Vol. 64, No. 9

Identification and Activities In Situ of *Nitrosospira* and *Nitrospira* spp. as Dominant Populations in a Nitrifying Fluidized Bed Reactor

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Bacterial aggregates from a chemolithoautotrophic, nitrifying fluidized bed reactor were investigated with microsensors and rRNA-based molecular techniques. The microprofiles of O_2, NH_4^+, NO_2^-, and NO_3^- demonstrated the occurrence of complete nitrification in the outer 125 μm of the aggregates. The ammonia oxidizers were identified as members of the *Nitrosospira* group by fluorescence in situ hybridization (FISH). No ammonia- or nitrite-oxidizing bacteria of the genus *Nitrosomonas* or *Nitrobacter*, respectively, could be detected by FISH. To identify the nitrite oxidizers, a 16S ribosomal DNA clone library was constructed and screened by denaturing gradient gel electrophoresis and selected clones were sequenced. The organisms represented by these sequences formed two phylogenetically distinct clusters affiliated with the nitrite oxidizer *Nitrospira moscoviensis*. 16S rRNA-targeted oligonucleotide probes were designed for in situ detection of these organisms. FISH analysis showed that the dominant populations of *Nitrospira* spp. and *Nitrosospira* spp. formed separate, dense clusters which were in contact with each other and occurred throughout the aggregate. A second, smaller, morphologically and genetically different population of *Nitrospira* spp. was restricted to the outer nitrifying zones.

Lithoautotrophic nitrification, the sequential transformation of NH_4^+ via NO_2^- to NO_3^-, is typically catalyzed by two phylogenetically distinct groups of bacteria, i.e., the ammonia-oxidizing bacteria and the nitrite-oxidizing bacteria. All characterized freshwater ammonia oxidizers belong to a coherent group within the β subclass of the class *Proteobacteria*, comprising the genera *Nitrosomonas* (formerly *Nitrosococcus mobilis* and *Nitrosomonas*), and *Nitrosospira* (formerly *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*) (14, 30). For a long time, all nitrite oxidizers isolated from freshwater habitats belonged to the genus *Nitrobacter* within the α subclass of *Proteobacteria* (7). Recently, *Nitrospira moscoviensis*, a member of an independent phylum (13), was isolated from a corroded iron pipe in Moscow.

Although nitrification is the central aerobic process of microbial nitrogen cycling, only limited knowledge about the ecological relevance of its various protagonists is available. Medium selectivity and the ability of some *Nitrosomonas* strains to outcompete other ammonia oxidizers in liquid cultures (6) made *Nitrosomonas europaea* the most commonly isolated and best investigated ammonia oxidizer. However, there is increasing evidence of dominance by other species or genera in particular environments (24). This situation might be similar for the *Nitrobacter* spp., the best-investigated nitrite oxidizers. The introduction of molecular techniques into microbial ecology has enabled the detection and reliable quantification of natural populations of nitrifiers. Based on comparative 16S rRNA sequence analysis, PCR primers (15, 20, 31, 35) and oligonucleotide probes (16, 21, 33) specific for ammonia oxidizers from the β subclass of the class *Proteobacteria* have been developed. Application of specific PCR in combination with the construction of clone libraries (28) or denaturing gradient gel electro-

phoresis (DGGE) (18) revealed a high genetic diversity within the ammonia oxidizers of the β subclass of *Proteobacteria* and a wide distribution of *Nitrosospira*-like sequences in natural samples like lake water, sediments, or soils. On the other hand, quantitative hybridizations with oligonucleotide probes showed that in ammonium-rich systems like activated sludge (33) or biofilm reactors (21, 26), the dominant ammonia oxidizers were members of the genus *Nitrosomonas*.

DNA from the genus *Nitrobacter* has been amplified from soil by using specific PCR primers (12), and oligonucleotide probes have been designed for use in quantitative hybridizations (34). However, with the exception of two biofilm reactors (21, 26), no *Nitrobacter* spp. could be detected in activated sludge, eutrophic and oligotrophic biofilms, river water (21, 34), or aquaria (16) by using molecular techniques. It was therefore suggested that other nitrite oxidizers are present and active in these systems. Consistent with this hypothesis, Hovanec et al. recently reported *Nitrospira* spp. to be associated with nitrite oxidation in freshwater aquaria (17).

