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IN SITU ANALYSIS OF NITRIFYING BACTERIA IN SEWAGE TREATMENT PLANTS

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ABSTRACT

Autotrophic microbial nitrification is the key process in the removal of ammonia from wastewater. To avoid the limitations of traditional microbiological methods an *in situ* identification technique for ammonia- and nitrite-oxidizing bacteria was developed. Based on comparative sequence analyses we designed a collection of 16S ribosomal RNA-targeted oligonucleotide probes for all validly described members of the genus *Nitrobacter*. Whole cell hybridizations of target and reference cells with fluorescent probe derivatives were used to determine the optimal hybridization stringency for each of the probes. These probes were applied together with a recently developed probe for important members of the genus *Nitrosomonas* for simultaneous identification of ammonia- and nitrite-oxidizing bacteria in natural and engineered systems. Ammonia-oxidizing bacteria were identified *in situ* in river water, epiphytic biofilms from eutrophic wetlands, oligotrophic biofilms, a nitrifying trickling filter biofilm as well as in all analyzed nitrifying activated sludge samples. In none of these samples could *Nitrobacter* cells be detected *in situ*. However, all hitherto described *Nitrobacter* species and a strain of *Nitrobacter* sp. isolated from one of the analyzed nitrifying activated sludge samples showed bright hybridization signals with all *Nitrobacter* specific probes. Possible reasons for the absence of *in situ* detectable *Nitrobacter* cells are discussed.

KEYWORDS

Activated sludge; biofilm; *in situ* hybridization; scanning confocal laser microscopy; nitrification; *Nitrobacter*; *Nitrosomonas*; ribosomal RNA.

INTRODUCTION

Nitrification, the oxidation of ammonia to nitrate via nitrite, is an important step in the full treatment of wastewaters. It prevents the discharge of ammonium salts into receiving waters which leads to oxygen consumption and is more toxic to fish than nitrate (Painter, 1986). Furthermore it is an integral part for total N-removal from sewage via denitrification, an important prerequisite for limiting eutrophication. In the first step of nitrification, obligate autotrophic ammonia-oxidizing bacteria convert ammonia to nitrite, subsequently nitrite-oxidizing bacteria catalyze the oxidation of nitrite to nitrate. Nitrifying bacteria have slow growth rates and are sensitive to toxic shocks, pH- and temperature swings. This explains why many sewage treatment plants fail to establish stable nitrification. Specific and rapid identification methods for nitrifiers in activated sludge or trickling filter biofilms are necessary to learn more about mechanisms involved in loss of

nitrification. Nitrifying bacteria in activated sludge and natural environments have commonly been enumerated by a most probable number technique (MPN, e.g. Matulevich *et al.*, 1975) or selective plating (Ford *et al.*, 1980). These methods however require incubation times of up to several months and often show poor counting efficiencies (Belser, 1979).

Fluorescent *in situ* hybridization (FISH) with rRNA-targeted nucleic acid probes is a new molecular tool for rapid, reliable and cultivation-independent monitoring of phylogenetically defined bacterial populations in environmental samples (for a review, see Amann *et al.*, 1995). This technique was recently applied for direct quantitative analyses of the structure and dynamics of microbial populations in activated sludge. Comparison of the *in situ* probing results with the heterotrophic saprophyte flora obtained on different media demonstrated that traditional plate count techniques reflect much more the selectivity of a certain medium for distinct bacteria than the actual composition of the monitored microbial community (Wagner *et al.*, 1993, 1994a, Wagner and Amann, in prep.). Combining FISH with scanning confocal laser microscopy (SCLM) and digital image analysis made it possible to analyze the spatial distribution of bacteria in activated sludge flocs and to remove disturbing autofluorescent signals (Wagner *et al.*, 1994c). The optical sectioning properties of SCLM also significantly improved the *in situ* detectability of *Zoogloea ramigera* (Rosselló-Mora *et al.*, 1995) and of gram-negative filamentous bacteria (Wagner *et al.*, 1994b) in activated sludge.

