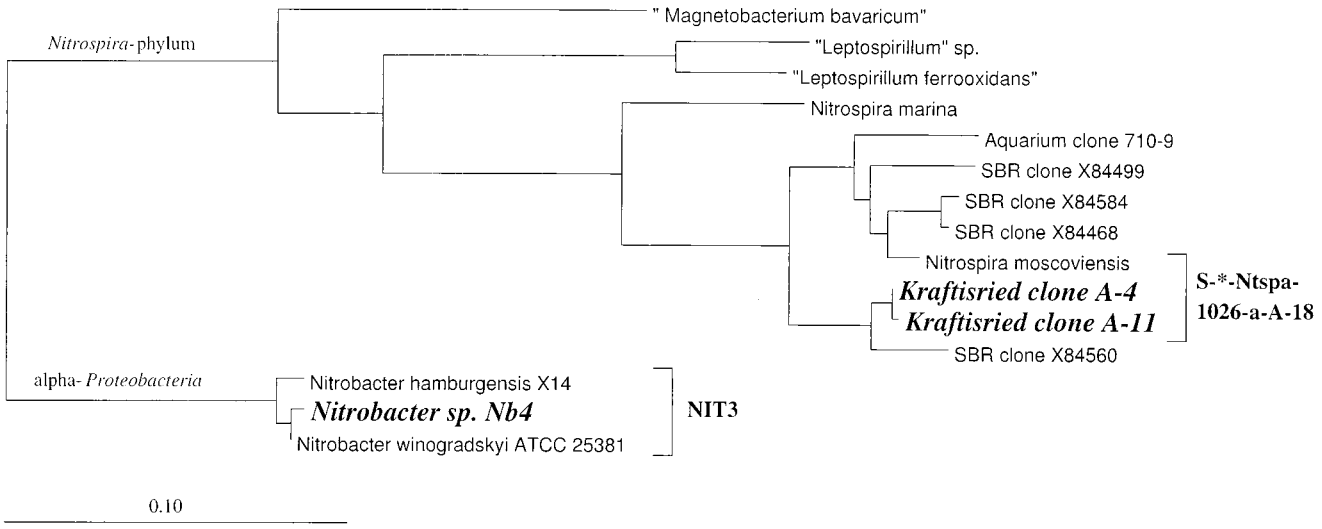

FIG. 1. Phylogenetic tree showing the relationships of the *Nitrospira*-like organisms represented by 16S rDNA clones A-4 and A-11 from activated sludge, *Nitrobacter* isolate Nb4, and their closest relatives. The tree is based on the results of maximum-likelihood analysis. Together with a 16S rDNA clone retrieved from an SBR (GenBank accession no. X84560; 8), both clone sequences form a separate lineage within the nitrite-oxidizing bacteria of the *Nitrospira* group, which is also supported by maximum-parsimony and neighbor-joining analyses. Target organisms for probes S-*-Ntspa-1026-a-A-18 and NIT3 are indicated by brackets. Due to partial sequencing of the SBR clones (8), no information about their sequence in the target region of probe S-*-Ntspa-1026-a-A-18 is available. The bar represents 10% estimated sequence divergence.

DNA-DNA hybridization. DNA similarities were estimated by photometric determination of thermal renaturation rates (13) as described by Koops and Harms (24).

Nucleotide sequence accession numbers. The sequences obtained in this study are available in GenBank under accession no. AF037105 (Nm93; 16S rRNA), AF037106 (Nm103, 16S rRNA), AF037107 (Nm103; *amoA*), AF037108 (Nm93; *amoA*), and AF043707 to AF043719 (*amoA* activated sludge clones 1 to 13).

RESULTS

The Kraftisried wastewater treatment plant receives sewage with exceptionally high NH_4^+ concentrations (up to 5,000 mg liter^{-1}) which stem from the decay of protein-rich material handled by the adjoining animal waste processing facility. Due to intermittent aeration and the high nitrifying and denitrifying capacity of its activated sludge, the Kraftisried plant was able to remove more than 90% of the N compounds from the sewage during the sampling period (October 1996 to October 1997).

Activated-sludge-derived 16S rDNA sequences affiliated with the genus *Nitrospira*. Three different methods were used for DNA extraction of activated sludge sampled in October 1996. For each DNA preparation, 16S rDNA products were separately amplified and used for the generation of 16S rDNA libraries. A total of 60 clones were randomly selected from the libraries for comparative sequence analysis. Interestingly, none of the 16S rDNA sequences analyzed grouped within the monophyletic clade of the beta subclass of ammonia oxidizers (data not shown). However, 2 of the 60 sequences analyzed were unambiguously affiliated with nitrite-oxidizing bacteria of the genus *Nitrospira* (Fig. 1). Both sequences were found in the 16S rDNA library generated after DNA extraction by the method of Chan and Goodwin (11). Within the genus *Nitrospira*, these almost identical clone sequences showed about 94 and 88% 16S rRNA sequence similarity to *N. moscoviensis* and *N. marina*, respectively (Table 2). Based on these two activated-sludge-derived 16S rDNA sequences, oligonucleotide probe S-*-Ntspa-1026-a-A-18 was designed (Table 1).