In this study, the nitrifying community of a chemolithoautotrophic fluidized bed reactor was investigated. The reactor, originally operated with high ammonia concentrations (11), was for this study operated with low ammonia concentrations, so that it rather resembled a natural freshwater habitat than a wastewater treatment system (24). The construction of a 16S rRNA clone library from directly isolated DNA was combined with fluorescence in situ hybridization (4). Microsensor measurements to relate the localization of the nitrifying populations to functional zones within the biofilm were performed.

MATERIALS AND METHODS

Nitrifying aggregates. The lab-scale fluidized bed reactor used as a source for nitrifying aggregates has been described in detail before (11). However, the recirculation rate was increased from 1 to 1.8 ml/s, resulting in ammonium limitation in the reactor instead of oxygen limitation; i.e., ammonium was depleted 50 cm above the inlet but oxygen was still present. At the time of sampling, the reactor had been running for more than 8 months under constant conditions. The

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TABLE 1. Oligonucleotide probes

Probe	Specificity	Probe sequence (5′-3′)	Target site[a] (rRNA positions)	% FA[b]	NaCl (mM)[c]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)	20	225	3
ALF1b	α Subclass of the class *Proteobacteria*, several members of the δ subclass of *Proteobacteria*, genus *Nitrospira*, most spirochetes	CGTTCGYTCTGAGCCAG	16S (19–35)	20	225	19
BET42a	β Subclass of *Proteobacteria*	GCCTTCCCACTTCGTTT	23S (1027–1043)	35[d]	80	19
GAM42a	γ Subclass of *Proteobacteria*	GCCTTCCCACATCGTTT	23S (1027–1043)	35[e]	80	19
NSO190	Ammonia-oxidizing β-subclass *Proteobacteria*	CGATCCCCTGCTTTTCTCC	16S (190–208)	55	20	21
NSV443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCC	16S (444–462)	30	112	21
NSM156	*Nitrosomonas* spp.	TATTAGCACATCTTTCGAT	16S (653–670)	40	56	21
NIT3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	16S (1035–1048)	40[f]	56	34
NSR826	Freshwater *Nitrospira* spp.	GTAACCCGCCGACACTTA	16S (826–843)	20	225	This study
NSR1156	Freshwater *Nitrospira* spp.	CCCGTTCTCCTGGGCAGT	16S (1156–1173)	30	112	This study
NSR447	Clones g6, o9, and o14	GGTTTCCCGTTCCATCTT	16S (447–464)	30	112	This study

[a] *E. coli* numbering (9).
[b] Percentage formamide in the hybridization buffer.
[c] Millimolar concentration of sodium chloride in the washing buffer.
[d] Used with an equimolar amount of unlabeled competitor oligonucleotide GAM42.
[e] Used with an equimolar amount of unlabeled competitor oligonucleotide BET42a.
[f] Used with an equimolar amount of unlabeled competitor oligonucleotide CNIT3 (34).

temperature was kept at 30°C, and the pH was adjusted to 8 in the aeration tank. Chemical parameters at the sampling point, 30 cm above the inlet, were as follows: $[O_2]$, 150 μM; $[NH_4^+]$, 40 μM; $[NO_2^-]$, 6.7 μM; $[NO_3^-]$, 5.5 mM; pH 7.7. No organic carbon source was added to the reactor. The nitrifying aggregates were irregularly shaped conglomerates with diameters of 1 to 2 mm and consisted of spheroid subunits with ca. 50-μm diameters.

Microsensor measurements. Clark-type O_2 microsensors with internal references and guard cathodes were prepared and calibrated as described previously (25). Tip diameters were <10 μm, and stirring sensitivities were <2%.

Liquid ion-exchanging membrane (LIX) microsensors for NH_4^+, NO_2^-, and NO_3^- were prepared with solidified tips and protein coatings as described by de Beer et al. (10). The tip diameters were 5 μm for NH_4^+ and NO_3^- microsensors and 15 μm for NO_2^- microsensors. Calibration was done in a dilution series of NH_4^+, NO_2^-, and NO_3^- in the medium used for the measurements.

Aggregates were attached to insect needles, placed in a flow cell, and perfused with medium as described previously (11). $[O_2]$, $[NH_4^+]$, $[NO_2^-]$, pH, and temperature in the medium were adjusted to in situ conditions; $[NO_3^-]$ was 100 μM. After 15 min of incubation, sufficient time to reach steady state, microprofiles were recorded by moving the sensors with a motor-driven micromanipulator at depth steps of 25 μm from the bulk liquid into the aggregate. The position relative to the aggregate surface was determined with a dissection microscope.