Phylogenetic relationships of ammonia- and nitrite-oxidizing bacteria have been studied by comparative 16S rRNA sequencing (Head *et al.*, 1993, Orso *et al*, 1994, Teske *et al.*, 1994). Based on these results a 16S rRNA-targeted oligonucleotide probe for some lithoautotrophic ammonia-oxidizers was developed and successfully applied for the *in situ* detection of these bacteria in samples from sewage treatment plants (Wagner *et al.*, 1995). Here we describe the development of a set of probes for members of the genus *Nitrobacter*. Simultaneous application of these probes could yield important insights in the relative spatial distribution and abundance of nitrifying bacteria.

MATERIALS AND METHODS

Organisms, culture conditions and cell fixation. All *Nitrobacter* species were cultivated on sterile mineral medium as outlined by Bock *et al.* (1990). *Blastobacter denitrificans* LMG 8443^T, *Bradyrhizobium japonicum* LMG 6138^T, *Bradyrhizobium lupini* DSM 30140, *Rhodopseudomonas palustris* LMG 4314 were grown as recommended in the catalogue of strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), and from the Laboratorium voor Microbiologie of the Universiteit Gent (Ghent, Belgium). Cells were fixed from cultures of heterotrophic bacteria and nitrite-oxidizers in the logarithmic growth phase with paraformaldehyde as previously reported (Amann *et al.*, 1990b).

Enrichment and isolation of nitrite oxidizing bacteria. A nitrite oxidizer was enriched and isolated from a sample of the plant at Kraftisried (Germany) according to Bock *et al.* (1990). Purity of the nitrite-oxidizing isolated strain was checked by inoculation of an organic culture medium (containing 0.5 g yeast extract, 0.5 g peptone, 0.5g beef extract and 0.584g NaCl per litre; pH 7.4) and by microscopic phase contrast observation.

Sampling. Grab samples of mixed liquor were collected from the aeration stage of a treatment plant receiving wastewater from an animal waste processing facility (Tierkörperbeseitigungsanstalt Kraftisried, Kraftisried, Germany; 6,000 PE, [1 PE = 60 g biological oxygen demand d^{-1}). In addition, samples were collected from the aeration stages of the municipal wastewater treatment plants München I (Großlappen, Germany; stage 1 and 2; 1 million PE); München II (Gut Marienhof, Germany; stage 1 and 2; 1 million PE), Freising (Germany; 110,000 PE), Grüneck (Germany; stage 1; 100,000 PE), Gersthofen (Germany; 45,000), Moosburg (Germany; 34,000 PE), Hirblingen (Germany; 30,000 PE) and Haag (Germany; 3,000 PE). Biofilms of a trickling filter (Grüneck, Germany, stage 2, 100,000 PE) were recovered on glass slides placed for 1 to 10 days in a vertical position between the lava rocks of the trickling filter at a depth of about 30 cm. The water sample of river Elbe was taken in the harbour basin Teufelsbrück (Germany). At this location effluent of a municipal sewage treatment plant is released into the river Elbe. For *in situ* hybridization river water, activated sludge samples and glass slides with attached biofilm were fixed with 4% paraformaldehyde as described by Amann *et al.*, (1990b).

Nitrifying drinking water biofilms were sampled from annular reactors fed with Sydney drinking water amended with monochloramine to normal disinfectant levels of 1.7 mg l^{-1} (located at laboratories of AWT-Ensight; research division of Sydney Water). The annular reactors were inoculated by adding 5-10g of sediment flushed from drinking water pipes. Biofilms were grown in the reactor for 4 weeks on removable polycarbonate slides. Polycarbonate slides were removed from the reactor and fixed with fresh 4% paraformaldehyde for 1h. After this procedure the biofilm side of the slide was covered in Leica cryo-embedding media and placed in the -80°C freezer. When frozen, the slide was flexed to snap of the frozen biofilm. The base of the frozen biofilm was then covered in embedding media and frozen again at -80°C. 10 µm thick sections of the embedded biofilm were obtained with a Reichert-Jung Cryostat at -24°C. Sections were finally placed onto microscope slides coated in 3-aminopropylsilane (Sigma, Deisenhofen, Germany) and stored at -50°C.
Epiphytic biofilms from wetland reeds were sampled from a constructed wetland system receiving secondary treated municipal sewage (located adjacent to the sewage treatment plant at Richmond, Australia). At the sampling time the system had been running for two years. The submerged region of a *Phragmites* sp. plant stem was cut with scissors and placed into a centrifuge tube filled with the trench water for transport to the laboratory. Concentrated fresh paraformaldehyde solution was added to achieve a final concentration of 4% paraformaldehyde. Samples were fixed for 1h at 4°C. Then the stem was removed and a sliver was cut from the stem. The sliver was embedded in cryo-embedding media. At no time during the whole sampling was the biofilm exposed to air. The embedded piece was frozen at -80°C and sectioned in a Reichert-Jung cryostat at -24°C. 10 µm thick sections were placed on microscopic slides coated in 3-aminopropylsilane and stored at -50°C.

