

In Situ Characterization of *Nitrospira*-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants

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Uncultivated *Nitrospira*-like bacteria in different biofilm and activated-sludge samples were investigated by cultivation-independent molecular approaches. Initially, the phylogenetic affiliation of *Nitrospira*-like bacteria in a nitrifying biofilm was determined by 16S rRNA gene sequence analysis. Subsequently, a phylogenetic consensus tree of the *Nitrospira* phylum including all publicly available sequences was constructed. This analysis revealed that the genus *Nitrospira* consists of at least four distinct sublineages. Based on these data, two 16S rRNA-directed oligonucleotide probes specific for the phylum and genus *Nitrospira*, respectively, were developed and evaluated for suitability for fluorescence in situ hybridization (FISH). The probes were used to investigate the in situ architecture of cell aggregates of *Nitrospira*-like nitrite oxidizers in wastewater treatment plants by FISH, confocal laser scanning microscopy, and computer-aided three-dimensional visualization. Cavities and a network of cell-free channels inside the *Nitrospira* microcolonies were detected that were water permeable, as demonstrated by fluorescein staining. The uptake of different carbon sources by *Nitrospira*-like bacteria within their natural habitat under different incubation conditions was studied by combined FISH and microautoradiography. Under aerobic conditions, the *Nitrospira*-like bacteria in bioreactor samples took up inorganic carbon (as HCO₃⁻ or as CO₂) and pyruvate but not acetate, butyrate, and propionate, suggesting that these bacteria can grow mixotrophically in the presence of pyruvate. In contrast, no uptake by the *Nitrospira*-like bacteria of any of the carbon sources tested was observed under anoxic or anaerobic conditions.

Nitrification, the oxidation of ammonia to nitrate catalyzed by bacteria, is a key part of global nitrogen cycling (37). In the first step of nitrification, chemolithoautotrophic ammonia oxidizers transform ammonia to nitrite, which is subsequently oxidized to nitrate by the nitrite-oxidizing bacteria (8). All isolated chemolithoautotrophic, nitrite-oxidizing bacteria belong to one of four different genera (7) *Nitrobacter* (alpha subclass of *Proteobacteria*), *Nitrococcus* (gamma subclass of *Proteobacteria*), *Nitrospina* (delta subclass of *Proteobacteria*), and *Nitrospira* (phylum *Nitrospira*). While species of the genus *Nitrobacter* have been isolated from a variety of environments, including soil and fresh water, it was long assumed that the other three genera were confined to marine environments (7). In recent studies, however, bacteria related to the genus *Nitrospira* were also found to occur in different nonmarine habitats. While the first described species of this genus, *Nitrospira marina*, was isolated from ocean water (49), the second isolated species, *N. moscoviensis*, was cultured from an iron pipe of a heating system in Moscow, Russia (16). These two species are the only cultivated representatives of the genus *Nitrospira*, but numerous related bacteria have recently been detected by comparative analysis of 16S rRNA sequences obtained from nitrifying bioreactors (11, 22), rhizosphere (30), a freshwater aquarium filter (21), groundwater contaminated with livestock wastewater (12), deltaic sediment (45), and deep-sea sediments (25). These sequences indicate considerable phyloge-

netic diversity within the genus *Nitrospira*; however, a thorough phylogenetic analysis of the phylum *Nitrospira* considering all of these sequences has not been performed. Furthermore, the retrieval of *Nitrospira*-related sequences from the above-mentioned environments demonstrates that these bacteria are widely distributed in nature and probably contribute significantly to global nitrite oxidation. However, the lack of pure cultures of *Nitrospira*-related bacteria from soil, water, sediment, and wastewater treatment plants restricts our knowledge of their physiology and genetics.

In contrast to textbook knowledge (5, 19), *Nitrospira*-like bacteria, not *Nitrobacter* spp., are the dominant nitrite oxidizers both in most full-scale wastewater treatment plants and in laboratory scale reactors (22, 36, 43, 48). Based on fluorescence in situ hybridization (FISH) combined with microelectrode measurements, it has been suggested that *Nitrospira*-like nitrite oxidizers represent K strategists adapted to low nitrite and oxygen concentrations, while *Nitrobacter* sp., as an r strategist, thrives if nitrite and oxygen are present in higher concentrations (42). Since many wastewater treatment plants suffer from repeated breakdowns of nitrification performance, more insight into the physiology of *Nitrospira*-like bacteria is required to find measures by which to stabilize this important step of nutrient removal in modern biological sewage treatment.

In this study, we investigated structural and functional features of *Nitrospira*-like bacteria in nitrifying biofilms and activated sludges from different wastewater treatment plants. Based on an in-depth analysis of the phylogeny of the phylum *Nitrospira*, 16S rRNA-directed oligonucleotide probes for the phylum and genus *Nitrospira* were developed. These probes

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were used to detect *Nitrospira*-like bacteria in situ in wastewater treatment plants and to study the morphology of their microcolonies by using a confocal laser scanning microscope (CLSM) and digital image analysis. Furthermore, the uptake of different carbon sources by *Nitrospira*-like bacteria in the bioreactor samples was examined under aerobic and anaerobic conditions by combining FISH and microautoradiography (MAR) (24).

(A preliminary report of this study was presented at the IAWQ Conference on Biofilm Systems [New York, N.Y., 17 to 20 October 1999] and at the IWA 2nd International Symposium on Sequencing Batch Reactor Technology [Narbonne, France, 10 to 11 July 2000].)

MATERIALS AND METHODS

Cultivation of reference organisms. *Bacillus stearothermophilus* (DSM 22) and *Leptospirillum ferrooxidans* (DSM 2705) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and cultivated in accordance with the supplier's instructions. Paraformaldehyde-fixed *N. moscoviensis* cells were kindly provided by Eberhard Bock and Gabriele Timmermann (University of Hamburg, Hamburg, Germany).

Bioreactor data, biofilm and activated-sludge sampling, and cell fixation. Samples of nitrifying biofilm were retrieved from an aerated sequencing batch biofilm reactor (SBBR 1) at a pilot wastewater treatment plant near Ingolstadt, Germany. Additional samples were obtained from a second nitrifying, continuously operated biofilm reactor (Biofor 2) at the same plant. The biofilm grew in both reactors on Biolite expanded clay beads (grain size, 4 to 8 mm) forming a fixed bed with an average volume of 10 m³. Reactor SBBR 1 received reject water from sludge dewatering by the municipal wastewater treatment plant at Ingolstadt with NH₄-N concentrations of 300 to 500 mg · liter⁻¹, an average total chemical oxygen demand of 300 mg · liter⁻¹, and an average conductivity of 5,000 to 6,000 μS · cm⁻¹. At the end of each cycle, NH₄-N at 40 to 50 mg · liter⁻¹ and NO₂-N at up to 70 mg · liter⁻¹ were detected at the outlet of the reactor. The cycle time was 4 to 8 h, and the fixed bed was backwashed every 10 to 48 h. Reactor Biofor 2 received municipal wastewater with an average NH₄-N concentration of 13 mg · liter⁻¹, an average chemical oxygen demand of 191 mg · liter⁻¹, and an average conductivity of 500 to 1,000 μS · cm⁻¹. The outlet of the reactor contained NH₄-N at 0.3 to 0.8 mg · liter⁻¹ and NO₂-N at less than 1 mg · liter⁻¹. The fixed bed was back washed every 10 to 48 h. Activated-sludge samples were taken from the nitrification stage of the Aalborg West (AAV) wastewater treatment plant (Aalborg, Denmark; 275,000 population equivalents). In addition, samples were retrieved from the nitrification stage of the Munich II wastewater treatment plant (Munich, Germany; 10⁶ population equivalents). The average NH₄-N concentration in the inlet of the nitrification stage of the AAV plant was 25 mg · liter⁻¹, while the outlet contained NH₄-N and nitrite at less than 1 mg · liter⁻¹. The sludge age was 20 days. The average NH₄-N concentration was 12 mg · liter⁻¹ at the inlet of the nitrification stage of the Munich II plant and 0.08 mg · liter⁻¹ at the outlet. Nitrite concentrations at the outlet of the nitrification stage are not available for this plant. The sludge age was 7 to 10 days.