In situ characterization of the population structure of nitrifying bacteria. The composition of the nitrifying consortium in the activated sludge was analyzed by fluorescent in situ hybridization with a set of previously published probes (33, 38, 55, 56). By searching for ammonia oxidizers in both samples, no hybridization signals could be obtained after application of *Nitrosospira* cluster-specific probe Nsv443, but 16 (October 1996) to 20% (September 1997) of the total number of cells stained with the intercalating dye DAPI could be assigned to the *Nitrosomonas* cluster of the beta subclass of ammonia oxidizers by

TABLE 2. Similarity ranking of clones A-4 and A-11 retrieved from activated sludge and members of the *Nitrospira* phylum

rDNA source	% Similarity to rDNA of:							
	A-4	A-11	710-9	SBR clone X84560	*N. moscoviensis*	*N. marina*	*Leptospirillum* sp.	*L. ferrooxidans*
A-4 sequence								
A-11 sequence	99.8							
710-9 sequence	92.2	92.0						
SBR clone X84560	97.0	96.5	92.1					
N. moscoviensis	94.0	93.8	94.7	91.6				
N. marina	88.2	88.0	87.2	83.0	88.8			
Leptospirillum sp.	80.9	80.7	79.9	76.5	80.0	81.3		
L. ferrooxidans	81.6	81.4	79.2	77.3	80.8	81.4	93.0	
M. bavaricum	80.3	80.2	78.5	73.9	79.9	80.8	79.7	81.4

simultaneous binding of probes Nso190, Nso1225, Nsm156, and NEU. In an additional hybridization experiment performed on both samples, more than 99% of the ammonia oxidizers detectable with probe NEU could simultaneously be visualized with *N. mobilis*-specific probe NmV. However, a few bacterial microcolonies did not show simultaneous binding of probes NEU and NmV but, instead, hybridized exclusively with either probe NEU or probe NmV. Looking for nitrite-oxidizing bacteria within the activated sludge, we were not able to detect any *Nitrobacter* cells by in situ hybridization with probe NIT3. However, in situ hybridization with probe S-*-Ntspa-1026-a-A-18, under stringent hybridization conditions (20% formamide in the hybridization buffer), revealed significant numbers (in both samples, approximately 9% of the total number of cells stained by DAPI) of *Nitrospira*-like cells to be present in the samples analyzed. Hybridization of the two activated sludge samples with probes NmV and S-*-Ntspa-1026-a-A-18 demonstrated that *Nitrospira*-like cells occurred as small microcolonies in close proximity to *N. mobilis* (Fig. 2).

Isolation of nitrifying bacteria. To obtain cultures of the numerically dominant nitrifying bacterial species, enrichments were initiated by inoculating small amounts of activated sludge into a series of different enrichment media. After plating of the enrichments on isolation agar and picking of colonies about 8 weeks later, eight ammonia oxidizer isolates and one nitrite oxidizer isolate were obtained. Using whole-cell hybridization of the ammonia oxidizer isolates with the respective probe set, we could distinguish between two types of ammonia oxidizers. Four of the isolates (Nm94, Nm100, Nm103, and Nm106) showed positive hybridization signals with probes Nso190, Nso 1225, Nsm156, and NEU but were not detectable with probe NmV. The remaining isolates (Nm93, Nm99, Nm104, and Nm107) showed a hybridization pattern identical to that observed for the *N. mobilis*-like cells present in the activated sludge. Nearly complete 16S rDNA sequences were determined for Nm93 and Nm103, representing the two types of ammonia oxidizers isolated. The 16S rRNA primary structures of isolates Nm93 and Nm103 shared overall similarities of more than 98% with *N. mobilis* Nc2 and *N. europaea* Nm50, respectively (Table 3; Fig. 3). Since the 16S rRNA similarities of both isolates to validly described species were greater than 97%, we performed DNA-DNA reassociation studies to clarify their species affiliations (46). The DNA similarity value measured for isolate Nm 93 and *N. mobilis* Nc2 was 82.7%, while the respective value for isolate Nm103 and *N. europaea* Nm50 was 84.8%. Based on the 16S rRNA sequence of isolate Nm103, oligonucleotide probe S-*-Nse-1472-a-A-18 was developed. Whole-cell hybridization experiments demonstrated that this probe also hybridized to *N. europaea* Nm50, *N. halophila* Nm1, and *N. eutropha* Nm 57 under stringent conditions (Table 4). Simultaneous in situ hybridization of the activated sludge with probes NEU, NmV, and S-*-Nse-1472-a-A-18, each labeled with a different fluorescent dye, revealed that all NEU-positive, NmV-negative cells were stained with probe S-*-Nse-1472-a-A-18.

The only nitrite-oxidizing isolate obtained (Nb4) could be assigned to the genus *Nitrobacter* by successful whole-cell hybridization with probe NIT3 (56). Consistent with the probing result, phylogenetic analysis of a partial 16S rRNA sequence (676 bp) of Nb4 revealed high similarity (99.8%) to the 16S rRNA of *N. winogradskyi*.