Oligonucleotide probes. The following 16S rRNA-targeted oligonucleotides were used: (i) NIT1 5′-CACCTCTCCCGAACTCAA-3′ complementary to a region characteristic for some members of the alpha-subclass of *Proteobacteria* including all *Nitrobacter* species, (ii) NIT2 5′-CGGGTTAGCGCACCGCCT-3′ and NIT3 5′-CCTGTGCTCCATGCTCCG-3′ complementary to a region of all hitherto sequenced *Nitrobacter* species, (iii) CNIT3 5′- CCTGTGCTCCAGGCTCCG-3′ complementary to a region of *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, *Afipia clevelandis* and *Afipia felis*, (iv) NEU, complementary to a region of some litoautotrophic ammonia-oxidizing bacteria (Wagner *et al.*, 1995), (v) CTE, complementary to a region of *Comamonas testosteroni*, *Brachymonas denitrificans*, *Rhodocyclus purpureus* and *Leptothrix discophora* (Schleifer *et al.*, 1992), (vi), EUB, complementary to a conserved region of the 16S rRNA of all bacteria (Amann *et al.*, 1990a). Oligonucleotides were synthesized with a C6-TFA aminolinker [6-(trifluoracetylamino)-hexyl-(2-cyanoethyl)-(N,N-diidopropyl)phosphoramidite] at the 5' end (MWG Biotech, Ebersberg, Germany). Labeling with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) and with the hydrophilic sulphoindocyanine dye CY3 (monofunctional CY3.29-OSu; Biological Detection Systems, Pittsburgh, Pa.) and purification of the oligonucleotide-dye conjugates was performed as described by Amann *et al.*, (1990a). For labeling with the CY3 dye the fluorochrome was suspended in a 1:1 mixture of 200 mM sodiumcarbonate buffer (pH 9.0) and dimethyl formamide.

In situ hybridization. Optimal hybridization conditions were determined for probes NIT1, NIT2 and NIT3. using the hybridization buffer and procedure described by Manz *et al.* (1992). Optimal hybridization stringency required the addition of formamide to a final concentration of 30% (probe NIT1) or 40% (probes NIT2, NIT3). The specificity of probe NIT3 was further enhanced by addition of an equimolar amount of unlabeled probe CNIT3 as competitor oligonucleotide. Specific hybridization with probe NEU required the addition of formamide to a final concentration of 40% to the standard hybridization buffer and addition of an equimolar amount of unlabeled oligonucleotide CTE as a competitor (Wagner *et al.*, 1995). All hybridizations were performed at a hybridization temperature of 46°C. A stringent washing step was performed for 10min at 48°C in a buffer containing 56 mM NaCl, 20 mM Tris/HCl (pH 8.0) and 0.01% SDS.

Microscopy. Slides were examined with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with Zeiss filter sets 09, 15 (Carl Zeiss) and HQ-CY3 filter set (Chroma Tech. Corp., Brattleboro, Vt, USA). Black and white photomicrographs were taken with Kodak Tmax 400 film. Exposure times were 0.06 s for phase contrast micrographs and 15 to 30 s for epifluorescence micrographs. Scanning confocal laser microscopy was performed using a Zeiss LSM 410 as described elsewhere (Wagner *et al.*, 1994c).