Chemical analysis. $[NH_4^+]$, $[NO_2^-]$, and $[NO_3^-]$ in the reactor column were determined colorimetrically (Spectroquant; Merck, Darmstadt, Germany). $[O_2]$ and pH were measured by an O_2 microsensor and a pH electrode (Radiometer, Copenhagen, Denmark), respectively, which were positioned in the reactor at the sampling point.

DNA extraction. Aggregate samples were homogenized by vigorous vortexing and subjected to three freeze-thaw cycles. An aliquot of 200 μl was suspended in 500 μl of AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA), and 1 ml of hot phenol-chloroform-isoamyl alcohol (25:24:1) and 100 μl of 25% (wt/vol) sodium dodecyl sulfate (SDS) were added. The mixture was shaken at 60°C for 30 min and then chilled on ice. The subsequent hot phenol extraction was done as described in the protocol of Wawer et al. (36). Nucleic acids were resuspended in distilled, sterile water, and their quality was checked by agarose gel electrophoresis (1%, wt/vol).

Amplification and cloning of 16S rDNA. Almost-full-length bacterial 16S rRNA gene (rDNA) fragments were amplified from extracted DNA by the PCR. According to Muyzer et al. (23), primer pair GM3F (*Escherichia coli* 16S ribosomal DNA positions 8 to 24 [9])-GM4R (*E. coli* positions 1492 to 1507) was used. One microliter of the PCR product was directly ligated into the pGEM-T vector cloning system (Promega, Mannheim, Germany) and transformed into competent cells (high-efficiency *E. coli* JM109 [Promega]) as described in the manufacturer's instructions.

DGGE screening of the gene library. A 550-bp-long 16S rDNA fragment for DGGE analysis was directly amplified from each 16S rDNA clone by using 1 μl of cell suspension in the PCR. PCR and DGGE were performed as described by Muyzer et al. (22). For DGGE, a gradient of 35 to 65% denaturant at 60°C and an electrophoresis time of 17 h at 100 V were used.

16S rDNA sequencing. Plasmids were extracted and purified from clones of interest with the Wizard Plus Minipreps DNA purification system (Promega) in accordance with the manufacturer's instructions. Sequencing of the 16S rDNA inserts was done with a LICOR automated sequencer by MWG-Biotech, Ebersberg, Germany. Partial sequences (ca. 900 bases) and almost full sequences (ca. 1,500 bases) were determined on one strand.

Data analysis. Sequences were added to the 16S rRNA database of the Technical University Munich by use of the program package ARB (29). The tool ARB_EDIT was used for sequence alignment. The 16S rRNA-based phylogenetic tree was calculated by using distance matrix, maximum-parsimony, and maximum-likelihood analyses, and the topologies of the resulting trees were compared. Branchings not supported by all three methods are displayed as multifurcations. Only sequences that were at least 90% complete were used for tree reconstruction. Partial sequences were added by using maximum parsimony without changing the overall topology of the tree.

Based on the newly retrieved *Nitrospira*-like sequences, oligonucleotide probes were designed. Their specificity was evaluated by using the ARB tool PROBE_FUNCTIONS and the rRNA database of the Technical University Munich (version 3/97).

