Microbial Ecology

Genera-Specific Immunofluorescence Labeling of Ammonia Oxidizers with Polyclonal Antibodies Recognizing Both Subunits of the Ammonia Monooxygenase

C. Fiencke and E. Bock

Institut für Allgemeine Botanik, Universität Hamburg, D–22609 Hamburg, Germany

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Abstract

Polyclonal antibodies that recognize the two subunits AmoA and AmoB of the ammonia monooxygenase (AMO) were applied to identify ammonia-oxidizing bacteria by immunofluorescence (IF) labeling in pure, mixed, and enriched cultures. The antibodies against the AmoA were produced using a synthetic peptide of the AmoA of *Nitrosomonas eutropha*, whereas the antibodies against the AmoB had been developed previously is against the whole B-subunit of the AMO [Pinck et al. (2001) Appl Environ Microbiol 67:118–124]. Using IF labeling, the AmoA antibodies were specific for the detection of all species of the genus *Nitrosomonas*. In contrast, the antiserum against AmoB labeled all genera of ammonia oxidizers of the β-subclass of Proteobacteria (*Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*). The fluorescence signals of the AmoA antibodies were spread all over the cells, whereas the signals of the AmoB antibodies were associated with the cytoplasmic membranes. The specificity of the reactions of the antisera with ammonia oxidizers were proven in pure and mixed cultures, and the characteristic IF labeling and the morphology of the cells enabled their identification at the genus level. The genus-specific IF labeling could be used to identify ammonia oxidizers enriched from various habitats. In enrichment cultures of natural sandstone, cells of the genera *Nitrosomonas*, *Nitrosovibrio*, and *Nitrosospira* were detected. Members of the genus *Nitrosovibrio* and *Nitrosolobus* were most prominent in enriched garden soil samples, whereas members of the genus *Nitrosomonas* dominated in enriched activated sludge. The antibodies caused only slight background fluorescence on sandstone and soil particles compared to oligonucleotide probes, which could not be used to detect ammonia oxidizers on these materials because of strong nonspecific fluorescence.

Correspondence to: C. Fiencke; E-mail: Claudia@Fiencke.de

Introduction

Ammonia-oxidizing bacteria play an essential part in the cycling of reduced nitrogen compounds throughout marine, freshwater, and soil environments. Chemolithoautotrophic bacteria convert ammonia to nitrite by two key enzymes and assimilate CO_2 as the major carbon source. The first enzyme, the membrane-bound ammonia monooxygenase (AMO) catalyzes the oxidation of ammonia to hydroxylamine [22, 59], which is then further oxidized to nitrite by hydroxylamine oxidoreductase (HAO) [3, 46]. Two proteins were sequenced and described as possible subunits of the AMO, a 27 kDa sized membrane-associated protein (AmoA) containing, most likely, the active site of the AMO [23, 24] and a protein of 41 kDa (AmoB) [8, 33].

All known ammonia oxidizers are members of two lineages within the β- and γ-subclasses of Proteobacteria according to comparative 16S rRNA sequence analysis [30]. The marine species of the genus *Nitrosococcus* cluster within the γ-subclass of Proteobacteria [58]. Members of the four other genera *Nitrosomonas* (including *Nitrosococcus mobilis*, which belongs phylogenetically to *Nitrosomonas*), *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*, form a monophyletic assemblage within the β-subclass of the Proteobacteria [57]. The ammonia oxidizers have been divided into separate genera based on phenotypic characteristics, in particular cell shape and the arrangement of intracytoplasmic membranes (ICMs) [29]. The coccoid cells of the marine *Nitrosococcus* species have a centrally located stack of flattened ICMs, whereas the coccoid and rod-shaped cells of *Nitrosomonas* possess paired membranes running along the periphery of the cells. The pleomorphic lobes of *Nitrosolobus* are divided

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into cell compartments by cytomembranes [54]. No ICMs are found in the spiral and slender curved rods of members of the genera *Nitrosospira* and *Nitrosovibrio*, respectively [18]. Irrespective of the gross morphological differences of the genera, it has been suggested [19] and subsequently questioned [47] that the genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* should be reclassified in the single genus *Nitrosospira* because of the high levels of homology in their 16S rRNA.