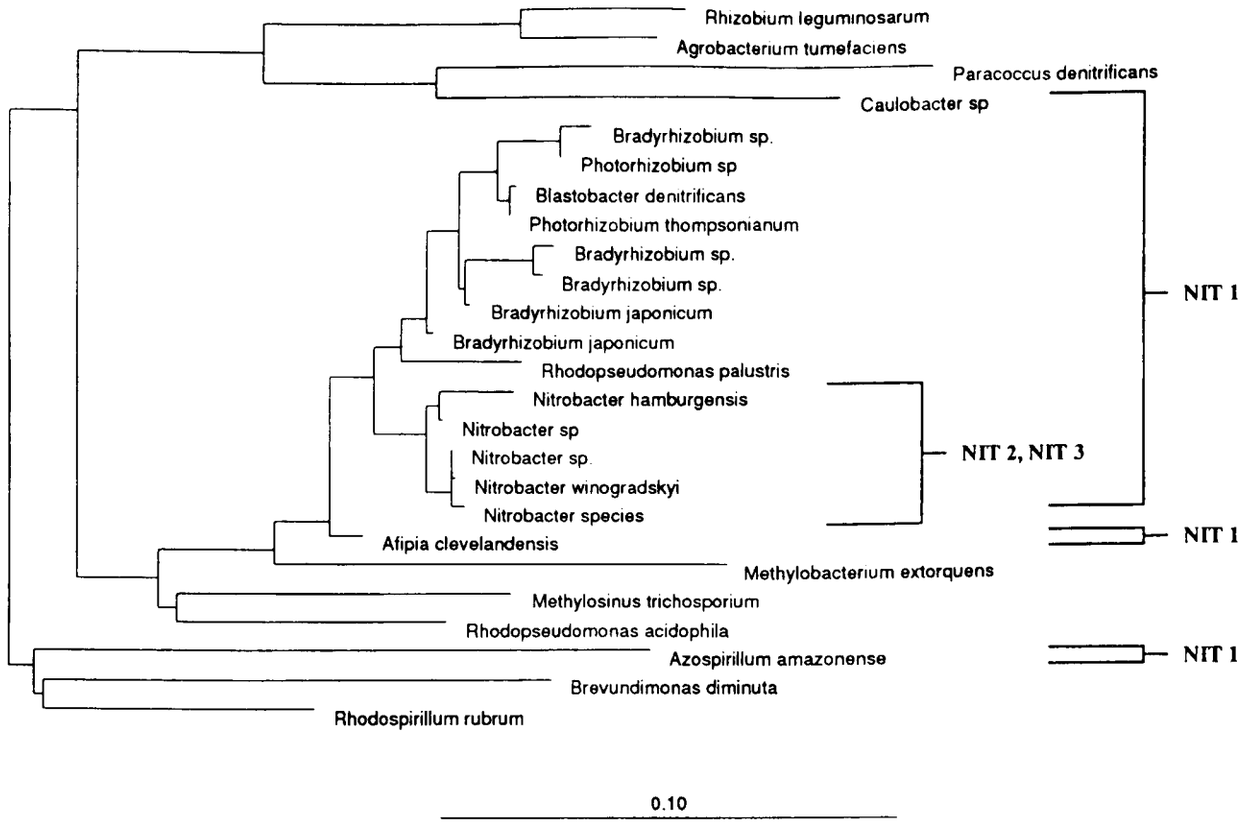

Fig. 1. Phylogenetic tree inferred from comparison of the 16S rRNA sequences of some characterized members of the alpha-subclass of *Proteobacteria*. The bar represents 0.1 estimated changes per nucleotide position. Target organisms for probes NIT1, NIT2 and NIT3 are indicated by brackets.