Biofilm and activated sludge were fixed for 5 h at 4°C in 3% paraformaldehyde as described by Amann (2) immediately after sampling (the biofilm was first detached from the expanded clay beads by gentle swirling). Unfixed sample aliquots used for DNA extraction were centrifuged (10 min, 4,550 × g), the supernatant was discarded, and the biomass was stored at -20°C. Pure bacterial cultures were harvested by centrifugation (10 min, 10,000 × g), resuspended in phosphate-buffered saline (PBS), and centrifuged again (10 min, 10,000 × g). The supernatant was removed prior to fixation of the cells in 3% paraformaldehyde. *B. stearothermophilus* cells were fixed with ethanol (40). Fixed samples and fixed pure cultures were stored in PBS-ethanol (1:1) at -20°C.

PCR amplification, cloning, sequencing, and phylogenetic analysis of 16S rDNAs. DNA was extracted from frozen biofilm samples in accordance with the protocol described by Zhou et al. (51). Almost complete (1,497 to 1,533 nucleotides) bacterial 16S rDNAs were amplified by PCR, cloned, and sequenced as detailed by Juretschko et al. (22). The 16S rDNA sequences obtained were added to the ARB 16S rRNA sequence database (<http://www.arb-home.de>). The sequences were aligned by the ARB_EDIT module of the program, and the alignments were refined by visual inspection. Nucleic acid similarities were calculated by using the respective tool of the ARB program. Phylogenetic trees were

computed by application of the ARB neighbor-joining and maximum-parsimony tools and by maximum-likelihood analysis of different sets of data. Treeing analyses were performed with and without application of a 50% conservation filter for the *Nitrospira* phylum. This filter was based on all sequences affiliated with the phylum *Nitrospira* that were longer than 1,400 nucleotides and was used to exclude highly variable alignment columns that are not conserved in at least 50% of the *Nitrospira* phylum sequences. For calculation of consensus trees, all sequences longer than 1,300 nucleotides were first processed by the maximum-likelihood method to determine the basic topology of the tree. The shorter sequences were added subsequently by use of the ARB_PARSIMONY function of the ARB program without changing the tree topology (26). Bootstrap values were determined by 100 iterations of maximum-parsimony bootstrapping analysis without application of a conservation filter. Neighbor-joining trees were calculated based on different sequence sets to verify the results obtained by the other treeing methods. Different sets of outgroup sequences that represented members of several other phyla of the domain *Bacteria* and were longer than 1,500 nucleotides were used in all treeing calculations. Checks for chimeric sequences were performed by independent phylogenetic analyses of the first 513 5' base positions, the middle 513 base positions, and the last 513 3' base positions of the sequences.

Incubation of activated sludge and biofilm with radioactive substrates. Substrate uptake experiments were performed with living activated-sludge and biofilm samples. The radioactively labeled substrates used were [³H]acetate, [¹⁴C]pyruvate (Amersham, Little Chalfont, United Kingdom), [¹⁴C]butyrate, [¹⁴C]propionate, and [¹⁴C]bicarbonate (NEN Life Science, Boston, Mass.). Fresh activated-sludge and biofilm samples were harvested the day before the experiments were performed, kept at 4°C, and brought to the laboratory. The dry-matter content (suspended solids) of activated sludge and biofilm was adjusted to a concentration of 1 g of suspended solids · liter⁻¹ with sterile-filtered supernatant from the respective bioreactors as described by Lee et al. (24). For incubation with all of the substrates but bicarbonate, 3 ml of diluted activated sludge or biofilm was transferred to 9-ml glass serum vials. For incubation with bicarbonate, 5 ml of a diluted sample was transferred to 25-ml serum vials. The incubation conditions applied were (i) aerobic, (ii) anoxic (i.e., anaerobic in the presence of 1 mM nitrate), and (iii) anaerobic. The nitrate concentrations in the samples were estimated by use of a nitrate test kit (Merckoquant Nitrate-test; Merck, Darmstadt, Germany) prior to the anaerobic incubations to ensure that these experiments were not influenced by nitrate present in the sludge or biofilm liquid. No nitrate was detected in the samples from the AAV plant and from reactor Biofor 2, but biofilm from reactor SBBR1 contained nitrate at approximately 500 mg · liter⁻¹. This biofilm was centrifuged (10 min, 5,000 × g), the supernatant containing nitrate was discarded, and the biofilm was resuspended in sterile-filtered, nitrate-free biofilm liquid. This biofilm liquid had previously been obtained by anaerobic overnight incubation of an aliquot of the same biofilm sample. Following this pretreatment, no nitrate was detected in the biofilm liquid by the nitrate test kit. All serum vials for anoxic and anaerobic incubations were closed with thick, gas-tight butyl rubber stoppers and flushed with pure nitrogen gas prior to incubation, and all following steps were performed by using strict anaerobic techniques. Unlabeled organic substrates were added to a final concentration of 1 mM. This concentration is slightly higher than the concentrations of fatty acids in most wastewater treatment plants, but it was used to ensure maximal substrate uptake rates and to avoid starvation of the bacteria in the incubated samples due to substrate depletion. The respective labeled substrates were then added to a final activity of 10 μCi (the specific activities of the purchased radioactive tracers were 30, 60, 58, 10, and 5 mCi · mmol⁻¹ for [¹⁴C]pyruvate, [¹⁴C]propionate, [³H]acetate, [¹⁴C]butyrate, and [¹⁴C]bicarbonate, respectively). Aerobically incubated preparations were vigorously shaken for 3 h, while anoxically and anaerobically incubated preparations were incubated for 4 h without agitation. All samples containing [¹⁴C]bicarbonate, however, were incubated for 5 h to compensate for the possibly lower fixation rates of inorganic carbon and because preliminary experiments (data not shown) demonstrated that 5 h of incubation was more suitable than 3 h for monitoring of the uptake of inorganic carbon by *Nitrospira*-like bacteria. Moreover, 1 mM NH₄Cl was added to these samples as a substrate for the indigenous ammonia oxidizers. Nitrite resulting from their activity could serve as an energy source for the nitrite oxidizers during incubation with bicarbonate. The incubated samples were centrifuged (10 min, 5,000 × g) and fixed in 3% (wt/vol) paraformaldehyde as described above. The samples were then resuspended in a 1:1 mixture of PBS and 96% (vol/vol) ethanol and stored at -20°C.

The amounts of organic substrates taken up by the biomass were determined to confirm that the substrates were not depleted during incubation. Aliquots (0.5 ml) of the incubated samples were taken before and after the experiments. The aliquots were immediately cooled on ice, centrifuged (5 min, 10,000 × g), and

TABLE 1. 16S rRNA-targeted oligonucleotide probes developed in this study^a

Probe	Sequence (5'–3')	Target site (16S rRNA position) ^b	% formamide/NaCl concn (mM)
S-G-Ntspa-0662-a-A-18	GGAATTCGCGCTCCTCT	662–679	35/80
Comp-Ntspa-0662	GGAATTCGCGCTCCTCT	662–679	— ^c
S-*-Ntspa-0712-a-A-21	CGCCTTCGCCACCGGCTTCC	712–732	50/28
Comp-Ntspa-0712	CGCCTTCGCCACCGGCTTCC	712–732	— ^d

^a The names, sequences, and target sites of the probes, the formamide concentrations in the hybridization buffer, and the salt concentrations in the wash buffers required for specific in situ hybridization are specified.

^b *E. coli* numbering (10).

^c Used as unlabeled competitor together with probe S-G-Ntspa-0662-a-A-18.