The presence of certain species of ammonia oxidizers within any given environmental niche, e.g., wastewater treatment plant or agricultural soil, gives evidence of environmental conditions. However, the detection and identification of these bacteria is difficult because of their slow growth rate and aggregation to biofilms [28, 43]. Traditional counting methods like the most-probable-number (MPN) technique [31] are time-consuming and often underestimate the numbers of ammonia oxidizers in the natural environment [7, 27]. Antibodies or 16S rRNA-targeted oligonucleotide probes are used for *in situ* analyses in order to avoid the limitations of the MPN technique. In previous studies antibodies had been developed using whole cells of ammonia oxidizers that recognize epitopes of the cell wall [6, 36, 41, 42, 48, 52]. These antibodies were applied in ecological studies to detect and count ammonia oxidizers in bacterial communities using fluorescence microscopy, and 10 to 1000-fold greater cell numbers were found compared to MPN techniques [27, 48, 51]. However, this approach is not entirely culture-independent because ecologically relevant strains have to be isolated prior to antibody development. As ammonia oxidizers show high serological diversity within even one genus [6, 52], unculturable bacteria are consequently not detectable using antibodies raised against whole cells, and a variety of antibodies are required. In contrast to antibodies to cell envelopes, antibodies to key enzymes of N-cycling bacteria such as ammonia oxidizers [38], nitrite oxidizers [1, 4, 5], and denitrifying bacteria [10, 11, 26, 53] could be successfully applied for the detection of these bacteria in culture-independent approaches.

In this study, polyclonal antibodies were produced against the AmoA subunit of the AMO. The specificity of AmoA antibodies was tested using immunoblotting of pure cultures of members of all genera of ammonia oxidizers, and immunofluorescence (IF) labeling with the AmoA antibodies and the previously described AmoB antibodies [38] was developed to visualize and identify ammonia oxidizers in pure, mixed, and enrichment cultures.

Materials and Methods

Bacterial Strains and Culture Conditions. All bacterial strains used in this study are listed in Table 1. The 21 strains of ammonia oxidizers and 7 strains of nitrite oxidizers were derived from soil, sewage, a biowaste fermenter, cattle manure, a heating system, sandstone of historical buildings and sea water (for more details see [38]). All strains including the methane oxidizers *Methylococcus capsulatus* Bath, *Methylomonas methanica* Oo52006, and *Methylocystis parvus* 4a and the chemoorganotrophic bacteria *Bacillus subtilis* 019, *Escherichia coli* K12/067 (ATCC 23716), *Methylobacterium radiotolerans*, *Paracoccus denitrificans* 001 (ATCC 19367), and *Pseudomonas* sp. AM1 are stored in the culture collection of the Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg. All other strains were obtained from C. Coeur (University of Lyon I, Villeurbanne, France).

Terrestrial and freshwater ammonia oxidizers were grown at 28°C in mineral salts medium [28] in the presence of 10 mM ammonium. *Nitrosomonas cryotolerans* Nm 55, *Nitrosomonas halophila* Nm 1, and *Nitrosococcus mobilis* Nc 2 were grown in the same medium containing 10 g NaCl L^{-1}. *Nitrosomonas marina* Nm 22, *Nitrosococcus oceani* Nc 1, and *Nitrosococcus halophilus* Nc 4 were cultivated in seawater medium of the following composition: 10 mM NH_4Cl, 0.4 mM KH_2PO_4, 3 g HEPES, and 1 mL 0.05% (w/v) cresol red solution per liter of 40% seawater.

Nitrobacter hamburgensis X_{14}, *Nitrobacter winogradskyi* Engel, and *Nitrobacter vulgaris* K_{48} were grown mixotrophically in the presence of 2 g of $NaNO_2$ L^{-1} [9]. *Nitrospira moscoviensis* M-1 was cultivated in mineral medium with 0.2 g of $NaNO_2$ L^{-1} [13]. *Nitrospina gracilis* 3, *Nitrospina* sp. 347, and *Nitrococcus mobilis* 231 were cultivated in seawater media according to Watson and Waterbury [55]. The cultures were incubated at 28°C expect *Nitrospira moscoviensis* M-1, which was incubated at 37°C.

