Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and *in situ* physiology

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Abstract  The frequency and distribution of putatively nitrite-oxidizing, *Nitrospira*-like bacteria in nitrifying biofilms from two reactors receiving wastewater with different ammonia and salt concentrations were observed by fluorescent *in situ* hybridization. For this purpose, new 16S rRNA-directed oligonucleotide probes targeting the bacterial phylum *Nitrospira* and the three main lineages within this phylum were developed and evaluated. The diversity of *Nitrospira*-like bacteria in the reactors was additionally investigated by retrieval and comparative analysis of full 16S rRNA sequences from the biofilms. We found that, despite of the differences in the influent composition, *Nitrospira*-like bacteria form dominant populations in both reactors. In addition, first insights into the physiology of these still unculturable bacteria were obtained by the incubation of active biofilm samples with radioactively labeled substrates followed by the combined application of fluorescent *in situ* hybridization and microautoradiography. The results are discussed in consideration of the frequently observed dominance of *Nitrospira*-like bacteria in nitrifying bioreactors. Consequently, high priority should be assigned to future studies on the ecology and physiology of these organisms in order to increase our fundamental understanding of nitrogen cycling and to enable knowledge-driven future improvements of nitrifying wastewater treatment plants.

Keywords  Nitrification; nitrite oxidation; *Nitrospira*; fluorescent *in situ* hybridization; microautoradiography

Introduction  The removal of nitrogen from sewage is one of the most important requirements in modern wastewater treatment. Increased concentrations of total nitrogen do not only disturb the nutrient balance in receiving waters, but ammonia and nitrite are also toxic to aquatic life and must therefore be eliminated from wastewater. Nitrogen is removed in bioreactors in two sequential steps, which are both catalysed by bacteria: nitrification (the oxidation of ammonia to nitrite and subsequently to nitrate, performed by ammonia- and nitrite-oxidizing bacteria, respectively), and denitrification (the anoxic reduction of nitrate or nitrite to NO, N₂O and N₂). While various bacteria belonging to different phylogenetic groups are involved in denitrification, the recognized nitrifying bacteria are restricted to a few bacterial lineages. The chemolithoautotrophic ammonia-oxidizing bacteria dominant in wastewater treatment belong to a monophyletic lineage within the beta-subclass of the *Proteobacteria* (Wagner et al., 1995; Juretschko et al., 1998). Members of the genus *Nitrobacter* have traditionally been regarded as the key nitrite oxidizers in wastewater treatment, but recent molecular studies demonstrated that relatives of the nitrite-oxidizing bacterium *Nitrospira moscoviensis* occur more frequently and in higher amounts in nitrifying reactors than *Nitrobacter*, leading to the assumption that these *Nitrospira*-like bacteria are the actual important nitrite oxidizers in these systems (e.g. Wagner et al., 1996; Juretschko et al., 1998; Schramm et al., 1998). These organisms belong to the distinct bacterial phylum “*Nitrospira*”, which contains, in addition to the nitrite-oxidizers *N. moscoviensis* and *N. marina*, an assemblage of slow-growing microorganisms with different physiological properties, for example the genera *Leptospirillum* and *Thermodesulfovibrio* (Ehrich et al.,
Nitrospira-like bacteria have been detected in wastewater treatment plants exclusively by retrieval of their 16S rRNA sequences, but no representatives of these bacteria could be isolated from this habitat up to now due to our insufficient knowledge of their physiology. In this study we applied a recently developed combination of fluorescent in situ hybridization (FISH) with microautoradiography, MAR (Lee et al., 1999), in order to investigate in a cultivation-independent way the distribution and physiology of Nitrospira-like bacteria in biofilms from two nitrifying reactors (one sequencing biofilm batch reactor, “SBBR1” receiving sewage water with high ammonia and salt concentrations, and one continuous reactor, “Biofor2” receiving municipal wastewater with moderate ammonia and salt concentrations) of a pilot wastewater treatment plant at Ingolstadt, Germany. After incubation of subsamples of the biofilms with several radioactively labeled substrates under different conditions, members of the phylum and genus Nitrospira, respectively, were specifically stained by FISH with 16S rRNA-targeted gene probes. Subsequently, confocal laser scanning microscopy allowed us to monitor the substrate uptake profiles of the Nitrospira-like bacteria in situ. Moreover, the 16S rRNA genes of Nitrospira-like bacteria living in these biofilms were retrieved and subjected to phylogenetic analysis to extend our current knowledge on their diversity.