^d Used as unlabeled competitor together with probe S-*-Ntspa-0712-a-A-21.

filtered (0.2- μ m-pore-size sterile filters; Millipore), and the supernatant was frozen for later analysis. Amounts of pyruvate, acetate, propionate, and butyrate in the supernatants of the respective incubations were measured by high-performance liquid chromatography on a Dionex ion chromatograph with a suppressed conductivity detector, 1 mM NaOH as the mobile phase, and an IonPac AS11-HC column. The uptake of [¹⁴C]bicarbonate (as HCO₃⁻ or CO₂) was estimated by measuring the amount of [¹⁴C]bicarbonate bound by the biomass after incubation. The radioactive bicarbonate in the biomass was quantified by liquid scintillation counting (Tri-Carb Analyzer 1600TR; Packard Instrument) of an aliquot. Unbound CO₂ had been removed from these aliquots prior to measurement by lowering of the pH to less than 1 with 1 N HCl and thorough flushing with N₂ for 30 min. Aliquots of pasteurized biofilm and sludge were incubated with the respective radioactive substrates to test for adsorption and precipitation phenomena in all experiments as described by Lee et al. (24).

16S rRNA-directed oligonucleotide probes. The program ARB and the current version of the ARB 16S rRNA sequence database (approximately 15,000 entries) were used to develop new 16S rRNA-directed oligonucleotide probes (Table 1). The probes used for in situ hybridization were 5' labeled with the dye FLUOS [5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester] or with the sulfoindocyanine dye Cy3 or Cy5. Labeled probes and unlabeled competitor oligonucleotides were obtained from Thermo Hybaid (Interactiva Division, Ulm, Germany) or MWG (Ebersberg, Germany). The following oligonucleotide probes were used in addition to the probes developed in this study: (i) NEU, which is specific for halophilic and halotolerant *Nitrosomonas* spp. and *Nitrosococcus mobilis* (47); (ii) Nso1225, which is specific for ammonia oxidizers in the beta subclass of *Proteobacteria* (33), except for *N. mobilis* (38); (iii) NIT3, which is complementary to a sequence region of all *Nitrobacter* species (48); (iv) the EUB338 probe mixture, which consists of probes EUB338 (3), EUB338-II, and EUB338-III (14) covering the domain *Bacteria*; and (v) NON338, which is complementary to probe EUB338 (29) and was used as a negative control in all FISH experiments. All of the probes developed in this study were named in conformance with the standard introduced by Alm et al. (1), while the names of previously published probes were left unchanged to avoid confusion.

FISH and MAR. To determine oligonucleotide probe dissociation profiles, 5- μ l samples of fixed reference cells from pure cultures were spotted onto microscope slides (Paul Marienfeld, Bad Mergentheim, Germany) and dried for 10 min at 46°C. Thereupon, in situ hybridization was performed as detailed by Manz et al. (29). Different concentrations of formamide in the hybridization buffers and of sodium chloride in the washing buffers were used to measure probe binding at increasing hybridization stringencies.

Initially, all biofilm and activated-sludge samples were hybridized with probe NON338 derivatives labeled with FLUOS, Cy3, and Cy5 to exclude nonspecific probe binding. In none of the samples was nonspecific labeling of cells observed.

A modified FISH protocol was used to preserve the three-dimensional structure of bacterial aggregates in biofilm and activated-sludge flocs. Silicone tube segments with a diameter of 5 mm and a length of 5 to 8 mm were glued onto microscope coverslips by using bicomponent glue. These hybridization chambers were filled with 20 μ l of the biofilm or activated-sludge samples. After sedimentation (by gravity) of the biomass, approximately 10 μ l of the supernatant was removed and replaced with 20 μ l of 1% (wt/vol) molten agarose (Gibco BRL ultraPure agarose; Life Technologies, Paisley, Scotland) at a temperature of approximately 37°C. After solidification of the agarose, the embedded samples were dehydrated by dipping the slips successively into 50, 80, and 96% (vol/vol) ethanol for 3 min each. Subsequently, FISH was performed as described previously (29) but 20 μ l of hybridization buffer and increased amounts of probes (60 ng of probes labeled with Cy3 or Cy5 and 100 ng of probes labeled with FLUOS) were applied. Following the hybridization and washing steps, the slips were

immersed for 10 s in ice-cold deionized water to remove buffer salts from the slip surface.

Fixed biofilm or activated-sludge samples that had been incubated with radioactive substrates were embedded in cryoembedding compound (catalog no. 350100; Microm, Walldorf, Germany) and sliced with a microtome (model HM 505E; Microm). Sections with thicknesses of 5 to 10 μ m were applied to microscope coverslips, and FISH was performed with suitable oligonucleotide probes. MAR was performed as described by Lee et al. (24). Different exposure times (2, 5, and 7 days) before the development of the radiographic film emulsion were tested for all samples incubated with the different substrates (shorter exposure resulted in weaker signals, while prolonged exposure caused increased background noise). Coverslips with developed film were stored at 4°C until microscopic analysis.

Microscopy and digital image analysis. All samples hybridized with oligonucleotide probes were embedded in CitiFluor (CitiFluor, Canterbury, United Kingdom) prior to microscopic observation. Alternatively, biofilm was embedded in a 1:1 mixture of CitiFluor and a 0.01% (wt/vol) fluorescein solution for negative staining. Fluorescence signals were recorded with an LSM 510 CLSM (Zeiss, Oberkochen, Germany) equipped with two HeNe lasers (543 and 633 nm, respectively) for detection of Cy3 and Cy5 and one Ar ion laser (450 to 514 nm) for detection of FLUOS. Probe dissociation profiles were obtained as described by Daims et al. (14). Diameters of cell aggregates were determined by using the measurement tools of the software delivered with the CLSM (LSM 510, version 2.01). For this purpose, optical sections were acquired in those planes where the colonies to be measured were largest and the diameters of the colonies in these images were determined. The average diameters of the *Nitrospira* colonies in the different samples were obtained by processing of 50 to 100 colonies per sample. Three-dimensional reconstructions of *Nitrospira* cell aggregates were generated from stacked optical sections through biofilm samples. The optical sections with thicknesses of 0.5 to 0.7 μ m were acquired with the CLSM and saved as monochrome 8-bit tagged image file format files. The single images of these image stacks were imported in consecutive order by a three-dimensional image analysis and visualization program (H. Daims, unpublished data). During this step, the intensities of the image pixels were stored in a three-dimensional array in computer memory. Thereupon, background noise was reduced by three-dimensional median filtering and the pixel intensity data were analyzed to distinguish cell material from voids within cell clusters. Isosurfaces (i.e., surfaces connecting points of the same intensity) were extracted from the pixel intensity array by a modified implementation of the marching-tetrahedron algorithm (18) to display the surfaces of cell clusters. The isosurface calculation requires that the intensity level of the isosurface be supplied by the user, who must find appropriate intensity levels for visualization of the real surfaces of an object as precisely as possible. These intensity levels were determined with the aid of a second visualization method that displayed the real interfaces between the cell aggregates, the surrounding medium, and the internal voids as semitransparent layers in one image. This method is based on a modification of a volume-rendering algorithm published elsewhere (13) that draws surfaces automatically and independently of user-supplied intensity levels. The results of the two visualization techniques were evaluated by generating isosurfaces with different intensity levels and by comparing these isosurfaces visually with the surfaces drawn by the volume-rendering algorithm. During this evaluation, the most suitable intensity levels were selected for drawing of the cell clusters as isosurfaces in the final images. All voids within cell clusters were drawn colored and semitransparent to visualize the cavities and channels in the cell aggregates.

RESULTS

Phylogenetic affiliation of *Nitrospira*-like bacteria in the sequencing batch biofilm reactor. Biofilm was taken from nitrifying sequencing batch biofilm reactor SBBR 1, total DNA was extracted, and a 16S rDNA clone library was established. Among the 129 cloned and phylogenetically analyzed 16S rDNA sequences, 6 almost identical sequences shared high overall similarities (greater than 97%) with 16S rRNA sequences of *Nitrospira*-like bacteria obtained from nitrifying bioreactors in other studies (Fig. 1). None of the 129 16S rRNA sequences analyzed that were retrieved from reactor SBBR 1 grouped with the genus *Leptospirillum* and the *Thermodesulfobivrio*-“*Magnetobacterium*” lineage.