The methane oxidizers were cultivated in nitrate mineral salts medium [56] containing 0.25 μM $CuSO_4$ with a 3% methane synthetic air atmosphere. The methylotrophs were grown in mineral medium with 0.15% (w/v) methanol [15]. All other bacterial strains were cultivated according to the instructions of the American Type Culture Collection (ATCC).

Enrichment Cultures. For the enrichment of ammonia oxidizers, activated sludge, ground natural sandstone, and garden soil were added to mineral salts medium [28] containing 10 mM ammonium and incubated for 2 weeks at 28°C. The activated sludge samples originated from the aeration stage of the sewage treatment plant in Dradenau near Hamburg, Germany, and had a pH of 8.0. Garden soil samples with a pH of 7.6 were obtained from the new botanical garden in Hamburg, Germany. Stone samples were taken from specially cut specimens (5 × 5 × 1 cm) from Baumberger sandstone, which had been exposed for 5 years in the new

Table 1. Reactivity of AmoA- and AmoB-antibodies and 16S rRNA probes with ammonia oxidizers, methane oxidizers, nitrite oxidizers and different chemoorganotrophic bacteria by using immunoblot, immunofluorescence, and FISH techniques

		Immunoblot		*IF labeling*		*FISH*			
Species	*Strain*	*AmoA-Abs*	*AmoB-Abs*	*AmoA-Abs*	*AmoB-Abs*	*EUB 338*	*Nso 190*	*NEU*	*Nsv 443*
Ammonia oxidizers of the β-subclass of Proteobacteria									
Nitrosomonas communis	Nm 2	−	+	+	+	+	−[40]	−[40]	−[40]
Nitrosomonas cryotolerans	Nm 55	−	+	+/−	+	+	+[40]	−[40]	−[40]
Nitrosomonas europaea	Freitag	+	+	+	+	+	+	+	−
Nitrosomonas eutropha	N904	+	+	+	+	+	+	+	−
Nitrosomonas eutropha	Dave	+	+	+	+	+	n.t.	n.t.	n.t.
Nitrosomonas halophila	Nm 1	+	+	+	+	+	+[40]	+[40]	−[40]
Nitrosomonas marina	Nm 22	−	+	+/−	+	+	+[40]	−[40]	−[40]
Nitrosomonas nitrosa	Nm 90	−	+	+	+	+	−[40]	−[40]	−[40]
Nitrosomonas oligotropha	Nm 45	−	+	+	+	+	−[40]	−[40]	−[40]
Nitrosomonas sp.	Nm R1.24	−	+	+	+	+	−	−	−
Nitrosomonas ureae	Nm 10	−	+	+	+	+	−[40]	−[40]	−[40]
Nitrosococcus mobilis	Nc 2	−	+	−	+	+	−[40]	−[40]	−[40]
Nitrosospira sp.	Nsp 1	−	+	−	+	+	+	−	+
Nitrosospira sp.	Nsp G1.6	−	+	−	+	+	+	−	−
Nitrosospira sp.	Nsp M1.3	−	+	−	+	+	+	−	−
Nitrosospira sp.	Nsp R6.2	−	+	−	+	+	+	−	−
Nitrosovibrio sp.	Nv G1.3	−	+	−	+	+	n.t.	n.t.	n.t.
Nitrosovibrio sp.	Nv K7.1	−	+	−	+	+	n.t.	n.t.	n.t.
Nitrosolobus multiformis	Nl 13	−	+	−	+	+	+[40]	−[40]	+[40]
Ammonia oxidizers of the γ-subclass of Proteobacteria									
Nitrosococcus halophilus	Nc 4	−	−	−	+/−	n.t.	n.t.	n.t.	n.t.
Nitrosococcus oceani	Nc 1	−	−	−	+/−	n.t.	n.t.	n.t.	n.t.
Methane oxidizers									
Methylococcus capsulatus	Bath	−	−	−	+/−	n.t.	n.t.	n.t.	n.t.
Methylomonas methanica	Oo52006	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Methylocystis parvus	4a	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrite oxidizers									
Nitrobacter hamburgensis	X_{14}	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrobacter vulgaris	K_{48}	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrobacter winogradskyi	Engel	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrospina gracilis	3	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrospina sp.	347	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrospira moscoviensis	M-1	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Nitrococcus mobilis	231	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Chemoorganotrophic bacteria									
Agrobacterium tumefaciens	GM 19023	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Alcaligenes faecalis	ATCC 8750	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Azorhizobium sp.	24	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Azospirillum lipoferum	ATCC 29707	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Bacillus azotoformans	ATCC 29788	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Bacillus subtilis	019	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Escherichia coli	ATC 23716	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Methylobacterium radiotolerans	ATC 19367	−	−	−	−	n.t.	n.t.	n.t.	n.t.
Pseudomonas sp.	AK15	−	−	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Pseudomonas sp.	AM1	−	−	−	−	n.t.	n.t.	n.t.	n.t.