Fixation and sectioning of aggregates. For in situ hybridization, aggregates were fixed in 4% paraformaldehyde for 1 h at 4°C, washed in phosphate-buffered saline, and stored in a 1:1 mixture of phosphate-buffered saline and 96% ethanol at −20°C (2). Prior to the cryosectioning, aggregates were embedded in OCT compound (Tissue-Tek II; Miles, Elkhart, Ind.) overnight and subsequently frozen in a cryomicrotome (Mikrom, Walldorf, Germany) at −35°C. Semithick cryosections (5 to 20 μm) were cut at −17°C, and the single sections were placed on poly-L-lysine (0.01% solution; Sigma, Deisenhofen, Germany)-coated micro-
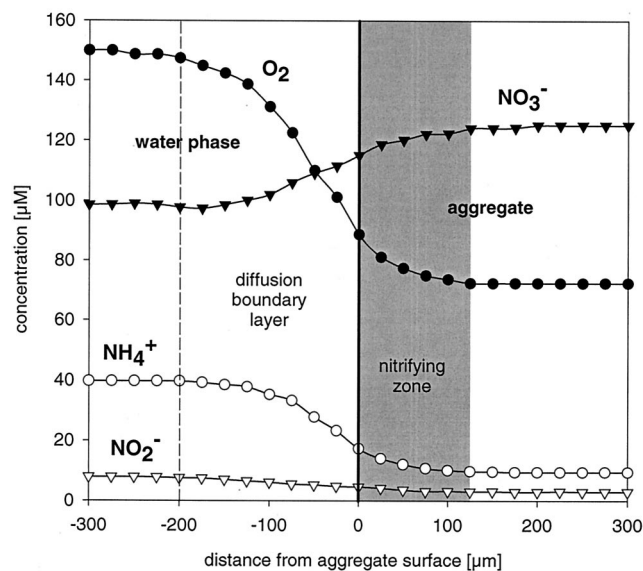

FIG. 1. Typical microprofiles of $[O_2]$, $[NH_4^+]$, $[NO_2^-]$, and $[NO_3^-]$ in nitrifying aggregates. The gray area marks the zone with nitrifying activity.

TABLE 2. Similarity matrix of clones affiliated with *Nitrospira* spp.

Species or clone	% Similarity to:						
	N. mosco-viensis	*N. marina*	b2	b18	b30	g6	o9
N. moscoviensis							
N. marina	89.3						
b2	92.8	87.8					
b18	92.1	87.5	97.1				
b30	92.8	88.2	98.9	97.5			
g6	95.8	87.6	91.7	92.6	91.6		
o9	95.8	87.6	90.1	92.3	90.1	99.5	
Clone 710-9 (17)	94.7	87.7	90.4	90.1	90.3	92.3	89.9

scopic slides. After air drying overnight to allow optimal attachment of the sections to the slides, the OCT compound was removed by dissolving it in a drop of distilled water and carefully dipping the slide in distilled water. Again the slides were allowed to air dry. Finally, the sections were dehydrated in an ethanol dilution series (50, 70, and 96%) and stored at room temperature.

Oligonucleotide probes. The following rRNA-targeted oligonucleotides were used (probe nomenclature according to Alm et al. [1] is given in parentheses): (i) EUB (S-D-Bact-0338-a-A-18); (ii) ALF1b (S-Sc-aProt-0019-a-A-17), BET42a (L-Sc-bProt-1027-a-A-17), and GAM42a (L-Sc-gProt-1027-a-A-17); (iii) NSO190 (S-*-Ntros-0190-a-A-19); (iv) NSV443 (S-*-Ntros-0443-a-A-19); (v) NSM156 (S-*-Nsom-0156-a-A-19); (vi) NIT3 (S-G-Nbac-1035-a-A-18); (vii) NSR1156 (S-*-Nspir-1156-a-A-18); (viii) NSR826 (S-*-Nspir-0826-a-A-18); and (ix) NSR447 (S-*-Nspir-0447-a-A-18). Oligonucleotides were synthesized and fluorescently labeled with a hydrophilic sulfoindocyanine dye, CY3 or CY5, or with 5(6)-carb-oxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) at the 5′ end by Interactiva

Biotechnologie GmbH (Ulm, Germany). All probe sequences, their hybridization conditions, and references are given in Table 1. The respective target organisms of probes specific for nitrifying bacteria are indicated in the phylogenetic tree (see Fig. 2).

In situ hybridization. All hybridizations were performed as described by Manz et al. (19) at 46°C for 90 min in hybridization buffer containing 0.9 M NaCl, formamide at the percentage shown in Table 1, 20 mM Tris-HCl (pH 7.4), and 0.01% SDS. The probe concentration was 5 ng/μl. Hybridization was followed by a stringent washing step at 48°C for 10 min in washing buffer containing 20 mM Tris-HCl (pH 7.4), NaCl at the concentrations listed in Table 1, and 0.01% SDS. Washing buffer was removed by rinsing the slides with distilled water. The slides were air dried, stained with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) for 10 min in the dark on ice, and finally rinsed again with distilled water. The slides were mounted in Vectashield (Vector Laboratories Inc., Burlingame, Calif.) to avoid bleaching and examined with a Zeiss Axioplan epifluorescence microscope equipped with filter sets CY3-HQ and CY5-HQ (Chroma Technology Corp., Brattleboro, Vt.) and 01 and 09 (Carl Zeiss, Jena, Germany). A Zeiss LSM 510 confocal laser scanning microscope, equipped with an Ar ion laser (488 nm) and two HeNe lasers (543 nm, 633 nm), was used to record optical sections as described by Wagner et al. (32).