Materials and methods

Cultivation of reference organisms, biofilm sampling, and cell fixation

Bacillus stearothermophilus (DSM 22), Leptospirillum ferrooxidans (DSM 2705), and Desulfovibrio desulfuricans (DSM 574) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultivated following the DSMZ instructions. Paraformaldehyde-fixed cells of Nitrospira moscoviensis were provided by Dr. Gabriele Timmermann (University of Hamburg, Germany). For sampling of biofilm, expanded clay beads covered with biofilm were taken from the nitrifying reactors and were swirled in order to detach the biofilm. Loosened biofilm and cells from the pure cultures were fixed in 3% (w/v) paraformaldehyde or 50% (v/v) ethanol and stored at –20°C.

Fluorescent in situ hybridization, microautoradiography, and confocal microscopy

Group-specific oligonucleotide probes targeting Nitrospira-like bacteria were designed using the software package ARB and the TU Munich rRNA database (Dr. Ludwig, Technical University Munich). Probes labeled with one of the sulfoindocyanine dyes Cy3 and Cy5 and unlabeled competitor oligonucleotides were obtained from Interactiva (Ulm, Germany). Nitrospira-directed probes designed in this study were S-*Ntspa-0712-a-A-21 (5’-CGCCTTCGCCACCCCGCCTCC-3’), specific for most members of the phylum Nitrospira; S-G-Nts-pa-0662-a-A-18 (5’-GGAA TTCCGCTCTCCTCT-3’), specific for the genus Nitrospira; S-G-Ltspi-1459-a-A-18 (5’-GCCATACCTTGGGCGGCT-3’), specific for the genus Leptospirillum; and S-*-Tdsulfo-0848-a-A-18 (5’-TTTCCCTTCGGCACA-GAG-3’), specific for the Thermodesulfovibrio/Magnetobacterium cluster. Probe S-*-Ntspa-0712-a-A-21 was used in a 1:1 ratio together with the competitor Comp-Ntspa-0712 (5’-CGCCTTCGCCACCCCGCCTCC-3’), and probe S-G-Nts-pa-0662-a-A-18 was applied with Comp-Nts-pa-0662 (5’-GGAA TTCCGCTCTCCTCT-3’). Additional probes used were NIT3 (Wagner et al., 1996) and Nso1225 (Mobarry et al., 1996), specific for nitrite-oxidizers of the genus Nitrobacter and for the ammonia-oxidizers of the beta-subclass of Proteobacteria, respectively. In situ hybridization of pure cultures and biofilm samples was performed as described by Juretschko et al. (1998). Images were recorded using a LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped...
with one Argon laser (450 to 514 nm), two Helium-Neon lasers (543 and 633 nm, respectively), and one UV laser (351 and 364 nm).

Fresh, unfixed biofilm was incubated separately with each of the radioactively labeled substrates \( ^3 \)H-acetate and \( ^{14} \)C-bicarbonate under aerobic, anoxic and anaerobic conditions. After incubation the biofilm was fixed with paraformaldehyde, cryosectioned and spread onto microscopic cover slips. Thereupon, FISH and MAR were performed in combination as described by Lee et al. (1999). The principle of MAR and the steps performed to combine FISH with MAR are illustrated in Figure 1. Cover slips were subsequently examined by confocal laser scanning microscopy in order to identify the organisms which had taken up the radioactive substrates.