+ = bright reaction, +/− = slight reaction, − = no reaction.
Abs: antibodies; n.t.: not determined.
[40] results of Purknold et al. (2000).

botanical garden of Hamburg, Germany. After this exposure, the sandstones were covered with green algae biofilms and other microorganisms and showed visible signs of weathering. The sandstone had a pH of 8.4 and was ground by pestle and mortar.

Development of Antibodies. A 15-residue synthetic peptide corresponding to the AmoA N-terminal peptide sequence of *Nitrosomonas eutropha* Nm 57 was produced and conjugated to keyhole limpet hemocyanin (KHL)-protein (Biotrend, Cologne, Germany). The

SwissProt accession numbers for *N. eutropha* Nm 57 *amoA1* and *amoA2* are U51630 and U72670, respectively. Antiserum against this synthetic peptide was raised in rabbits (Biotrend). AmoB-antibodies were recently produced by Pinck et al. [38] using the whole subunit of *Nitrosomonas eutropha* N904 for immunization of chickens.

Immunoblotting. Protein extracts of pure cultures (1.5 mg protein mL^{-1}) were separated by SDS-PAGE, electroblotted onto a cellulose nitrate membrane using a discontinuous buffer system, and subsequently applied to the immunostaining. The 27-kDa AmoA protein and the 41-kDa AmoB protein were detected in separate immunoblots. Alkaline phosphatase conjugated anti-rabbit antibodies (Sigma) and alkaline phosphatase conjugated anti-chicken antibodies (Sigma) were used as secondary antibodies for the detection of AmoA and AmoB antibodies, respectively. For the enzyme reaction 0.005% 5-bromo-4-chloro-3-indolyl phosphate and 0.001% 4-nitro-blue-tetrazolium was employed. The detailed procedures of SDS-PAGE, electroblotting, and immunostaining were given by Pinck et al. [38].

IF Labeling. For IF labeling cells of pure and enrichment cultures were harvested by centrifugation and stored overnight in phosphate-buffered saline (PBS)–ethanol (1:1) at −20°C. The samples were placed on slides and dehydrated by using 50, 80, and 96% ethanol (3 min each) [17]. The cells were then treated with lysozyme to enhance the permeation of the antibodies [16]. All samples were blocked with PBS containing 3% bovine serum albumin (BSA) for 30 min at room temperature. The samples were then simultaneously incubated overnight with the AmoA- and AmoB-antibodies diluted 1:10 in PBS containing 0.05% BSA and 0.025% Tween 20. Afterward, the cells were incubated with secondary antibodies diluted 1:100 in PBS containing 0.05% BSA and 0.025% Tween 20 for 1 h in the dark at room temperature. The secondary antibodies were Cy2-labeled anti-rabbit (Biotrend) and Cy3-labeled anti-chicken antibodies (Biotrend) for binding AmoA and AmoB antibodies, respectively. The reactions were stopped by washing the slides in PBS. Control preparations without primary antibodies were included in every experiment. Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) (10 μg mL^{-1}) for 5 min to detect total cells.