16S rRNA-directed oligonucleotide probes for in situ detection of the genus and the phylum *Nitrospira*. In this study, two 16S rRNA-directed oligonucleotide probes were developed for in situ identification of members of the genus and phylum *Nitrospira*, respectively (for details, see Table 1). The organisms target by each probe are indicated by brackets in Fig. 1.

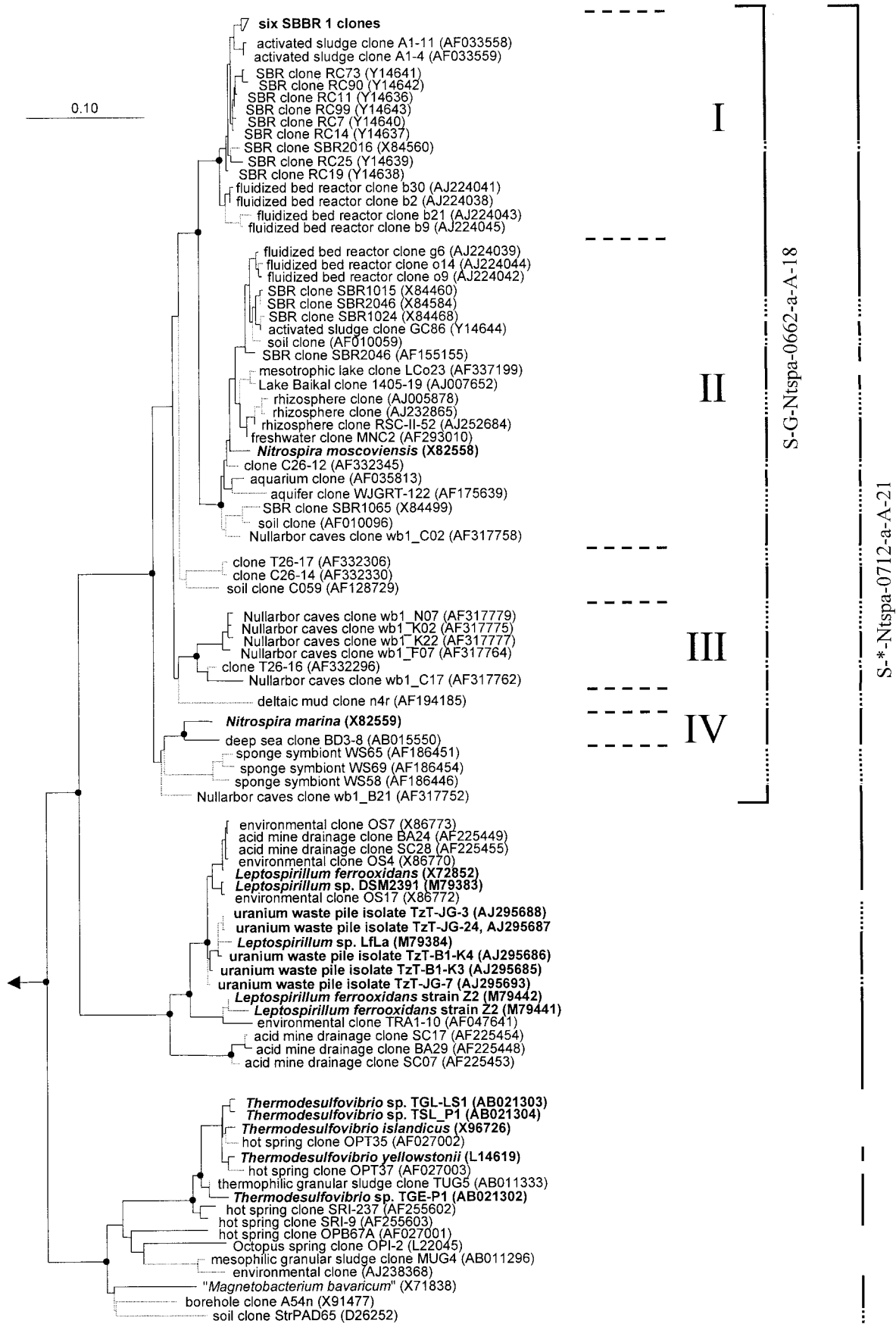
Probe S-G-Ntspa-0662-a-A-18 targets the described species *N. moscoviensis* and *N. marina*, as well as all environmental 16S rRNA sequences clustering with the genus *Nitrospira* line of descent for which the probe target site has been sequenced (Fig. 1). The 16S rRNA sequences of these organisms are fully complementary to probe S-G-Ntspa-0662-a-A-18 at the probe binding site, while all members of the other main lineages in the phylum *Nitrospira* possess at least one central mismatch with this probe (Fig. 2A). Several additional nontarget bacteria, for example, *B. stearothermophilus*, have a single C→A transversion at *Escherichia coli* position 669 (Fig. 2A). The probe dissociation profile of probe S-G-Ntspa-0662-a-A-18 indicates that this single mismatch does not prevent binding of the probe to the 16S rRNA (Fig. 3A). Fluorescence emitted by probe-stained cells of target species *N. moscoviensis* was clearly visible after FISH with up to 55% formamide in the hybridization buffer. However, cells of nontarget organism *B. stearothermophilus* were also stained by the probe and appeared even brighter. The probe-conferred fluorescence of the *Bacillus* cells was probably stronger, because these heterotrophic cells contained more ribosomes than the slow-growing, autotrophic cells of *N. moscoviensis*. Therefore, a competitor oligonucleotide (Comp-Ntspa-0662) was designed that is fully complementary to the 16S rRNAs of all nontarget organisms with the same sequence as *B. stearothermophilus* between *E. coli* positions 662 and 679. This competitor completely suppressed the hybridization of probe S-G-Ntspa-0662-a-A-18 with *B. stearothermophilus* if applied in equimolar amounts together with the probe in a hybridization buffer containing at least 35% formamide (Fig. 3A). Under these conditions, probe S-G-Ntspa-

0662-a-A-18 also did not bind to *Leptospirillum ferrooxidans* and *Thermodesulfobivrio yellowstonii* (data not shown). Bacterial probe EUB338 stained *N. moscoviensis* and *B. stearothermophilus* at formamide concentrations between 0 and 60% without any visible decrease in the fluorescence signal intensity (data not shown).

Probe S*-Ntspa-0712-a-A-21 targets all members of the phylum *Nitrospira* except *T. islandicus*; *Thermodesulfobivrio* sp. strains TGL-LS1 and TSL-P1; hot-spring clone OPB67A; environmental sequences OPT35, OPT37, MUG4, AJ238368, and OS17; mesotrophic-lake clone LCo23 (Fig. 2B); and Octopus Spring clone OPI-2. *N. marina* has one G→A transition at position 712 (Fig. 2B). We assume that this single mismatch does not prevent the binding of probe S*-Ntspa-0712-a-A-21 to *N. marina* because it is located marginally at the 5' end of the probe target region. Target species *N. moscoviensis* and *L. ferrooxidans* were stained by probe S*-Ntspa-0712-a-A-21 with up to 70% formamide in the hybridization buffer (Fig. 3B). Probably due to the length (21 nucleotides) and high G+C content of this probe (76.2%), no clear dissociation of the probe from the target organisms was observed, even at the highest formamide concentration tested. In addition, the competitor Comp-Ntspa-0712 (see below), which was applied together with probe S*-Ntspa-0712-a-A-21 in these experiments, did not prevent hybridization of the probe to the target organisms. Figure 3B also contains the dissociation profile of probe S*-Ntspa-0712-a-A-21 with *Desulfobivrio desulfuricans*. This species represents a group of nontarget organisms with two mismatches in the binding region of the probe (Fig. 2B). These mismatches did not prevent hybridization of the probe, but instead, this species was stained as efficiently as *N. moscoviensis* with up to 60% formamide in the hybridization buffer and detectable signals were still observed when more formamide was applied (Fig. 3B). Therefore, a competitor oligonucleotide (Comp-Ntspa-0712) complementary to the 16S rRNAs of all organisms with the same sequence as *D. desulfuricans* between *E. coli* positions 712 and 732 was designed to improve the specificity of probe S*-Ntspa-0712-a-A-21. When Comp-Ntspa-0712 was added in equimolar concentrations to the hybridization buffer, probe S*-Ntspa-0712-a-A-21 did not stain *D. desulfuricans*, even under conditions of low stringency (Fig. 3B).