Nucleotide sequence accession numbers. 16S rDNA sequence data obtained in this study will appear in the EMBL, GenBank, and DDBJ databases under accession no. AJ224038 to AJ224047.

RESULTS

Microsensor measurements. All investigated aggregates showed consumption of O_2, NH_4^+, and NO_2^- and production of NO_3^- (Fig. 1). The consumed NH_4^+ was completely converted to NO_3^- without an intermediate NO_2^- peak, and even NO_2^- present in the bulk water was consumed. This indicates that nitrite oxidation was at least as fast as ammonia oxidation.
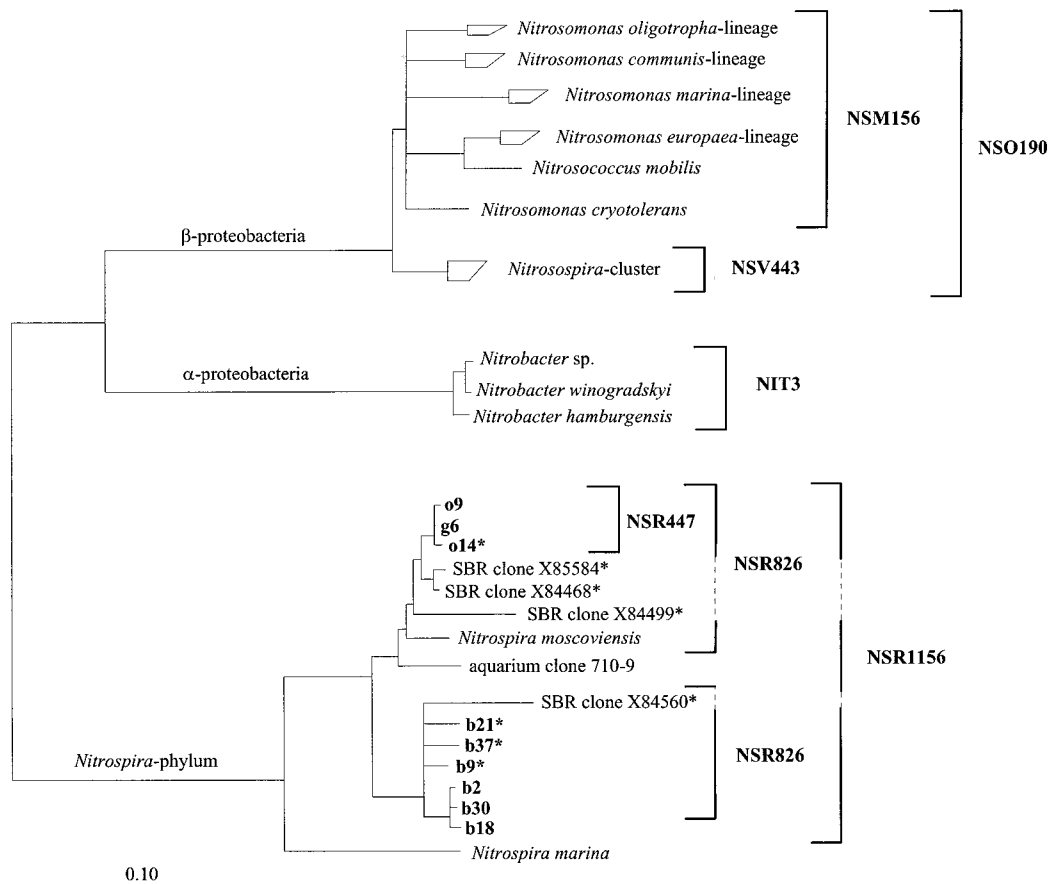