RESULTS AND DISCUSSION

Design and optimization of probes for *Nitrobacter*

Bacteria of the genus *Nitrobacter* belong to the alpha-2 branch of *Proteobacteria*. They are closely associated with *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Afipia clevelandensis*, *Afipia felis* and *Blastobacter denitrificans* (e.g. Seewaldt *et al.*, 1982, Willems and Collins, 1992, Orso *et al.*, 1994, Teske *et al.*, 1995). By comparative analysis of the 16S rRNA sequences of all hitherto sequenced *Nitrobacter* strains (Orso *et al.*, 1994; Teske *et al.*, 1994) with all other accessible 16S rRNA sequences (3,000 complete or almost complete sequences, [Maidak *et al.*, 1994; VandePeer *et al.*, 1994]) we designed a set of oligonucleotide probes. Probe NIT1 is complementary to *E. coli* pos. 652-669 of the 16S rRNA of some bacteria of the alpha-subclass of *Proteobacteria* (see Figure 1) including all *Nitrobacter* strains. In addition probes NIT2 and NIT3, which are complementary to *E. coli* pos. 1434-1451 and 1035-1048, respectively, of the 16S rRNA of all *Nitrobacter* strains included in the databases, were developed (Figure 1). A recent database check (June 1995) showed that all non-target bacterial 16S rRNA sequences had at least one mismatch with the NIT2 and NIT3 probe sequences. The difference alignments in Table 1 display locations of mismatches in the 16S rRNA sequence of some phylogenetically closely related bacteria.

CY3-labeled derivatives of probes NIT1, NIT2 and NIT3 were used for whole cell hybridization with *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*. All *Nitrobacter* cells showed

strong hybridization signals with all probes. In a negative control experiment all *Nitrobacter* reference cells were hybridized with a CY3-labeled ammonia-oxidizer probe NEU. No unspecific binding of probe NEU to *Nitrobacter* cells was observed. Consequently, (i) the rRNA molecules in fixed *Nitrobacter* cells were accessibile for *in situ* hybridization, (ii) the examined *Nitrobacter* species contained relative high levels of rRNA despite their low growth rates and (iii) the recently described *Nitrobacter vulgaris* (Bock *et al.*, 1990), for which the 16S rRNA sequence has not yet been determined, is also detected by the developed probes.

Probe NIT1 binds not only to *Nitrobacter*, but is also complementary to other bacteria. It should only be used as an indicator for potential presence of these nitrite-oxidizing bacteria. The specificities of the more specific probes NIT2 and NIT3 were further evaluated by hybridization to mixtures of whole fixed cells of selected reference strains. Hybridization of the nonnitrite-oxidizing bacteria *Blastobacter denitrificans* and *Bradyrhizobium japonicum* with probe NIT2 could not be prevented by applying the highest possible hybridization stringency (40% formamide in the hybridization buffer). The 16S rRNA target sites of *Blastobacter denitrificans*, *Bradyrhizobium japonicum* and some other members of the alpha-subclass of *Proteobacteria* have only single marginal mismatches to the target sequence of probe NIT2 (Table 1). In contrast to NIT2 probe NIT3 hybridized at high stringency exclusively to members of the genus *Nitrobacter*. Binding of NIT3 to *Bradyrhizobium japonicum, Bradyrhizobium lupini, Rhodopseudomonas palustris*, which all have one central T-C mismatch within the target site of probe NIT3, was prevented by the addition of 40% formamide and an equimolar amount of competitor oligonucleotide CNIT3 to the hybridization buffer (Figure 2, panel A). Hybridization with FLUOS-labeled probe EUB served as positive control for probe permeability and rRNA content of fixed cells.

TABLE 1. DIFFERENCE ALIGNMENTS OF THE 16S rRNA TARGET REGIONS OF PROBES NIT2 AND NIT3

Organisms	Target of probe NIT2
Nitrobacter hamburgensis	5´-AGGCGGUGCGCUAACCCG-3´
Nitrobacter winogradskyi	5´-.....-3´
Blastobacter denitrificans	5´-.....A.-3´
Bradyrhizobium japonicum	5´-..A.....-3´
Blastobacter natatorius	5´-.....C.....-3´

Organisms	Target of probe NIT3
Nitrobacter hamburgensis	5´-CGGAGCAUGGAGCACAGG-3´
Nitrobacter winogradskyi	5´-.....-3´
Bradyrhizobium lupini	5´-.....C.....-3´
Bradyrhizobium japonicum	5´-.....C.....-3´
Rhodopseudomonas palustris	5´-.....C.....-3´