Comparative *amoA* sequence analysis. For isolates Nm93 and Nm103, a 665-bp fragment of the *amoA* gene was amplified with the primer set described by Sinigalliano et al. (44). Nucleic acid sequence analysis of both *amoA* fragments revealed that the sequence of isolate Nm103 was identical to that of the corresponding *amoA* fragment of *N. europaea* Nm50 (32) and displayed only a single base substitution compared to the sequence of isolate Nm93 (99.8% sequence similarity). For fine-scale diversity analysis of environmental populations of *N. europaea* and *N. mobilis*, a fragment of the *amoA* gene was amplified from DNA extracted from activated sludge for comparative sequence analysis. Initial *amoA* amplification experiments were performed with the primer set published by Sinigalliano et al. (44). While this primer pair amplified the expected 665-bp fragment from *N. europaea* Nm50, Nm93, and Nm103, no such product was observed for the Kraftisried activated sludge when 25 or 50 ng of DNA was used for amplification (Fig. 4). After increasing the amount of DNA used for *amoA* PCR to 100 ng, small amounts of a 665-bp amplification product, as well as larger and smaller products, were observed (Fig. 4). We excised and purified the 665-bp product from the agarose gel for subsequent cloning. However, sequence analysis of 20 randomly selected clones revealed that none of them had an *amoA*-related insert (data not shown). To enhance the specificity and sensitivity of the *amoA* PCR, we designed an additional inner primer pair for nested amplification of *amoA* from *N. europaea* and *N. mobilis*. By applying the nested PCR assay, we obtained a single amplification product with the expected length of 625 bp from both *N. europaea* Nm50 and Kraftisried activated sludge (Fig. 4). The nested PCR products retrieved from the activated sludge were used to generate an *amoA* gene library. A total of 13 clones were randomly selected for comparative sequence analysis. All of the nucleic acid sequences obtained were highly similar to each other (>99.8% sequence similarity) and to the partial *amoA* sequence of clone SP3 derived from activated sludge by Rotthauwe et al. (40; >99.7% sequence similarity). Six of the *amoA* clone nucleic acid sequences were identical to the *amoA* genes of *N. europaea* Nm50 and Nm103 and had a single base substitution in comparison with the *amoA* gene of the *N. mobilis* isolate Nm93. The remaining seven *amoA* clones had one to four base substitutions compared to the *amoA* sequences of *N. europaea* Nm50 (32), Nm103, and Nm 93. Consequently, the Kraftisried *amoA* sequences were lumped together with *N. europaea*, *N. mobilis*, and clone sequence SP3 in a phylogenetic tree for the *amoA* sequences reconstructed by the maximum-likelihood method (Fig. 5).

DISCUSSION

Recent studies of nitrifying bacteria in activated sludge systems by fluorescent in situ hybridization with rRNA-targeted oligonucleotides indicated that part of the numerically important ammonia oxidizer assemblage has not yet been characterized (33) and that the importance of nitrite oxidizers other than *Nitrobacter* spp. for sewage treatment might have been underestimated (56). To learn more about the nitrifying bacteria involved in sewage treatment, the nitrifying consortium present in the nitrifying-denitrifying activated sludge from an industrial sewage treatment plant was characterized by a combination of molecular and cultivation-based techniques. In situ diversity analysis with multiple probes demonstrated that at least three different types of ammonia-oxidizing bacteria were present in activated sludge from the Kraftisried plant. It was surprising to find that *N. mobilis*-like cells were of the numerically dominant ammonia oxidizer type, as this species was originally isolated from brackish water (23) and had not been reported to contribute to nitrification in wastewater treatment. A second, small population of cells showed an unexpected hybridization pattern indicative of a novel, as-yet-uncultured (or if cultured, then previously not characterized on the 16S rRNA