FIG. 2. Phylogenetic tree inferred from comparative analysis of 16S rRNA sequences. The tree is based on the results of maximum-likelihood analysis of sequences of >1,400 nucleotides. Partial sequences (marked by an asterisk) were added by maximum-parsimony analysis without changing the tree topology. Target organisms of probes used in this study are indicated by brackets; SBR clones are not sequenced in the probe target regions. The bar represents 0.1 estimated change per nucleotide.
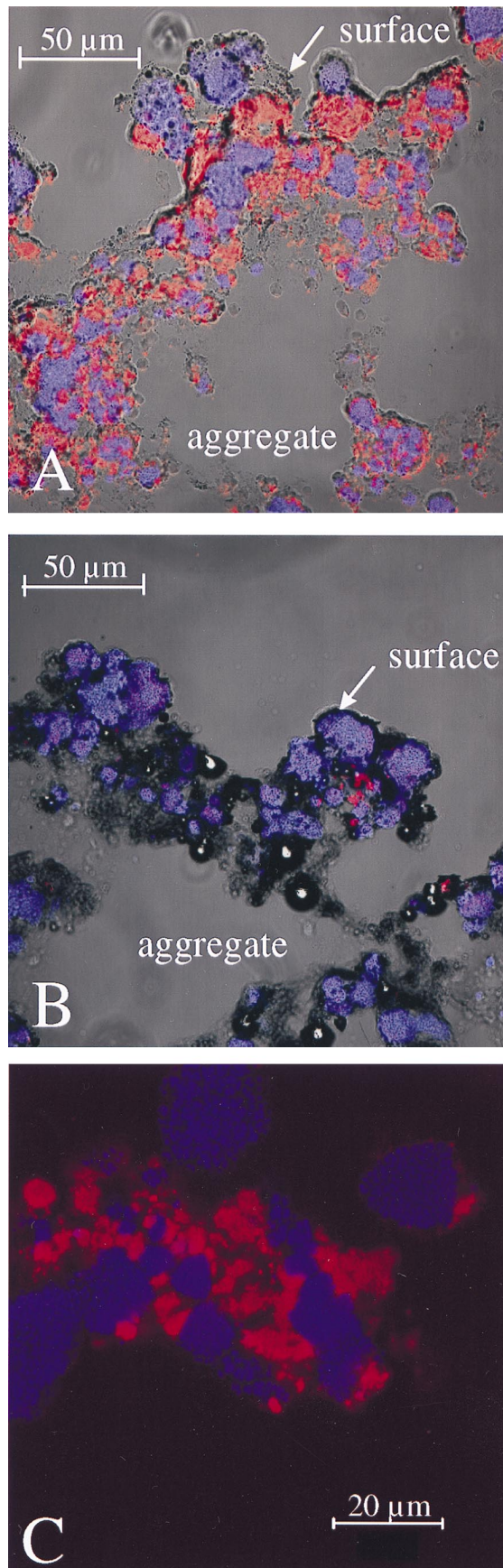
The active nitrifying zone was restricted to the outer 125 μm of the aggregates, although O_2 and NH_4^+ were both still present in the center of the aggregates at concentrations of about 75 and 10 μM, respectively.

FISH analysis. Phase-contrast light microscopy of the aggregate sections showed dense bacterial clusters, void spaces, and an irregular surface. If excited with blue light (488 nm), cells and extracellular material exhibited strong green autofluorescence, which made it difficult to reliably detect FLUOS-labeled oligonucleotide probes. Therefore, we exclusively applied CY3- or CY5-labeled probes for fluorescence in situ hybridization (FISH) analysis.

In situ hybridization with probe EUB proved that the ribosome content of most cells was sufficient for single-cell detection and that probes penetrated most, if not all, cell clusters. Only a few DAPI-stained cells in the central regions of aggregates were not detectable by FISH. Application of probes ALF1b, BET42a, and GAM42a indicated that the population consisted mainly of approximately equal fractions of ALF1b- and BET42a-positive cells. No cells hybridizing with probe GAM42a were detected. ALF1b-positive cells had diameters of below 0.6 μm and formed clusters 3 to 10 μm in size which were situated close to BET42a-positive cells that had diameters of ca. 1 μm and formed clusters 5 to 20 μm in size. Taken together, both populations made up >90% of all cells in the nitrifying zone as well as in the central region of the aggregates. Virtually all cells detected by probe BET42a also hybridized with probes NSO190 and NSV443 (Fig. 3), but no cells could be detected with probe NSM156. Thereby, the ammonia-oxidizing cells were identified as members of the genus *Nitrosospira*. In contrast, none of the cells detected by probe ALF1b hybridized with probe NIT3, which was specific for the nitrite-oxidizing bacteria of the α subclass of *Proteobacteria* of the genus *Nitrobacter*.