FISH. For fluorescence *in situ* hybridization (FISH) cells were fixed in 3% formaldehyde for 1 h on ice and stored in PBS–ethanol (1:1) at −20°C. The samples were placed on slides and dehydrated by using 50, 80, and 96% ethanol (3 min each) [17]. Afterward, they were incubated with the 16S rRNA directed oligonucleotide probe EUB338, Nso 190, NEU, or Nsv 443 in hybridization buffer (0.9 M NaCl, 20 mM M Tris/HCl pH 7.2, 0.01% SDS) with a final concentration of 30% formamide (EUB338, Nsv 443) or 40% formamide (NEU, Nso 190) at a temperature of 46°C for 2 h. As a competitor for NEU, an equimolar amount of unlabeled oligonucleotide CTE was added. Probe EUB338 was used to detect members of the domain Bacteria [12], and probe Nso 190 detects all ammonia oxidizers of the β-subclass of Proteobacteria [35] except some *Nitrosomonas strains* (Table 1, [40]). Probe NEU is specific for the detection of only a few members of the genus *Nitrosomonas* (Table 1 [40, 50]). Probe Nsv 443 is directed to *Nitrosolobus multiformis*, *Nitrosospira briensis* and *Nitrosovibrio tenuis* (Table 1 [35, 40]). The probes were labeled with the fluorochrome Cy3 (MWG-Biotech AG). The samples were then incubated 20 min at 48°C in washing buffer (50 mM NaCl, 20 mM Tris/HCl pH 7.2, 0.01% SDS). Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) (10 μg mL^{-1}) for 5 min to detect total cells.

Fluorescence Microscopy and Confocal Laser Scanning Microscopy. DAPI stained cells were visualized by using Leica filter set A (BP 340–380 exc.; RKP 400; LP 425 em.). The Cy2 labeling of secondary AmoA antibodies and the Cy3 labeling of secondary AmoB antibodies and 16S rRNA probes were visualized by using Leica filter set I 3 (BP 450–490 exc.; RKP 510; LP 515 em.) and Leica filter set N 2.1 (BP. 515–560 exc.; RKP 580; LP 590 em.), respectively.

IF labeling and FISH were additionally visualized with a confocal laser scanning microscope (CLSM) (model TCS 4D; Leica); excitation was supplied by an argon–krypton laser (488/568 exc.; LP 590, BP 520–560 em.). Image processing was performed with standard software (Scanware 5.1; Leica). No or only slight autofluorescence of biofilm, soil, or stone particles was found in the absence of fluorescent antibodies or probes.

Electron Microscopy. The methods used for cell fixation, embedding, ultrathin sectioning, and shadow casting were the methods described by Ehrich et al. [13]. Electron microscopy was performed with a Philips model 420 transmission electron microscope.

Results

Immunoblotting. Polyclonal antibodies were produced against a synthetic AmoA peptide, and their specificity was tested by immunoblotting of crude extracts. Various strains were investigated including 21 ammonia oxidizers, three methane oxidizers, seven nitrite oxidizers, and 14 strains of different chemoorganotrophic bacteria, which are all summarized in Table 1. In immunoblot studies the AmoA antibodies were highly specific for the detection of a 27-kDa protein in cell extracts of *Nitros-*