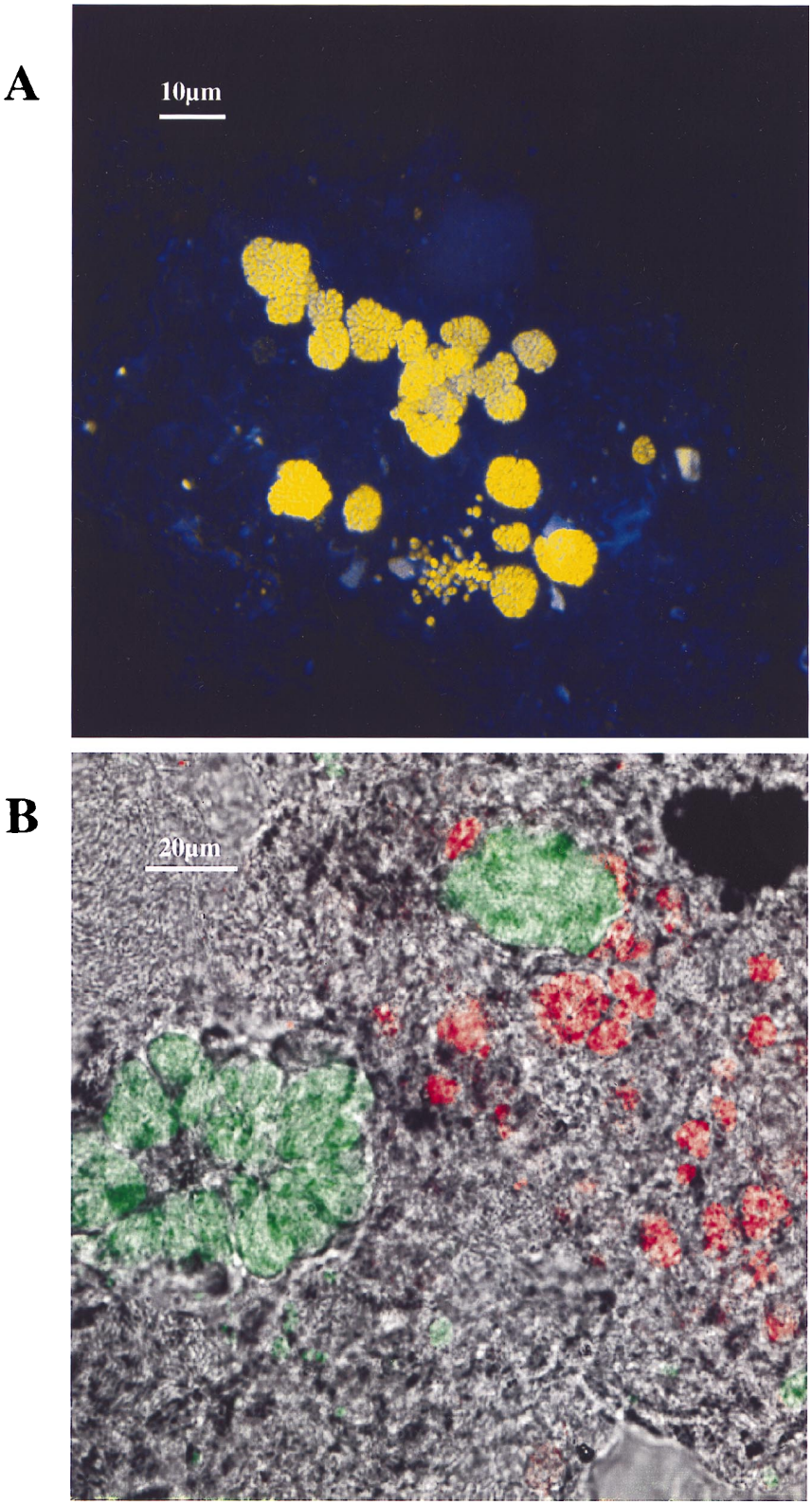

FIG. 2. In situ identification of nitrifying bacteria in activated sludge from the Kraftisried plant. (A) Simultaneous in situ hybridization with Cy3-labeled probe NmV and FLUOS-labeled probe NEU. *N. mobilis* cells appear yellow because of the overlapping labels. For visualization of the activated sludge floc, its autofluorescence was recorded with a 633-nm laser and colored blue by image analysis. (B) Simultaneous in situ identification of *N. mobilis* and *Nitrospira*-like bacteria after in situ hybridization with FLUOS-labeled probe NmV (green) and Cy3-labeled probe S-*-Ntspa-1026-a-A-18 (red). A phase-contrast image was superimposed for visualization of the floc material.

TABLE 3. 16S rRNA sequence similarities of ammonia-oxidizing bacteria Nm93 and Nm103 isolated from activated sludge and members of the *Nitrosomonas* cluster

rDNA source	% Similarity to rDNA of:				
	N. mobilis Nm93	*N. europaea* Nm103	*N. europaea*	*N. mobilis*	*N. eutropha*
N. mobilis Nm93					
N. europaea Nm103	95.4				
N. europaea	95.6	99.1			
N. mobilis	98.2	95.1	95.5		
N. eutropha	94.9	97.7	98.1	94.4	
N. halophila	96.1	97.3	98.1	95.6	96.6

TABLE 4. Reference strains analyzed by whole-cell hybridization with oligonucleotides NEU and S-*-Nse-1472-a-A-18

Organism	Strain[a]	Hybridization[b] with probe:	
		S-*-Nse-1472-a-A-18	NEU
Nitrosomonas europaea	Nm103	+	+
Nitrosococcus mobilis	Nm93	−	+
Nitrosomonas europaea	Nm50^T	+	+
Nitrosomonas halophila	Nm1^T	+	+
Nitrosomonas eutropha	Nm57^T	+	+
Nitrosomonas cryotolerans	Nm55^T	−	+
Nitrosomonas aestuarii	Nm36^T	−	+
Nitrosomonas marina	Nm22^T	−	+
Nitrosomonas oligotropha	Nm45^T	−	−
Nitrosomonas ureae	Nm10^T	−	−

[a] T = type strain.
[b] +, hybridized; −, failed to hybridize.

level) ammonia oxidizer. This population hybridized with probes Nso190, Nso1225, and Nsm156 and *N. mobilis*-specific probe NmV but did not hybridize with probe NEU, which targets halophilic and halotolerant members of the genus *Nitrosomonas*, including *N. mobilis*. A third population of ammonia oxidizers with a low in situ abundance in the Kraftisried plant hybridized with probes Nso190, Nso1225, Nsm156, NEU, and S-*-Nse-1472-a-A-18—a hybridization pattern which is indicative of *N. halophila*, *N. europaea*, and *N. eutropha*. Despite the high in situ abundance of ammonia oxidizers in the activated sludge (10 to 20% of the total number of cells), none of the 60 sequences analyzed from the three 16S rDNA gene libraries obtained from the activated sludge was affiliated with ammonia oxidizers belonging to the beta subclass of *Proteobacteria*. The 16S rDNA amplification procedure did, however, work well when DNA from the ammonia oxidizer isolates was used as the template (see below). Plausible explanations for the absence, or at least significant underrepresentation, of ammonia oxidizer 16S rDNA sequences in the gene libraries are that (i) the three different DNA extraction techniques applied were not sufficiently rigorous to lyse the dense microcolonies of ammonia oxidizers in the activated sludge and/or that (ii) PCR or cloning biases occurred. Since microcolonies of ammonia oxidizers similar in architecture to those in the Kraftisried plant have been observed in situ in many nitrifying sewage disposal plants (55) and natural systems (56), results of DNA extraction and PCR-based molecular techniques alone for detection and diversity analysis of ammonia-oxidizing bacteria have to be carefully interpreted. Such an analysis might overlook numerically dominant populations in the environment.