In situ monitoring of *Nitrosomonas* and *Nitrobacter* in natural and engineered environments

The abundance of *Nitrosomonas* and *Nitrobacter* was analyzed in samples from (i) 9 sewage treatment plants, (ii) river Elbe, (iii) a nitrifying oligotrophic drinking water biofilm and (iv) an epiphytic biofilm on reed stems grown in constructed wetlands (Table 2). Ammonia-oxidizers could be detected *in situ* with probe NEU in all samples from sewage treatment plants with nitrifying activity (Wagner *et al*, 1995), in river Elbe water and in the oligotrophic and epiphytic biofilms (Figure 2, panel B). The specifically stained ammonia-oxidizers in the oligotrophic and epiphytic biofilms were clustered in microcolonies formed by rod-shaped bacteria. They strongly resembled the clusters of ammonia-oxidizing bacteria observed in the activated sludge and trickling filter biofilms. The evidence that ammonia-oxidizing bacteria are able to grow in epiphytic biofilms on submerged reed stems points to the importance of plants for nitrification in wetlands. In contrast to all other

samples only non-clustered single ammonia-oxidizers were detected in the aggregates collected from the river Elbe.

TABLE 2. PRESENCE OF NITRIFIERS IN VARIOUS ENVIRONMENTS AS DETECTED BY *IN SITU* HYBRIDIZATION

Studied environment	nitrification observed	probe NIT1	probe NIT2	probe NIT3	probe NEU
river Elbe aggregates	ND	-	-	-	+
drinking water biofilm	+	-	-	-	+
epiphytic biofilm	+	-	-	-	+
Freising	+	-	-	-	+
Gersthofen	+	-	-	-	+
Grüneck	-	-	-	-	-
Grüneck, trickling filter	+	ND	ND	ND	+
Haag	+	+	-	-	+
Hirblingen	+	-	-	-	+
Kraftisried	+	+	-	-	+
Moosburg	+	-	-	-	+
München I stage 1	-	+	-	-	-
München I stage 2	+	-	-	-	+
München II stage 1	-	+	-	-	-
München II stage 2	+	-	-	-	+

ND Not determined

All samples were additionally hybridized with probes NIT1, NIT2 and NIT3 for *in situ* detection of *Nitrobacter*. Surprisingly only probe NIT1 stained cells in some of the samples. No hybridization signals could be observed using probes NIT2 and NIT3 in any of the samples (Table 2). In contrast, the isolation of a *Nitrobacter* strain (Nb4) from one of the examined activated sludge samples (Kraftisried, Germany) that hybridized with all *Nitrobacter* probes clearly demonstrated that some cells binding NIT2 and NIT3 were present in this plant.

There are several possible reasons for the failure of FISH to identify *Nitrobacter* cells in the examined samples. Firstly, actual cell numbers could be below 10^4 ml^{-1}, the detection limit of this technique (Amann *et al.*, 1995). In such low concentrations, members of the genus *Nitrobacter* could not be responsible for a major part of nitrite-oxidation in sewage treatment plants. Secondly, the cellular rRNA content of the *Nitrobacter* cells in the sample could be too low for *in situ* identification. Due to the fact that physiological active *Nitrobacter* cells possess high amounts of rRNA per cell (see above) a low cellular rRNA content would indicate a low physiological cell activity (Schaechter *et al.*, 1958; DeLong *et al.*, 1989). Thirdly, the *Nitrobacter* cells in the samples could be impermeable to probes. This is quite unlikely since all examined pure cultures of *Nitrobacter* were nicely permeabilized by the fixation procedure used in this study. Therefore, most probably, nitrite-oxidizing bacteria other than *Nitrobacter* could be present in higher numbers and/or activity in the examined samples. These nitrite-oxidizers could be members of of the genera *Nitrospina* (Watson and Waterbury, 1971), *Nitrococcus* (Watson and Waterbury, 1971) and *Nitrospira* (Watson *et al.*, 1986), heterotrophic nitrite-oxidizing bacteria or new still unknown autotrophic nitrite-oxidizers. The last possibility was already suggested by Watson *et al.* (1981): "Additional species of nitrifying bacteria probably exist that have not been either cultured, isolated, or described, since relatively few investigators have isolated nitrifying bacteria". Probes for *Nitrospina*, *Nitrococcus* and *Nitrospira* are under development. Future studies using these probes for FISH will help reveal more about nitrification as it occurs in nature.