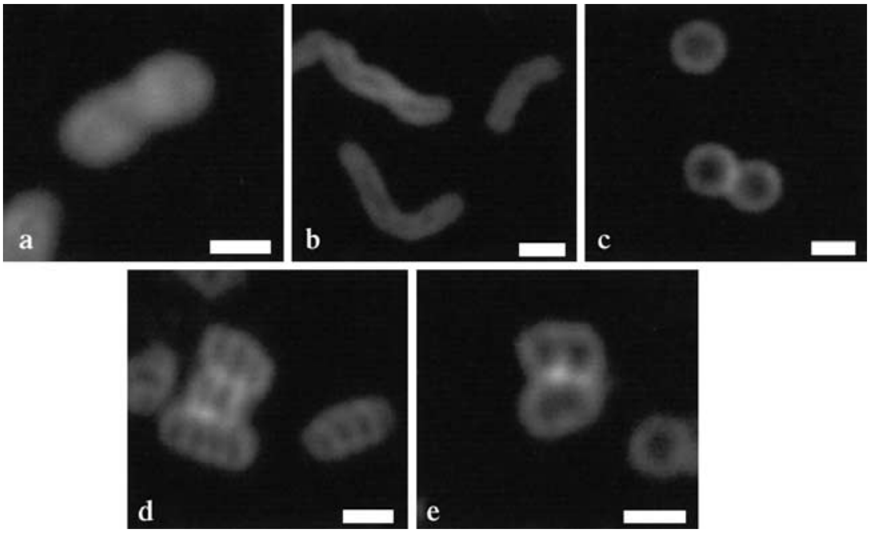

Figure 1. IF labeling with AmoA and AmoB antibodies of pure cultures of ammonia oxidizers belonging to the β-subclass of Proteobacteria (a) *Nitrosomonas eutropha* N904 (zoom step 16.4). (b) *Nitrosovibrio* sp. Nv K7.1 (zoom step 17.0). (c) *Nitrosococcus mobilis* Nc 2 (zoom step 15.8). (d) Four cells of *Nitrosospira* sp. Nsp R6.2 (zoomstep 20.0). (e) One single cell of *Nitrosolobus multiformis* Nl 13 in the center (zoom step 23.0). Bars = 1 μm. The objective used was a Neoflutar objective (100×/1.4 oil). The images were obtained with a CLSM (model TCS 4D microscope; Leica) by using different zoom steps. Excitation of the green Cy2-labeled anti-rabbit antibodies for the detection of AmoA and red Cy3-labeled anti-chicken antibodies for the detection of AmoB was provided by an argon–krypton laser (488/568 exc./LP 590 em. and BP 520–560 em.).

omonas strains *N. europaea* Freitag, *N. eutropha* N904, *N. eutropha* Dave, and *N. halophila* Nm 1. Cells of all the other strains of *Nitrosomonas* sp. including *Nitrosococcus mobilis* Nc 2 could not be detected by the AmoA antibodies. These results are in contrast to those previously reported results that showed AmoB antibodies detected the 41-kDa AmoB of all ammonia oxidizers of the β-subclass of Proteobacteria [38]. These results are included in Table 1 for completeness. The AmoA-antibodies did not react with the crude extracts of the tested ammonia oxidizers of the γ-subclass of Proteobacteria, the methane oxidizers, the nitrite oxidizers, and the different chemoorganotrophic bacteria.

IF Labeling of Pure Cultures. Immunofluorescence (IF) labeling using the AmoA antibodies and the previously described AmoB antibodies [38] was developed on pure cultures to visualize intact cells of ammonia oxidizers. To obtain successful antibody penetration into the bacteria, the cells had to be stored in PBS–ethanol at −20°C and were then treated with lysozyme. The AmoA antibodies detected all strains of the genus *Nitrosomonas* using IF labeling although fluorescence intensity varied (Table 1). Strains of *Nitrosomonas* sp. such as *N. eutropha*, *N. europaea*, and *N. halophila* that were successfully detected by immunoblotting were strongly stained, whereas cells of *N. cryotolerans* Nm 55 and *N. marina* Nm 22 showed weak fluorescence. In contrast to the AmoA antibodies, the AmoB antibodies had a broad specificity, as they detected all strains of ammonia oxidizers of the Proteobacteria that were tested (Table 1), all of which were stained strongly. Apart from weak IF labeling of *Nitrosococcus halophilus* Nc 1, *Nitrosococcus oceani* Nc 4, and *Methylococcus methanica* Oo52006 using the AmoB-antibodies, both antisera did not react with ammonia oxidizers of the γ-subclass of Proteobacteria, methane oxidizers, nitrite oxidizers, and different chemoorganotrophic bacteria. In control experiments without AmoA and AmoB antibodies the cells exhibited no fluorescence signals.