Another goal of the present study was to obtain a pure culture isolate for each of the in situ-detected ammonia ox-

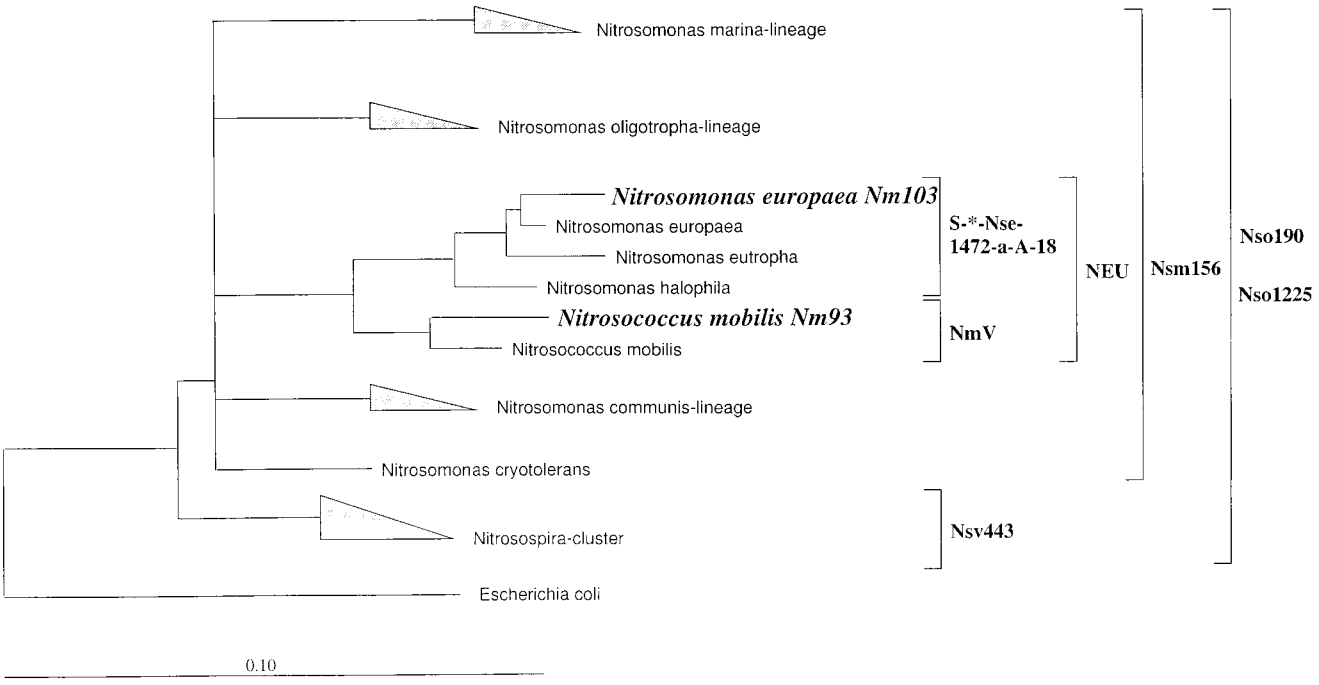

FIG. 3. Phylogenetic tree showing the relationships of ammonia oxidizer isolates *N. mobilis* Nm93 and *N. europaea* Nm103 and their closest relatives among the beta subclass of *Proteobacteria*. The tree is based on the results of maximum-likelihood analysis. Target organisms for probes Nso190, Nso1225, Nsm156, Nsv443, NEU, S-*-Nse-1472-a-A-18, and NmV are indicated by brackets. The bar represents 10% estimated sequence divergence.

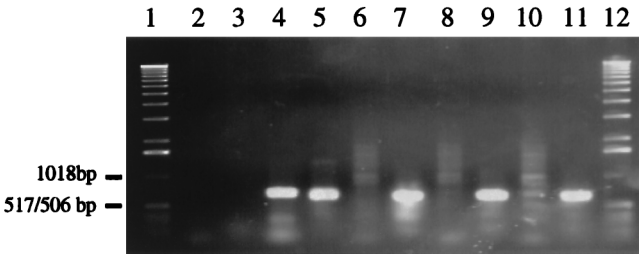

FIG. 4. Direct and nested PCR amplification of an *amoA* fragment from *N. europaea* Nm50 and from activated sludge (Kraftisried). Direct PCR amplification was performed with primers AMO-F and AMO-R (lanes 2, 4, 6, 8, and 10), and nested PCR amplification was performed with primers AMO-F and AMO-R, followed by primers AMO-F2 and AMO-R2 (lanes 3, 5, 7, 9, and 11). Lanes: 1 and 12, 1-kb DNA ladder; 2 and 3, water control; 4 and 5, 25 ng of *N. europaea* DNA; 6 and 7, 25 ng of activated sludge DNA; 8 and 9, 50 ng of activated sludge DNA; 10 and 11, 100 ng of activated sludge DNA.

idizer types. Screening with the ammonia-oxidizing bacterial probe set revealed that half of the ammonia-oxidizing isolates obtained by standard cultivation techniques were *N. mobilis* like, while the other half of the isolates could be assigned to the NEU-positive, NmV-negative *N. europaea* type. This distribution is in contrast to the abundance of both of the ammonia oxidizer populations found in the activated sludge and reflects the different salt requirements of the two ammonia oxidizer types. As most of our enrichments were performed with low-salt media (10 mM NaCl) composed for limnetic and terrestrial ammonia oxidizers, *N. mobilis*, which is characterized by an obligate salt requirement, was outnumbered in these enrichments by *N. europaea*. We failed to isolate the putative novel population of ammonia oxidizers observed in activated sludge by in situ hybridization. This cultivation failure may have been caused by low in situ abundance or by unique physiological properties not addressed by the enrichment strategies applied. It is also possible that the observed novel population could not be obtained by selective cultivation because it belongs to a hypothetical taxon closely related to the beta subclass ammonia oxidizer clade which does not oxidize ammonia. To date, no precedent for such a dramatic phenotype variation within the monophyletic beta-subclass ammonia oxidizers has been reported.