In situ detection and morphology of *Nitrospira* microcolonies in biofilm and activated sludge. Activated sludge and biofilm from four different nitrifying bioreactors were screened for *Nitrospira*-related bacteria by FISH with the newly developed oligonucleotide probes. The presence of *Nitrospira*-like organisms in reactor SBBR 1 was confirmed by FISH with probes S-G-Ntspa-0662-a-A-18 and S*-Ntspa-0712-a-A-21. Both probes exclusively stained the same cell aggregates with

FIG. 1. Phylogenetic tree of the phylum *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum-likelihood analysis of all sequences longer than 1,300 nucleotides. Shorter sequences were successively added by use of the ARB_PARSIMONY module of the ARB program without changing the overall tree topology. Branches leading to sequences shorter than 1,000 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high-parsimony bootstrap support above 90% based on 100 iterations. The scale bar indicates 0.1 estimated change per nucleotide. Sequences of *Nitrospira*-like bacteria retrieved in this study from reactor SBBR 1 and sequences that belong to isolated strains are in boldface. The four sublineages of the genus *Nitrospira* are delimited by horizontal dashed lines and numbered I to IV. The brackets illustrate the coverage of the 16S rRNA-targeted oligonucleotide probes developed in this study. Dotted bracket segments indicate that the corresponding partial sequences do not include the probe target site. Brackets are interrupted where sequences are not targeted by the respective probe.



A Probe S-G-Ntspa-0662-a-A-18

Probe target site sequence	5' AGAGGAGCGCGGAAUUC 3'
<i>Nitrospira moscoviensis</i>	5' ----- 3'
<i>Nitrospira marina</i>	5' ----- 3'
SBBR 1 clone B28	5' ----- 3'
<i>Leptospirillum ferrooxidans</i>	5' ----GAA-----U- 3'
<i>Thermodesulfobivrio yellowstonii</i>	5' -----G---N----- 3'
" <i>Magnetobacterium bavaricum</i> "	5' --G---UG----- 3'
<i>Bacillus stearothermophilus</i>	5' -----A----- 3'
<i>Bacillus denitrificans</i>	5' -----A----- 3'
<i>Rhodospirillum salinarum</i>	5' -----A----- 3'

B Probe S^{*}-Ntspa-0712-a-A-21

Probe target site sequence	5' GGAAGGCCGGUGGCCGAAGCG 3'
<i>Nitrospira moscoviensis</i>	5' ----- 3'
<i>Nitrospira marina</i>	5' A----- 3'
mesotrophic lake clone LCo23	5' -----A----- 3'
SBBR 1 clone B28	5' ----- 3'
<i>Leptospirillum ferrooxidans</i>	5' ----- 3'
environmental clone OS17	5' ----A-----U----- 3'
<i>Thermodesulfobivrio yellowstonii</i>	5' ----- 3'
<i>Thermodesulfobivrio islandicus</i>	5' -----A-U-U----- 3'
<i>Thermodesulfobivrio</i> sp. TGL-LS1	5' -----U----- 3'
<i>Thermodesulfobivrio</i> sp. TSL-P1	5' -----U----- 3'
hot spring clones OPT35, OPT37	5' ----A---U-----A--- 3'
granular sludge clone MUG4	5' -----U-U----- 3'
environmental clone AJ238368	5' -----U-U----- 3'
" <i>Magnetobacterium bavaricum</i> "	5' ----- 3'
<i>Desulfobivrio desulfuricans</i>	5' ----CA----- 3'
<i>Saccharopolyspora hirsuta</i>	5' ----CA----- 3'

FIG. 2. Target site sequences and corresponding 16S rRNA sequence regions of target and nontarget organisms for probe S-G-Ntspa-0662-a-A-18 (A) and probe S^{*}-Ntspa-0712-a-A-21 (B). Hyphens represent identical nucleotides. Mismatches between the rRNA sequences of organisms and the probe target site sequence are indicated by capital letters.

average diameters of $12.4 \pm 7.0 \mu\text{m}$ (the mean diameters with standard errors are specified) (Fig. 4B). Similar results were obtained for an additional biofilm reactor (Biofor 2), where the cell aggregates had an average diameter of $11.4 \pm 7.6 \mu\text{m}$. The smallest and largest measured diameters of the *Nitrospira* cell clusters were 4.9 and $38.1 \mu\text{m}$, respectively, in reactor SBBR 1

and 1.6 and $31.7 \mu\text{m}$, respectively, in reactor Biofor 2. Some of the *Nitrospira* microcolonies in these biofilms had a spherical appearance, but most of them were shaped more irregularly. Their most remarkable features were cavities and channels within the cell colonies. Negative staining of biofilm from reactor SBBR 1 with fluorescein after FISH with probe S-G-

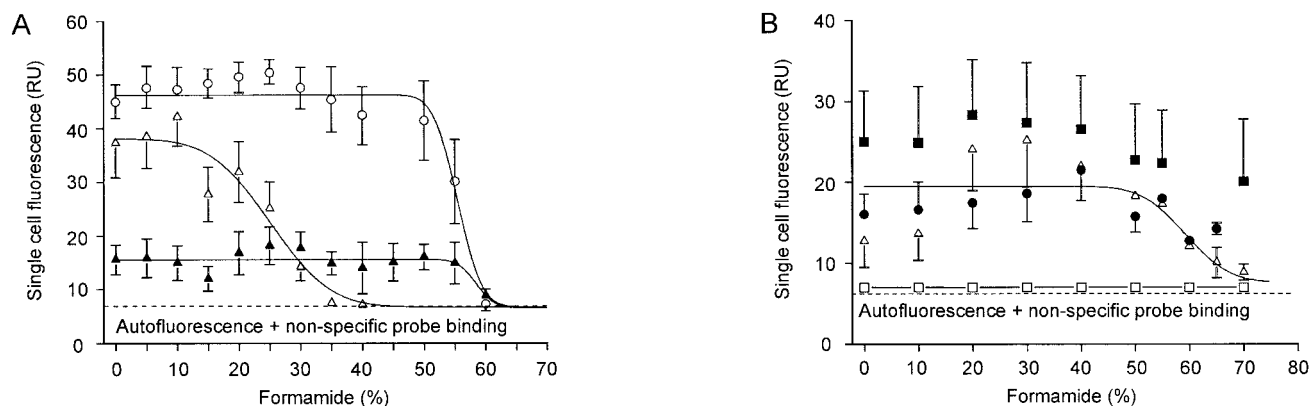


FIG. 3. Probe dissociation profiles of the oligonucleotide probes developed in this study with reference organisms under increasingly stringent hybridization and washing conditions. For each data point, the mean fluorescence intensity of at least 100 cells was determined. Regression curves were calculated by the plotting software based on a sigmoidal curve fit model. Error bars indicate 1 standard deviation. Error bars that are smaller than the marker symbols are not shown. (A) Hybridization of target organism *N. moscoviensis* with probe S-G-Ntspa-0662-a-A-18 in the presence of competitor Comp-Ntspa-0662 (▲). Hybridization of nontarget bacterium *B. stearothermophilus* with probe S-G-Ntspa-0662-a-A-18 without (○) and with (△) competitor Comp-Ntspa-0662. (B) Hybridization of target organisms *N. moscoviensis* (●) and *L. ferrooxidans* (■) with probe S^{*}-Ntspa-0712-a-A-21 in the presence of competitor Comp-Ntspa-0712. Hybridization of nontarget bacterium *D. desulfuricans* with probe S^{*}-Ntspa-0712-a-A-21 without (△) and with (□) competitor Comp-Ntspa-0712. The regression curve refers to the data points obtained for *D. desulfuricans* without addition of the competitor. RU, relative units.