Clone library. A 16S rDNA clone library was constructed from aggregate samples to identify the unknown nitrite oxidizers. Fifty-five clones were selected at random. To avoid redundant sequencing, PCR-amplified rDNA fragments of all clones were analyzed by DGGE. Ten distinct categories of bands could be identified, and one representative of each category was selected for comparative sequence analysis. The 16S rRNA sequences of 9 of the 10 clones were affiliated with those of the nitrite oxidizers of the genus *Nitrospira* (13). Three clone sequences (g6, o9, and o14) were closely related to *Nitrospira moscoviensis* (96% similarity), whereas the other six (b2, b9, b18, b21, b30, and b37) formed a distinct branch within the genus *Nitrospira* but were still more closely related to *Nitrospira moscoviensis* (92% similarity) than to *Nitrospira marina* (87% similarity). Table 2 shows the 16S rRNA similarity values for the five almost-full-length sequences determined in this study, and Fig. 2 illustrates their affiliation in a phylogenetic tree. The remaining partial sequence (g14) was related to the 16S rRNA gene of a *Hyphomonas* sp. of the α subclass of *Proteobacteria*.

FIG. 3. In situ identification of nitrifying bacteria in aggregate sections demonstrating their spatial distribution. Images are composed of overlays of phase-contrast microscopic images and two confocal microscopic images. (A) Simultaneous in situ hybridization with CY5-labeled probe NSV443 and CY3-labeled probe NSR1156. Cells of *Nitrosospira* spp. are shown in blue; cells of *Nitrospira*-like bacteria are red. (B) Simultaneous in situ hybridization with CY5-labeled probe NSV443 and CY3-labeled probe NSR447. Cells of *Nitrosospira* spp. are blue; a smaller subpopulation of *Nitrospira* spp. is visualized in red. (C) Simultaneous in situ hybridization with CY5-labeled probe NSV443 and CY3-labeled probe NSR1156 displaying the morphology and close association of ammonia- and nitrite-oxidizing bacteria.

No sequences affiliated with the genus *Nitrobacter* or with any known ammonia-oxidizing bacterium were retrieved.

Probe design and in situ detection of *Nitrospira*. Based on the retrieved sequences, two probes specific for all clones that included *Nitrospira moscoviensis*, NSR1156 and NSR826 (with one mismatch to clone b18), were developed. Another probe, NSR447, was designed to be complementary to clones g6, o9, and o14 only (Fig. 2). Hybridization conditions of the probes were evaluated in situ by using increasing concentrations of formamide in the hybridization buffer. Probes NSR1156 and NSR447 showed good signal and specificity in buffer with 30% formamide; probe NSR826 did so in buffer with 20% formamide. When applied to the aggregate sections, probes NSR1156 and NSR826 hybridized to virtually all ALF1b-positive cells. A 16S rRNA sequence database check revealed that, indeed, all *Nitrospira* spp. have the target sequence for probe ALF1b. The nitrifying community active in the fluidized bed reactor was shown to consist of members of the genera *Nitrosospira* and *Nitrospira* (Fig. 3A). Only a minor fraction of all NSR1156- and NSR826-positive cells also hybridized with probe NSR447. These cells were slightly bigger, formed smaller clusters than the major part of the *Nitrospira* sp. cells, and were located exclusively in the nitrifying part of the aggregates (Fig. 3B). Single-cell resolution of the very small *Nitrospira* spp. was almost impossible even with confocal laser scanning microscopy (Fig. 3C).

DISCUSSION

Microprofiles. The in situ activity measurements clearly indicate that both ammonia and nitrite oxidizers were active within a 125-μm zone at the aggregate surface (Fig. 1). In contrast, no activity was measured in the inner part of the aggregates, although dense populations of nitrifiers could be detected (Fig. 3) and oxygen and ammonium were present. One possible explanation is that the ammonium concentration in this zone (10 μM) was substantially below the half-saturation constant (K_s) for ammonia oxidation. Indeed, the lowest K_s values reported so far for ammonia-oxidizing bacteria are 30 to 75 μM NH_4^+ plus NH_3 (at pH 7.8) for strains of the *Nitrosomonas oligotropha* group, isolated from the River Elbe (27). No such data are currently available for members of the genus *Nitrosospira*.