Two isolates representing the two types of ammonia-oxidizing bacteria obtained were identified as *N. mobilis* and *N. europaea* by comparative 16S rDNA sequence analysis (Fig. 3) and DNA-DNA hybridization, thereby confirming the specificity of the probes used for in situ analysis. As *N. mobilis* isolate Nm93 grows significantly slower in low-salt media than does *N. europaea* isolate Nm103, it appears likely that this species has been overlooked in activated sludge in previous laboratory culture studies.

Rotthauwe et al. (40) found the topology of an *amoA*-based phylogenetic tree to be in good agreement with the topology of the corresponding 16S rDNA tree. Nucleotide sequence similarities of closely related ammonia oxidizers were significantly lower for the *amoA* genes than for the corresponding 16S rRNA. For example, *N. europaea* and *N. eutropha* share 98.2% sequence similarity on the 16S rRNA level, whereas the corresponding values for the 665-bp *amoA* fragment analyzed in the present study is only 87.3%. Since *N. europaea* and *N. mobilis* share 95.6% sequence similarity on the 16S rRNA level, it was surprising that we could detect one single base substitution only between the 665-bp *amoA* fragments of *N. europaea* isolate Nm103 and *N. mobilis* isolate Nm93. This is in accordance with the results of Böttcher (9), which revealed unexpected high sequence similarities among 613-bp *amoA* fragments amplified from different genera of ammonia-oxidizing bacteria. These results could indicate lateral *amoA* gene transfer events between species of ammonia oxidizers of different genera. This assumption implies that the *amoA* genes we sequenced fulfill the criterion of orthology. Since some ammonia oxidizers possess multiple copies of the *amoA* gene (34–36), it is possible that the primers we used amplified not all of the *amoA* copies of *N. mobilis* Nm93 and that its orthologous ammonia monooxygenase has been overlooked. This appears to be unlikely, however, as sequence variation between the multiple *amoA* copies of a single species analyzed so far was shown to be very low (34–36). Additional experiments have been initiated to provide evidence for or against the hypothesis of horizontal transfer of the *amoA* gene and, as a result, for or against the suitability of the *amoA*-encoded protein to accurately reflect the phylogenetic relationships between ammonia oxidizers.

amoA clone sequences were retrieved from activated sludge after combining a modified DNA extraction protocol with a newly developed nested PCR technique. Keeping in mind the possible inadequacy of the three DNA extraction techniques used to generate 16S rDNA gene libraries for efficient cell lysis of *N. mobilis* microcolonies (see above), we implemented several additional enzymatic pretreatments in a modified DNA extraction protocol. Nevertheless, none of the 13 *amoA* acti-

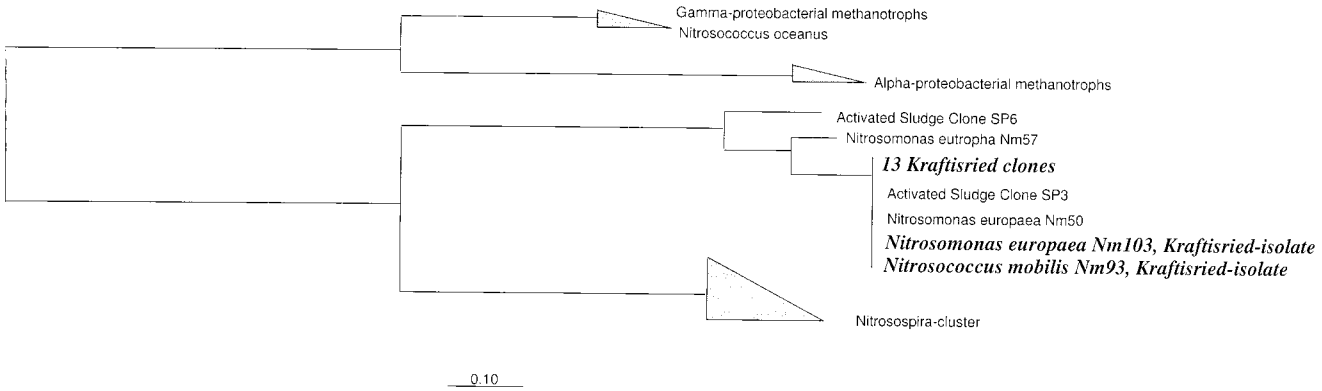

FIG. 5. Maximum-likelihood tree based on partial *amoA* gene sequences showing the phylogenetic positions of the *amoA* gene stretches from ammonia oxidizer isolates *N. mobilis* Nm93 and *N. europaea* Nm103 and the environmental sequences retrieved from activated sludge. The bar indicates 10% estimated sequence divergence.

vated sludge clones analyzed on the sequence level was identical to the *amoA* sequence of *N. mobilis* Nm93. Several *amoA* clones with sequence identity to the *amoA* sequence of *N. europaea* could be detected, however. This result, which is in apparent contrast to the in situ abundance of *N. mobilis* and *N. europaea*, and the fact that a nested PCR approach had to be used for successful *amoA* amplification from the activated sludge suggest that our modified DNA extraction protocol might still not be suitable for obtaining representative amounts of DNA from *N. mobilis* microcolonies. The development of more efficient DNA extraction protocols is a prerequisite for increasing the significance of DNA extraction-based molecular diversity analysis of ammonia oxidizers.