Cloning and sequencing of 16S rRNA genes, and phylogenetic analysis
Cloning, sequencing and phylogenetic analysis of 16S rDNA sequences retrieved from the biofilms was performed as detailed by Juretschko et al. (1998).

Results

In situ analysis of Nitrospira-like bacteria

For specific in situ identification of Nitrospira-like bacteria in complex samples we designed one probe that targets almost all known members of the phylum Nitrospira, S-\(^*\)-Ntspa-0712-a-A-21, and three probes which are directed against the three lineages within this phylum (Figure 2). Regarding the probe specificity we found that the 16S rRNA of some non-target organisms, including B. stearothermophilus, has only one weak mismatch to the probe sequence of S-G-Ntspa-0662-a-A-18 (the probe directed against the genus Nitrospira), and that the 16S rRNAs of D. desulfuricans and some other non-target organisms have only one mismatch to probe S-\(^*\)-Ntspa-0712-a-A-21. In order to ensure the desired probe specificity we additionally designed two competitor oligonucleotides which are able to block the binding of the Nitrospira-specific probes to non-target bacteria. Proper
Stringency for in situ hybridization was determined for both probes by applying labeled derivatives of them together with the respective unlabeled competitors to fixed cells of N. moscoviensis (target organism for both probes), L. ferrooxidans (target organism for S-*. Ntspa-0712-a-A-18), and to B. stearothermophilus as well as D. desulfuricans (non-target organisms for both probes) at increasing formamide concentrations in the hybridization buffer. At 35% (v/v) of formamide, both probes hybridized exclusively to their respective target organisms with no signal being observed for the non-target organisms. Using these stringent conditions for hybridization, high numbers of bacteria were detected by both probes in the two different nitrifying biofilms. These cells occurred mainly as clusters within dense biofilm areas and were frequently found in close vicinity to ammonia-oxidizing bacteria stained by probe Nso1225 (data not shown). While all cells hit by probe S-G-Ntspa-662-a-A-18 were simultaneously stained by probe S-*.Ntspa-0712-a-A-21 in both biofilms, in the SBBR1 biofilm some smaller cell aggregates exclusively showed signals with probe S-*.Ntspa-0712-a-A-21 suggesting the additional presence of members of the Nitrospira-phylum not affiliated to the genus Nitrospira. No signals were observed, however, after hybridization of this biofilm with the probes S-G-Ltspi-1459-a-A-18 (specific for the genus Leptospirillum) and S-*.Tdsulfo-0848-a-A-18 (specific for Thermodesulfovibrio and Magnetobacterium) even under conditions of low stringency (without formamide in the hybridization buffer).

Remarkably, Nitrobacter cells were detectable by in situ hybridization of the SBBR1 biofilm with probe NIT3 (albeit in lower numbers compared to Nitrospira), while hybridization of the Biofor2 biofilm with probe NIT3 yielded negative results only. Similar to the Nitrospira-like bacteria, the Nitrobacter microcolonies in the SBBR1 biofilm were frequently located in close vicinity to ammonia-oxidizing bacteria, suggesting that in this biofilm, both nitrite-oxidizers live in mutualistic relationships with the ammonia-oxidizers.

Sequencing of 16S rRNA genes and phylogenetic analysis

Up to now we have retrieved four full 16S rRNA sequences of Nitrospira-like bacteria from biofilm of the reactors Biofor2 and SBBR1, respectively. A phylogenetic tree of the phylum Nitrospira including these four sequences and two additional sequences retrieved from the activated sludge of a wastewater treatment plant belonging to an animal waste processing facility at Kraftisried, Germany (Juretschko et al., 1998) is shown in Figure 2. It is evident from this tree that the sequences from Biofor2, SBBR1, and the Kraftisried plant are closely related to each other and cluster together with N. moscoviensis and several sequences obtained from other nitrifying reactors or biofilters (Burrell et al., 1998; Hovanec et al., 1998; Schramm et al., 1998). For a more encompassing analysis of the diversity of Nitrospira-like bacteria in these biofilms we are currently retrieving and analyzing additional Nitrospira 16S rRNA sequences from the two reactors.