Ntspa-0662-a-A-18 revealed that the fluorescein, which did not cross the boundaries of the *Nitrospira* cells, could penetrate these voids (Fig. 4B). The absence of bacterial cells from the cavities was confirmed by additional hybridization with the EUB338 probe mixture (data not shown). The impression that the *Nitrospira* microcolonies could be interlaced with a network of microscopic channels was verified by three-dimensional reconstruction of a probe-stained *Nitrospira* microcolony (Fig. 4C and D). *Nitrospira* microcolonies with this intricate morphology occurred frequently in the biofilms but were rare in both activated-sludge samples. *Nitrospira* aggregates detectable with both probes (S-G-Ntspa-0662-a-A-18 and S-*Ntspa-0712-a-A-21) were abundant in these sludges, but compared to the biofilm samples, the cells were packed more tightly and the aggregates were significantly smaller and had a more spherical shape (Fig. 4A). The average diameter of a *Nitrospira* microcolony in the AAV plant was $2.8 \pm 1.6 \mu\text{m}$, with a minimum of $0.9 \mu\text{m}$ and a maximum of $7.5 \mu\text{m}$. In the activated-sludge sample from the Munich II plant, their average diameter was $3.9 \pm 2.3 \mu\text{m}$, with a minimum of $1.0 \mu\text{m}$ and a maximum of $13.6 \mu\text{m}$.

All samples were also screened by FISH with a set of probes targeting nitrifying bacteria other than *Nitrospira* spp. *Nitrobacter* spp. were detected in neither the biofilm from reactor Biofor 2 nor the two activated-sludge samples when probe NIT3 was used. In reactor SBBR 1, however, probe-stained *Nitrobacter* cells occurred frequently but were less abundant than *Nitrospira*-like bacteria (data not shown). Furthermore, ammonia oxidizers from the beta subclass of *Proteobacteria* were detected frequently in all samples when probes NEU and Nso1225 were used (Fig. 4I).

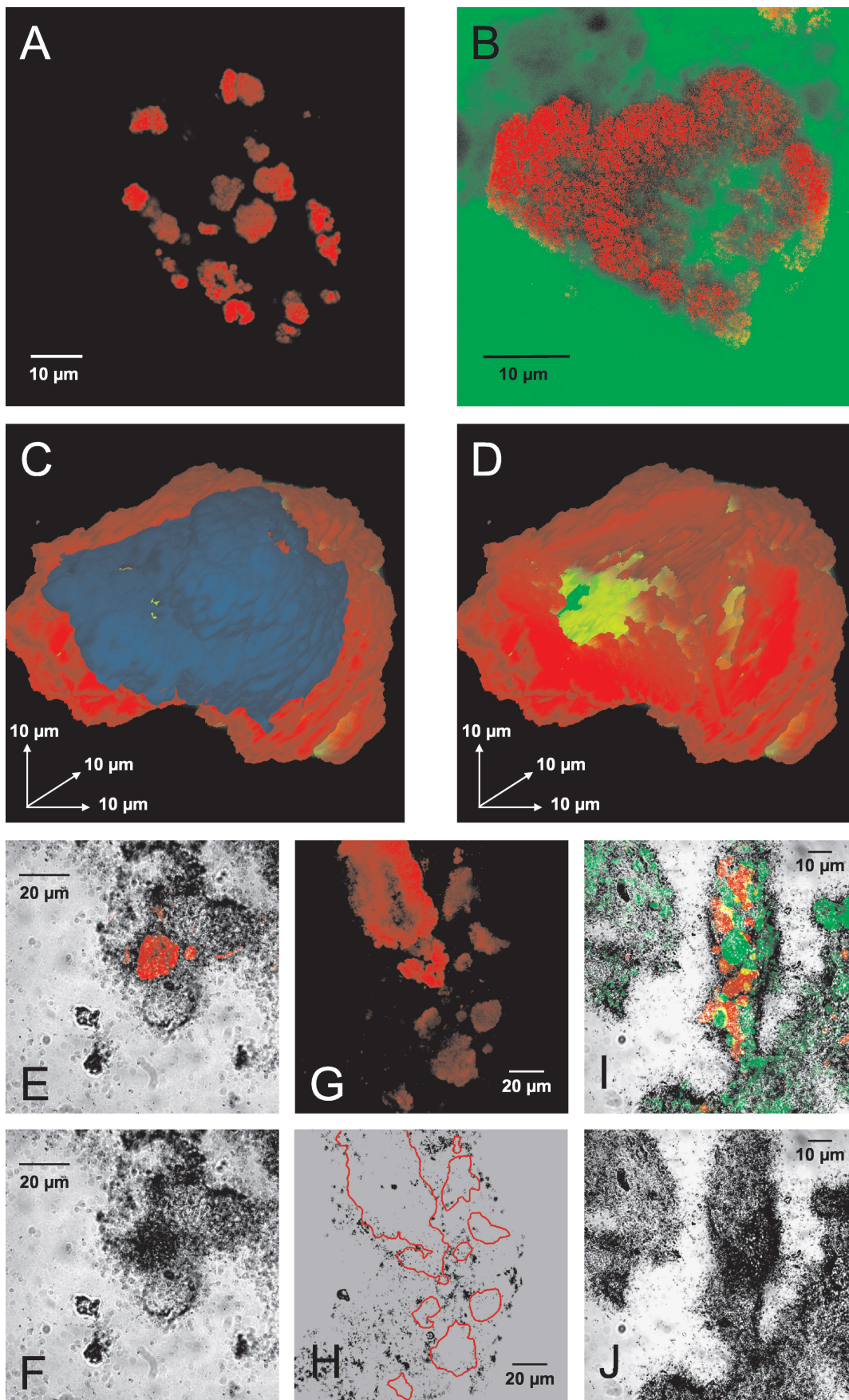
Uptake of carbon sources by *Nitrospira*-like bacteria in wastewater treatment plants. The uptake of substrates by *Nitrospira*-like bacteria in wastewater treatment plants was studied by incubation of living nitrifying biofilm and activated sludge with radioactive carbon sources, followed by FISH and MAR. Biofilm samples from reactors SBBR 1 and Biofor 2 and activated sludge from the AAV plant were incubated with different carbon sources. The liquid scintillation counts and high-performance liquid chromatography measurements performed before and after all incubations confirmed that the substrates were not depleted during the incubation time (data not shown). The incubated samples were hybridized with the *Nitrospira*-specific probes developed in this study and the EUB338 probe mixture. After completion of the FISH-MAR procedure, the samples were screened for probe-stained microcolonies of *Nitrospira*-like bacteria. Silver grain formation above these aggregates indicated that the cells took up the respective substrates during the incubation period. All substrate uptake experiments were qualitative, except for one experiment with bicarbonate and activated sludge from the AAV plant, which was evaluated quantitatively by counting MAR-positive and -negative *Nitrospira* microcolonies (see below). Under aerobic incubation conditions, most of the *Nitrospira* colonies detected in the biofilm sample from reactor SBBR 1 took up inorganic carbon (Fig. 4E and F). No uptake of acetate, propionate, or butyrate by *Nitrospira*-like bacteria was observed after aerobic incubation (Fig. 4G and H). Most of the *Nitrospira* colonies in the biofilm, however, were clearly MAR positive after aerobic incubation with ^{14}C -labeled pyruvate

(Fig. 4I and J). Uptake of pyruvate by the remaining *Nitrospira* colonies was uncertain because only weak silver grain formation was observed above these aggregates. Results similar to those obtained with reactor SBBR 1 were obtained with the biofilm from reactor Biofor 2 (data not shown). *Nitrospira*-like bacteria did not take up any substrate tested in all three samples under anoxic or anaerobic conditions. Other bacteria in the samples, which were stained by the EUB338 probe mixture, took up the different substrates under the anoxic incubation conditions and, to a much lesser extent, under the anaerobic incubation conditions (data not shown). These bacteria were not identified by FISH with more specific probes but served as a positive control for the incubation experiments and the FISH-MAR procedure. In contrast, no MAR-positive cells were detected in the control experiments performed with pasteurized biofilm (data not shown). Therefore, adsorption of labeled substrates to cells or other organic components did not account for the MAR signals observed with living biofilm and activated sludge.