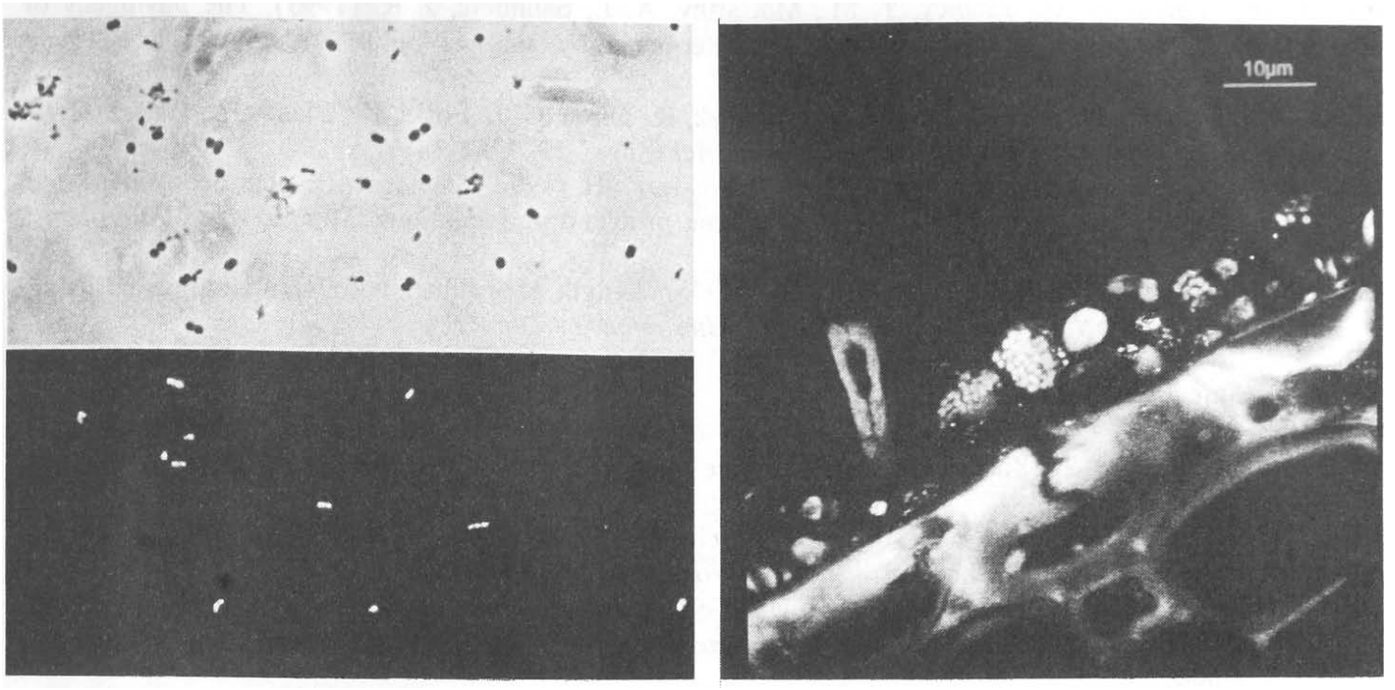

Fig. 2. Whole cell identification of *Nitrobacter* sp. and ammonia-oxidizers. (A) Artificial mixture of fixed cells of *Rhodopseudomonas palustris* and *Nitrobacter hamburgensis*. hybridized with CY3-labeled probe NIT3. An identical field was viewed by phase contrast microscopy (top) and epifluorescence microscopy (bottom). (B) 2-d SCLM-image of ammonia-oxidizing cells growing in an epiphytic biofilm on reed stems, specifically stained with CY3-labelled probe NEU.

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