Combination of fluorescent in situ hybridization with microautoradiography

The availability of the newly developed set of Nitrospira-specific gene probes and the high numbers of Nitrospira-like bacteria in the investigated biofilms (see above) encouraged us to study the physiology of these bacteria by combining FISH and MAR. Biofilm samples were incubated with radioactively labeled bicarbonate and acetate under aerobic, anoxic and anaerobic conditions and, after FISH and MAR, we are currently monitoring the uptake profiles of the Nitrospira-like bacteria for additional substrates within the natural habitat of these bacteria by confocal laser scanning microscopy. First results demonstrated that the Nitrospira-like bacteria present in the reactors take up bicarbonate only under aerobic conditions, and that they do not take up acetate under the different conditions applied.
The Nitrospira-specific probes developed in this study allow for the first time a fast and reliable in situ monitoring of the genus and phylum Nitrospira within environmental samples. Our results obtained with the nitrifying biofilms from two different reactors, which receive sewage with high and moderate ammonia and salt concentrations, respectively, strongly support the recently raised hypothesis that Nitrospira-like bacteria are of general major importance for nitrite-oxidation in wastewater treatment (Juretschko et al., 1998).

Consistent with previous findings in other plants (Wagner et al., 1996; Juretschko et al., 1998) the number of Nitrobacter cells in the Biofor2 biofilm was below the detection limit of FISH (approximately 10^3 cells/ml) strongly suggesting a re-evaluation of the selection of Nitrobacter as model organism for nitrite-oxidation. More generally our findings illustrate the insufficient knowledge on the ecology of nitrite-oxidizing bacteria. Why do Nitrospira and Nitrobacter co-exist in reactor SBBR1, but not in most other investigated nitrifying bioreactors, where Nitrospira-like bacteria are dominant? Which factors select for Nitrobacter or Nitrospira, respectively, as the dominant nitrite-oxidizer? Which differences in the physiology of Nitrobacter and Nitrospira cause their unequal distribution in different nitrifying treatment plants? The answers on these questions are a prerequisite for the design

**Figure 2** Phylogenetic tree of the phylum Nitrospira, based on comparative analysis of 16S rRNA sequences. The sequences retrieved from the reactors Biofor2 and SBBR1 of the Ingolstadt pilot plant and from the Kraftisried treatment plant (Juretschko et al., 1998) are printed in bold. The braces indicate the coverage of the specified oligonucleotide probes. The tree was calculated using the Neighbour Joining treeing method. The scale bar indicates 0.1 changes per nucleotide.

†These organisms show mismatches to the sequence of probe S-* Nitrospira-0712-a-A-21 at the probe target site. All other known members of the phylum Nitrospira have full sequence complementarity with this probe at the target site.
‡This organism possesses mismatches to probe S-* Tdsulfo-0848-a-A-18 at the probe target site.

Abbreviations: env. environmental
of more efficient and stable nitrifying bioreactors as well as for the development of bioaugmentation strategies, which must be well-adapted to the bacterial population structures and the environmental conditions present in the treatment plants affected.

**Conclusions**

For optimal exploitation and modeling of the nitrite-oxidizing, yet uncultured *Nitrospira*-like bacteria in wastewater treatment, detailed knowledge of their physiology is urgently required. The combination of FISH and MAR allows the *in situ* investigation of key physiological parameters. First results obtained with this technique demonstrated that the *Nitrospira*-like bacteria, similar to the cultivated nitrite-oxidizer *N. moscoviensis*, are able to fix bicarbonate as carbon source. Additional MAR-FISH experiments with other substrates will almost certainly yield valuable information on other physiological traits of *Nitrospira*-like bacteria, for example whether they are able to grow mixotrophically or under oxygen-limited conditions. Such fundamental knowledge will be extremely useful for the design of suitable enrichment and isolation media for *Nitrospira*-like bacteria and will help us to more completely understand the microbiology of nitrification in sewage treatment.

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**References**