An additional experiment was performed to determine the fraction of detectable *Nitrospira* microcolonies taking up bicarbonate in activated sludge from the AAV wastewater treatment plant. The sludge was incubated with [^{14}C]bicarbonate under aerobic conditions, and the subsequent steps of the FISH-MAR protocol were performed. Different microscope slips covered with the radiographic film emulsion were exposed for 5, 6, or 10 days. Following film development, 200 *Nitrospira* microcolonies were investigated for fixation of inorganic carbon at each exposure time in several microscope fields per slip to obtain the fraction of colonies with inorganic carbon uptake activity. After 5 days of exposure, 56% of the *Nitrospira* colonies counted were MAR positive. These colonies usually had diameters greater than $3.2 \mu\text{m}$, while most of the smaller colonies were MAR negative. Similar results were obtained after 6 days of exposure, when 51% of the colonies counted were MAR positive, with the same correlation of colony size and MAR signals. After 10 days of exposure, however, the percentage of clearly MAR-positive colonies had increased to 77%. The remaining cell aggregates were very small (diameters of less than $2.0 \mu\text{m}$), and silver grain formation above these colonies possibly indicating bicarbonate uptake could not be distinguished from side effects due to background radiation or radiation emitted by larger adjacent colonies.

DISCUSSION

Phylogenetic trees containing all of the publicly available 16S rRNA sequences related to the phylum *Nitrospira* were calculated by using the maximum-parsimony and maximum-likelihood treeing methods on different data sets. The resultant consensus tree of the phylum shown in Fig. 1 is almost identical in topology to the respective neighbor-joining tree (data not shown). Consistent with the original definition of the phylum *Nitrospira* proposed by Ehrich et al. (16), the phylum currently consists of three main monophyletic lineages that are supported by all treeing methods and high bootstrap values. All of the sequences in the first main lineage are affiliated with *N. moscoviensis* and *N. marina*, the sequences in the second main lineage are related to *L. ferrooxidans*, and the organisms in the third main lineage are relatives of *T. yellowstonii* and "*M.*



bavaricum". Our analyses demonstrate that the line of descent containing both *Nitrospira* species can be further subdivided into at least four monophyletic sublineages, which are supported by all treeing methods and high bootstrap values above 90% (I to IV; Fig. 1). The sequences grouping together in each sublineage share 16S rRNA similarities of at least 94.9%. In contrast, the similarities of sequences that belong to different sublineages are always below 94.0%. Therefore, it can be postulated, based on the suggestion by Stackebrandt and Goebel (44), that the genus *Nitrospira* contains, in addition to *N. marina* and *N. moscoviensis*, at least two new candidate species represented by sublineages I and III, respectively. However, valid description of these species must await the isolation and phenotypic characterization of these organisms. Comparison of sublineage affiliation and sampled environments suggests that sublineages I, III, and IV each encompass a specialized group of nitrite oxidizers adapted to a certain habitat while sublineage II contains nitrite oxidizers that can thrive in different systems. One might speculate that the apparently unequal distribution of the sublineages in nature is a direct consequence of evolutionary events or of limited searches that have been done for these organisms.

Specifically, sublineage I contains only uncultivated organisms found in nitrifying bioreactors. These bacteria were detected in a laboratory scale sequencing batch reactor (11), a laboratory scale fluidized bed reactor (43), and an industrial full-scale activated-sludge basin (22). In addition, all six of the *Nitrospira*-related 16S rRNA sequences that were obtained from reactor SBBR 1 in this study group with this sublineage (Fig. 1). Sublineage II contains the cultivated species *N. moscoviensis*, as well as 16S rRNA sequences of uncultivated bacteria retrieved from diverse habitats, including bioreactors (9, 11, 43), freshwater aquaria (21), soil, rhizosphere (30), and lake water. The topology of the tree implies that these two sublineages emerged from the same ancestor after the divergence of the other two sublineages (Fig. 1). Sequences retrieved from different nitrifying bioreactors occur in sublineages I and II, but no obvious correlation between the types or operational modes of the source reactors and the affiliation of the corresponding sequences to one of the sublineages exists. For example, each sublineage contains organisms found in sequencing batch reactors. Furthermore, the sequences retrieved from the same fluidized bed reactor by Schramm et al. (43) are distributed over both sublineages (clones b30, b2, b21,

b9, g6, o14, and o9; Fig. 1). Sublineage III consists mainly of sequences found in aquatic samples from Nullarbor caves in Australia (20) (Fig. 1). Sublineage IV hosts the cultivated species *N. marina* and a related organism detected in the deep sea. It should be noted that eight environmentally retrieved 16S rDNA sequences affiliated with the *Nitrospira* lineage are possibly not members of the above-described four sublineages. These sequences, however, are incomplete, and their exact phylogenetic affiliation with the other sublineages of the genus, thus, cannot be determined.

Based on the available 16S rRNA sequences, both a *Nitrospira* phylum-specific oligonucleotide probe and a *Nitrospira* genus-specific oligonucleotide probe suitable for FISH were developed. Probe S^{*}-Ntspa-0712-a-A-21 is specific for most known members of the phylum *Nitrospira*. A probe targeting the phylum *Nitrospira* has, to our knowledge, not been published before, and thus, probe S^{*}-Ntspa-0712-a-A-21 extends the current set of bacterial group-specific probes (28, 29, 32, 35, 40), which have found widespread application in microbial ecology (4, 46). Probe S-G-Ntspa-0662-a-A-18 covers the whole genus *Nitrospira*, including the four sublineages mentioned above. Nontarget organisms without nucleotide mismatches at the probe binding site, or elsewhere on the rRNA, are not known. A similar probe that targets positions 664 to 685 on the 16S rRNA of *Nitrospira*-like bacteria was described in a previous report (21). This probe is also suitable for in situ hybridization (36), but it does not cover the *Nitrospira*-like bacteria detected in an industrial activated-sludge plant by Juretschko et al. (22). Several other 16S rRNA-targeted oligonucleotide probes for the in situ detection of *Nitrospira*-like bacteria were published previously (22, 43). These probes, however, were designed to target only certain *Nitrospira*-related sequences retrieved from wastewater treatment plants and do not cover the whole genus *Nitrospira*.

Application of the newly developed *Nitrospira*-specific probes revealed that the microcolony architecture of the target organisms differed significantly between the activated-sludge and biofilm samples. Constant turbulence, shearing, and the limited sludge age most likely prevent large, morphologically complex cell aggregates of slowly growing nitrite oxidizers in activated-sludge flocs. Accordingly, the *Nitrospira* microcolonies in the activated-sludge samples were relatively small, compact, and almost spherical. In contrast, the *Nitrospira* aggregates in the biofilm samples were significantly larger and had a