Results of the full-cycle rRNA approach presented here clearly show that *Nitrospira*-like bacteria, which previously had been found exclusively in marine environments (59), a heating system in Moscow (14), and freshwater aquaria (20), occurred as a dominant population in Kraftisried activated sludge, while *Nitrobacter* numbers were below the detection limit of the in situ hybridization technique (10^3 to 10^4 cells ml^{-1}). In the activated sludge flocs, *Nitrospira*-like cells always were in the vicinity of *N. mobilis* microcolonies, which may reflect the syntrophic association between ammonia- and nitrite-oxidizing bacteria. Phylogenetic analysis of partial 16S rRNA sequences retrieved by Bond et al. (8) from a laboratory-scale sequencing batch reactor (SBR) in Australia demonstrated their association with the genus *Nitrospira* (Fig. 1), which indicates a widespread occurrence of *Nitrospira*-like cells in nitrifying activated sludge systems. Keeping in mind that low in situ *Nitrobacter* numbers have been reported for many nitrifying natural and engineered systems (56) and that the described species of *Nitrospira* grow significantly slower in pure culture than do *Nitrobacter* spp., it is tempting to speculate that *Nitrospira* spp. are responsible for nitrite oxidation in these environments but have previously been overlooked, as *Nitrobacter* outcompetes them during standard enrichment and isolation procedures. Consistent with this hypothesis is the fact that the only nitrite oxidizer isolate obtained from the Kraftisried plant could be assigned to the genus *Nitrobacter* by comparative 16S rRNA sequence analysis, while representatives of the in situ-dominant *Nitrospira*-like bacteria were missing among the isolates. It should be stressed, however, that comparative 16S rRNA analysis does not unambiguously prove that the molecular isolates of *Nitrospira*-like cells are indeed able to oxidize nitrite. For a detailed physiological characterization of *Nitrospira*-like organisms, representative isolates in pure culture will be required. Attempts to recover *Nitrospira*-like cells from activated sludge via probe-assisted isolation have been initiated. In the present report, we describe for the first time *N. mobilis* and *Nitrospira*-like bacteria as putative numerically dominant species of the nitrifying consortia in an industrial sewage treatment plant. The relevance of this finding lies in the importance of nitrifier activity for efficient nutrient removal in sewage treatment. Different species of ammonia- and nitrite-oxidizing bacteria most likely differ in their in situ growth kinetics, their ammonia and nitrite oxidation rates, their substrate and oxygen affinities, and their sensitivities to environmental perturbations. Probe-assisted isolation of numerically dominant nitrifying bacteria will allow a better understanding of the microbiology of the nitrification process and will help to improve the modeling, design, and operation of nitrifying wastewater treatment plants.

ACKNOWLEDGMENTS

This work was supported by the Körber Preis and DFG SFB 411.

We thank Sibylle Schadhauser for excellent technical assistance, as well as Heike Abicht, Sabine Gindhart, Anette Steidle, and Regina Schuhegger for their participation in these experiments. We are grateful to Martin Klotz for thoughtful comments and for providing sequence data on *amoA* genes. Tom Fritsche, Hilde Lemmer, and Andreas Schramm are acknowledged for critically reading the manuscript.

REFERENCES

1. **Alm, E., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557–3559.
2. **Amann, R. I.** 1995. In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, p. 1–15. *In* A. D. C. Akkerman, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Arthur, J. W., C. W. West, K. N. Allen, and S. F. Hedke.** 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bull. Environ. Contam. Toxicol. **38:**324–331.
4. **Belser, L. W.** 1979. Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. **3:**309–333.
5. **Bock, E., H. Sundermayer-Klinger, and E. Stackebrandt.** 1983. New facultative lithotrophic nitrite-oxidizing bacteria. Arch. Microbiol. **136:**281–284.
6. **Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert.** 1990. A new facultative nitrite-oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch. Microbiol. **153:** 105–110.
7. **Bock, E., and H.-P. Koops.** 1992. The genus *Nitrobacter* and related genera, p. 2302–2309. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes 2nd ed. Springer-Verlag, New York, N.Y.
8. **Bond, P. L., P. Hugenholtz, J. Keller, and L. L. Blackall.** 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Appl. Environ. Microbiol. **61:**1910–1916.
9. **Böttcher, B.** 1996. Untersuchungen zur Phylogenie des ammoniakoxidierenden Systems nitrifizierender Bakterien. Ph.D. thesis. Universität Hamburg, Hamburg, Germany.
10. **Brosius, J., T. L. Dull., D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
11. **Chan, J. W. Y. F., and P. H. Goodwin.** 1995. Extraction of genomic DNA from extracellular polysaccharide synthesizing gram-negative bacteria. BioTechniques **1:**419–422.
12. **Chandler, D. P., R. W. Schreckhise, J. L. Smith, and H. Bolton, Jr.** 1997. Electroelution to remove humic compounds from soil DNA and RNA extracts. J. Microbiol. Methods **28:**11–19.
13. **DeLey, J., H. Cattoir, and A. Reynaerts.** 1970. The quantitative measurements of DNA hybridization from renaturation rates. Eur. J. Biochem. **12:** 133–142.
14. **Ehrich, S., B. Behrens, A. Lebedeva, W. Ludwig, and E. J. Bock.** 1995. A new obligately chemolithoautotrophic nitrite-oxidizing bacterium *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. Arch. Microbiol. **164:** 16–23.
15. **Hastings, R. C., M. T. Ceccherini, N. Miclaus, J. R. Saunders, M. Bazzicalupo, and A. J. McCarthy.** 1997. Direct molecular biological analysis of ammonia oxidizing bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol. Ecol. **23:**45–54.
16. **Head, I. M., W. Hiorns, T. Embley, A. McCarthy, and J. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **13:**1147–1153.
17. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
18. **Hiorns, W. D., R. Hastings, I. M. Head, A. McCarthy, J. Saunders, R. Pickup, and G. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonium-oxidizing bacteria. Microbiology **14:**2793–2800.
19. **Hovanec, T. A., and E. F. DeLong.** 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl. Environ. Microbiol. **62:**2888–2896.
20. **Hovanec, T. A., L. T. Taylor, A. Blakis, and E. DeLong.** 1998. *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. Appl. Environ. Microbiol. **64:**258–264.
21. **Jones, R. D., R. Morita, H.-P. Koops, and S. Watson.** 1988. A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. Can. J. Microbiol. **3:**1122–1128.
22. **Kane, M. D., L. K. Poulsen, and D. A. Stahl.** 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. **59:**682–686.
23. **Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov. sp. Arch. Microbiol. **10:**277–282.
24. **Koops, H.-P., and H. Harms.** 1985. Desoxyribonucleic acid homologies