Ammonia oxidizers. By using FISH with a set of specific probes, it was shown that members of the genus *Nitrosospira* (in its extended definition [14] also including the former genera *Nitrosolobus* and *Nitrosovibrio*) were the only ammonia-oxidizing bacteria in the aggregates. As previously shown for members of the genus *Nitrosomonas* in biofilms (21, 26), *Nitrosospira* spp. also tended to form monospecies clusters in close association with clusters of nitrite oxidizers. Cluster formation could be disadvantageous due to the longer diffusion path of substrates. However, since the nitrifying bacteria rely on CO_2 fixation by ribulose bisphosphate carboxylase (RubisCO), it might be a mechanism for increasing the efficacy of CO_2 fixation by lowering the $[O_2]$ in the clusters. Cluster formation might also provide protection against hazardous environmental factors, or it might just be an ecologically neutral result of cell division. In our study, the clustering of *Nitrosospira* sp. was probably the reason that no 16S rDNA clone sequences of this genus were obtained. Intact clusters could still be detected after the DNA isolation procedure, indicating resistance against the DNA extraction protocol based on freeze-thawing and hot phenol and SDS extraction. This potential for formation of highly resistant clusters should be considered in all attempts to monitor ammonia-oxidizing bacteria with methods based on DNA extraction.

The in situ detection of *Nitrosospira* spp. in this ammonium-poor system is in agreement with observations that in natural systems that are low in ammonium, *Nitrosospira* spp. can be detected rather than *Nitrosomonas* spp. (15, 18, 28). However, these studies depend on DNA extraction and PCR while true quantitative in situ analyses of these habitats are missing. In contrast, *Nitrosomonas* spp., i.e., members of the *Nitrosomonas europaea* lineage, are more abundant in activated sludge, biofilms, and enrichments with millimolar concentrations of ammonium (21, 26, 28, 33). The main adaptations to the different niches are probably via the ammonia oxidation kinetics and the growth rate. In that respect, *Nitrosospira* and *Nitrosomonas* seem to be typical K and r strategists, respectively (5). However, the isolation of *Nitrosomonas oligotropha* and related strains with remarkably low K_s values (27) already indicates limits for such a generalizing statement.

Nitrite oxidizers. *Nitrospira*-like bacteria were identified in the nitrifying aggregates by a combination of comparative 16S rRNA sequence analysis and FISH. Their localization within the nitrifying zone and their association with the ammonia-oxidizing *Nitrosospira* species lend strong evidence that they are responsible for nitrite oxidation in our system. Two genetically different populations were distinguished in this study. The main population, which was more distantly related to *Nitrospira moscoviensis*, also occurred in the inactive center of the aggregates. The second, smaller population had a higher level of 16S rRNA similarity to *Nitrospira moscoviensis*. It was restricted to the active nitrifying zone. The genotypic differences obviously coincide with different physiological adaptations leading to these distinct spatial distributions. The exact description of the differing physiological properties of the new nitrite oxidizers, however, will require their isolation. A directed isolation can be monitored by the oligonucleotide probes developed in this study.

The detection of *Nitrospira* spp. also yields independent support for a recent molecular study reporting that *Nitrospira*-like bacteria were the main nitrite-oxidizing population in freshwater aquaria (17). Before, *Nitrospira*-like 16S rDNA sequences had also been isolated from activated sludge (8), but, at that time, reference sequences of cultured *Nitrospira* spp. with proven nitrite-oxidizing capability were not available. Therefore, it was impossible to link the genotype with a phenotype. This clearly shows the importance of cultivation of bacteria. Considering the rare in situ detection of *Nitrobacter* species in freshwater systems (16, 21, 34), *Nitrospira* spp. might be of more general importance for nitrite oxidation. The molecular detection of *Nitrospira*-like bacteria in habitats as diverse as activated sludge, freshwater aquaria, and an oligotrophic fluidized bed reactor and the initial isolation of *Nitrospira moscoviensis* from a corroded water pipe may indicate a wide distribution in nitrifying systems.

Conclusion. *Nitrosospira* and *Nitrospira* spp. were identified as main components of nitrifying aggregates. Their activity and spatial distribution could be shown by the combination of microsensor measurements and in situ hybridization. Whether the combination of these two genera is unique to the investigated system or is of general relevance in more oligotrophic freshwater habitats must be evaluated in the future.

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