FIG. 4. In situ analyses of *Nitrospira*-like bacteria within activated sludge and biofilms. (A) *Nitrospira* cell aggregates detected in activated sludge by FISH with probe S-G-Ntspa-0662-a-A-18 (red). (B) *Nitrospira* cell aggregate detected in biofilm from reactor SBBR 1 by FISH with probe S-G-Ntspa-0662-a-A-18 (red). All of the *Nitrospira* colonies detected in SBBR 1 with probe S-G-Ntspa-0662-a-A-18 also hybridized with probe S^{*}-Ntspa-0712-a-A-21 (data not shown). The biofilm was also stained with fluorescein (green). (C and D) Three-dimensional reconstruction of a *Nitrospira* cell aggregate from reactor SBBR 1 stained by FISH with probe S-G-Ntspa-0662-a-A-18. *Nitrospira* cells are red; the blue part of the microcolony in panel C was digitally removed to allow insight into the aggregate (D). Voids within the aggregate are green. (E and F) Uptake of bicarbonate by *Nitrospira*-like bacteria in biofilm from reactor SBBR 1 under aerobic incubation conditions. (E) *Nitrospira* cells stained by probe S-G-Ntspa-0662-a-A-18 (red) combined with the micrograph of the radiographic film at the same position. Panel F shows only the film to visualize the MAR signal at the position of the *Nitrospira* cells. Other MAR signals were caused by CO₂-fixing bacteria, which were not detected by the *Nitrospira*-specific probe. (G and H) No uptake of acetate by *Nitrospira*-like bacteria in biofilm from reactor SBBR 1 under aerobic incubation conditions. (G) *Nitrospira* cells stained by probe S-G-Ntspa-0662-a-A-18 (red). Panel H is a micrograph of the radiographic film at the same position. The localization of the *Nitrospira* microcolonies in panel G is indicated by red borderlines in panel H. (I and J) Uptake of pyruvate by *Nitrospira*-like bacteria (stained by probe S-G-Ntspa-0662-a-A-18; red) and by ammonia oxidizers (stained by probes NEU and Nso1225; green) in biofilm from reactor SBBR 1 under aerobic incubation conditions. The fluorescence recorded in a stack of images by the CLSM is combined by orthographic projection in panel I. Stacked cells of *Nitrospira*-like bacteria and ammonia oxidizers appear therefore yellow. The image stack was acquired to ensure that all of the nitrifiers that contributed to the MAR signal would be visible in the final image.

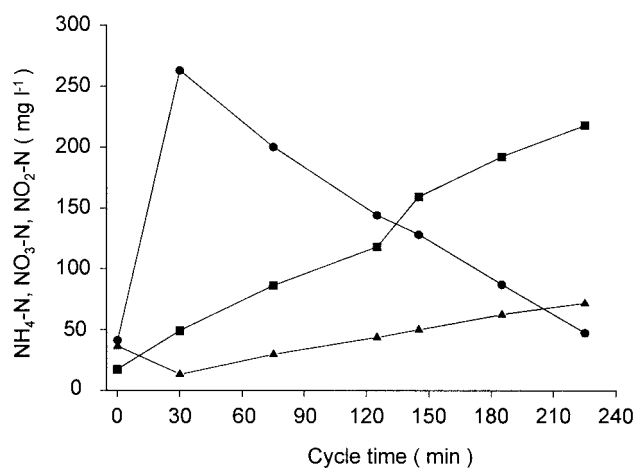


FIG. 5. Concentrations of NH₄-N (●), NO₃-N (■), and NO₂-N (▲) in reactor SBBR 1 during a representative reactor operating cycle.

more complex and irregular morphology with internal cavities and a network of cell-free channels. Structurally similar networks formed by channels and cavities between cell aggregates were previously detected by confocal laser scanning microscopy in biofilms of different origins and compositions (23, 31). Channels were also found in microbial granules and have been interpreted as facilitating the exchange of nutrients and gases between the surface and deeper regions (27, 39, 50). This idea was strongly supported by de Beer et al. (15), who showed that O₂ concentrations inside a biofilm are significantly higher in voids than in cell clusters. The network of channels and voids we observed within cell clusters of *Nitrospira*-like bacteria may have similar effects. Staining with fluorescein demonstrated that the channels are permeable for low-molecular-weight, water-soluble substances and thus could facilitate the diffusion of nitrite, gases, and metabolic waste compounds throughout the aggregates. Furthermore, the voids and channels in *Nitrospira* cell clusters indicate that the modern concept of biofilm architecture as a complex assembly of cell aggregates, organic polymers, and cavities may also apply on a smaller scale to single bacterial microcolonies.

The *Nitrospira*-specific probes were used together with a previously published probe for in situ detection of *Nitrobacter* bacteria (48) to investigate the community structure of nitrite-oxidizing bacteria in nitrifying activated-sludge and biofilm samples. While *Nitrospira* was present in significant amounts in all of the samples analyzed, *Nitrobacter* cells were detected only in sequencing batch biofilm reactor SBBR 1. Reactor SBBR 1 receives reject water from sludge dewatering, which is particularly rich in ammonia and dissolved salts. Due to the batch performance of the reactor, ammonia, nitrite, and nitrate concentrations vary significantly during an operating cycle (Fig. 5). The repeated, pronounced temporal nitrite concentration shifts within SBBR 1 create an ecological niche for nitrite oxidizers adapted to high nitrite concentrations that does not occur in continuously operated bioreactors. This niche is obviously filled by *Nitrobacter* sp., which, according to Schramm et al. (42), is a putative r strategist for nitrite and oxygen. In contrast, the *Nitrospira*-like bacteria were postulated to be K strategists that can grow with lower nitrite (and oxygen) con-

centrations and can thus coexist with *Nitrobacter* bacteria in SBBR 1.

The activity and in situ physiology of *Nitrospira*-like organisms in wastewater treatment plants were studied by a recently developed combination of FISH and MAR (24). The FISH-MAR experiments with radioactive bicarbonate demonstrated that *Nitrospira*-like bacteria growing in aerated bioreactors fix inorganic carbon. This finding was not unexpected, since pure cultures of *N. moscoviensis* and *N. marina* grow autotrophically under laboratory conditions (16, 49), but inorganic carbon fixation of the recently discovered *Nitrospira*-like bacteria has not been demonstrated previously. Furthermore, uptake of inorganic carbon can be exploited as a method by which to demonstrate the in situ activity of nitrifying bacteria. Such a method is urgently required, since positive FISH signals were also observed for starved or metabolically inhibited nitrifying bacteria (34, 47). In the municipal activated sludge analyzed, more than 75% of the *Nitrospira* microcolonies detected in situ were unambiguously active, as visualized by the incorporation of inorganic carbon. The high proportion of active *Nitrospira* cell aggregates in the activated sludge may result from the permanent removal of inactive, nonproliferating cells due to continuous reactor operation.

Qualitative microscopic observation of the samples from reactors SBBR 1 and Biofor 2 after MAR with radioactive pyruvate showed that most of the *Nitrospira* colonies took up pyruvate. As the parallel experiments with [¹⁴C]bicarbonate had shown that most of the *Nitrospira* colonies in these samples also took up inorganic carbon, this suggests that at least a fraction of these bacteria can grow mixotrophically (defined in this context as the ability to simultaneously incorporate inorganic and organic carbon sources). This ability might contribute to the competitiveness of *Nitrospira*-like bacteria in wastewater treatment plants. Growth with pyruvate as a carbon source has also been reported for pure cultures of *Nitrobacter* (6) and *N. marina* bacteria, which reached the highest cell densities in nitrite media containing pyruvate (49). However, since the FISH-MAR technique cannot be used to monitor the simultaneous uptake of two or more different substrates, the possibility cannot be excluded that different populations of *Nitrospira*-like bacteria with different abilities to take up inorganic carbon or pyruvate existed in the samples investigated. In contrast to the aerobic incubation experiments, no uptake of organic or inorganic carbon sources by *Nitrospira*-like bacteria was observed in the absence of oxygen. However, combined FISH and microsensor measurements revealed that high numbers of *Nitrospira*-like bacterial cells can persist in biofilm zones with low oxygen pressure (17, 36, 41). The ability to switch from aerobic respiration to an anaerobic metabolism should be advantageous in such habitats. According to our experiments, *Nitrospira*-like bacteria seemingly do not use acetate, propionate, butyrate, or pyruvate in the presence of nitrate and the absence of oxygen. Thus, these results provide no indication that the *Nitrospira*-like bacteria respired anaerobically with nitrate, but they might employ other strategies to survive periods of limited oxygen availability. Pure-culture experiments indicated that *N. marina* is obligately aerobic (49) while *N. moscoviensis* can oxidize H₂ with nitrate as the electron acceptor and CO₂ as the sole carbon source (16). Hydrogenase activity was not examined in this study, but we do not

exclude the possibility that the *Nitrospira*-like bacteria living in engineered systems can take advantage of this or other alternatives to aerobic nitrite oxidation.

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