among 96 strains of ammonia oxidizing bacteria. Arch. Microbiol. **141:**214–218.
25. **Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. **15:** 244–248.
26. **Koops, H.-P., B. Böttcher, U. Möller, A. Pommerening-Röser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. J. Gen. Microbiol. **13:**1689–1699.
27. **Koops, H.-P., and U. C. Möller.** 1992. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2263. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes 2nd ed. Springer Verlag, New York, N.Y.
28. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 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.
29. **Maidak, B. L., N. Larsen, M. McCaughey, R. Overbeek, G. Olsen, K. Fogel, J. Blandy, and C. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **2:**3485–3487.
30. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. **1:**593–600.
31. **McCaig, A. E., T. Embley, and J. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidizers. FEMS Microbiol. Lett. **12:**363–368.
32. **McTavish, H., J. A. Fuchs, and A. B. Hooper.** 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436–2444.
33. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
34. **Norton, J. M., J. M. Low, and M. G. Klotz.** 1996. The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol. Lett. **139:**181–188.
35. **Norton, J. M., and M. G. Klotz.** 1996. Nucleotide sequence of two gene copies encoding the subunit A of the ammonia monooxygenase from *Nitrosomonas eutropha* Nm57. GenBank Database, Bethesda, Md.
36. **Norton, J. M., J. Alzerreca, and M. G. Klotz.** 1997. Nucleotide sequence of two gene copies encoding the subunit A of the ammonia monooxygenase from *Nitrosolobus multiformis* C-71. GenBank Database, Bethesda, Md.
37. **Orso, S., M. Gouy, E. Navarro, and P. Normand.** 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. **44:**83–86.
38. **Pommerening-Röser, A., G. Rath, and H.-P. Koops.** 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Syst. Appl. Microbiol. **1:**344–351.
39. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
40. **Rotthauwe, J.-H., K.-P. Witzel, and W. Liesack.** 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.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **7:**5463–5467.
43. **Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K.-H. Schleifer.** 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **62:**4641–4647.
44. **Sinigalliano, C. D., D. N. Kuhn, and R. D. Jones.** 1995. Amplification of the *amoA* gene from diverse species of ammonia-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl. Environ. Microbiol. **61:**2702–2706.
45. **Smalla, K., N. Cresswell, L. C. Mendonca-Hagler, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74:**78–85.
46. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
47. **Stehr, G., B. Böttcher, P. Dittberner, G. Rath, and H.-P. Koops.** 1995. The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol. Ecol. **1:**177–186.
48. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147–4154.
49. **Strunk, O., and W. Ludwig.** 1997. ARB software program package; http://www.biol.chemie.tu-muenchen.de/pub/ARB/.
50. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
51. **Utåker, J. B., L. Bakken, Q. Jiang, and J. Nes.** 1995. Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. Syst. Appl. Microbiol. **1:**549–559.
52. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450.
53. **Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer.** 1993. Probing activated sludge with *Proteobacteria*-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
54. **Wagner, M., R. Amann, P. Kämpfer, B. Aßmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer.** 1994. Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. **1:**405–417.
55. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **1:** 251–264.
56. **Wagner, M., G. Rath, H.-P. Koops, J. Flood, and R. Amann.** 1996. *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Technol. **3:**237–244.
57. **Ward, B. B., M. A. Voytek, and K.-P. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers by specific PCR amplification. Microb. Ecol. **33:**87–96.
58. **Watson, S. W., and J. B. Waterbury.** 1971. Characteristics of two marine nitrite-oxidizing bacteria, *Nitrospina gracilis* nov. gen. nov. sp. and *Nitrococcus mobilis* nov. gen. nov. sp. Arch. Microbiol. **77:**203–230.
59. **Watson, S. W., E. Bock, F. W. Valois, J. B. Waterbury, and U. Schlosser.** 1986. *Nitrospira marina* nov. gen. nov. sp.: a chemolithotrophic nitrite-oxidizing bacterium. Arch. Microbiol. **144:**1–7.
60. **Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Ammonia-oxidizing bacteria, p. 1818–1834. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. III. The Williams & Wilkins Co., Baltimore, Md.
61. **Winogradsky, S.** 1892. Contributions à la morphologie des organismes de la nitrification. Arch. Sci. Biol. (St. Petersburg) **1:**88–137.
62. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of purple bacteria, the beta subdivision. Syst. Appl. Microbiol. **5:**327–336.
63. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of purple bacteria, the gamma subdivision. Syst. Appl. Microbiol. **6:**25–33.
64. **Zhou, J., M. A. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316–322.