IF labeling revealed that the AmoB antibodies were associated with the cytoplasmic membranes. As a result, the signals appeared at the cell periphery of cells of *Nitrosomonas* sp., *Nitrosovibrio* sp., and *Nitrosococcus* sp. (Fig. 1a–c). In cells of members of the genera *Nitrosospira* and *Nitrosolobus,* the fluorescence signals were also observed in the cytoplasm along the cytoplasmic membrane, which is vaulted to the center in tightly coiled cells of *Nitrosospira* sp. (Fig. 1d) and partially compartmentalizes the lobate cells of *Nitrosolobus* sp. (Fig. 1e).

Using both antibodies simultaneously, cells of *Nitrosomonas* sp. could be double-stained and showed bright red fluorescence of the secondary AmoB antibodies at the cell periphery as well as bright green signals of the secondary AmoA antibodies distributed over the whole cell (Fig. 1a). In contrast, cells of *Nitrosococcus mobilis* and cells belonging to the genera *Nitrosovibrio*, *Nitrosospira*, and *Nitrosolobus* were only labeled by the AmoB antibodies (Fig. 1b–e). The different IF labeling specificity of AmoA and AmoB antibodies together with the characteristic morphology of the cells enabled us to distinguish between the members of different genera of ammonia-oxidizers of β-Proteobacteria in pure cultures. Using IF labeling, the four new ammonia-oxidizing strains R1.24, G1.6, M1.3, and R6.2 isolated from historical building stones could be classified on the genus label. The straight rods of strain R1.24 were labeled with both antibodies (Table 1). In contrast the coiled rods of the strains G1.6, M1.3, and R6.2 could only detect by AmoB-antibodies (Table 1, Fig. 1d). Because of the specific IF labeling and typical morphology of the cells, strain R1.24 was classified as a member of the genus *Nitrosomonas* and the strains G1.6, Ml.3, and R6.2 were assigned to the genus *Nitrosospira.* This classification was underlined by electron microscopic investigations, where cells of strain R1.24 showed typical *Nitrosomonas* and cells of the strains G1.6, M1.3, and R6.2 typical *Nitrosospira* morphology (not shown).

In defined mixed cultures comprising members of the genus *Nitrosomonas* and *Nitrosococcus mobilis* or one strain of a *Nitrosospira* sp., a *Nitrosovibrio* sp., and a *Nitrosolobus* sp., respectively, cells could also be identified to the genus level by IF labeling (Fig. 2).

FISH of Pure Cultures. Using FISH, the 16S rRNA probe EUB338 detected all pure cultures of ammonia oxidizers that were tested (Table 1). Probes Nso 190, NEU, and Nsv 443 were only applied on pure cultures of *Nitrosomonas europaea* Freitag, *Nitrosomonas eutropha* N904, and *Nitrosospira* Nsp1 and the four isolated strains R1.24, G1.6, M1.3, R6.2 (Table 1). Probe Nso 190 labeled all the strains except strain R1.24. In contrast, probe NEU could only detect *Nitrosomonas europaea* Freitag and *Nitrosomonas eutropha* N904, and probe Nsv 443 detected *Nitrosospira* Nsp1, but none of the isolates (Table 1). For completeness, the specificity of probe Nso 190, NEU, and Nsv 443 for nearly all of the ammonia oxidizers used in this study was included in Table 1.

IF Labeling of Enrichment Cultures. Based on the results obtained from pure cultures, enrichment cultures either derived from activated sludge, natural sandstone or soil were analyzed using IF labeling and FISH.

In enrichment cultures from activated sludge from the sewage treatment plant in Dradenau, up to 90% of the free suspended cells and about 40% of biofilm-embedded cells could be labeled *in situ* by the antibodies. Almost all detected ammonia oxidizers could be identified as cells of the genus *Nitrosomonas* according to the specific IF labeling. Except for the cells associated with the biofilm, almost all ammonia oxidizers were labeled by AmoA and AmoB antibodies. The biofilm cells, however, were only detectable by AmoA-antibodies (Fig. 3b) because of nonspecific reactions of the AmoB antibodies with sludge particles (Fig. 3c), but could be identified as *Nitrosomonas* sp. cells. Inside the biofilm, loosely aggregated single cells (Fig. 3d) and dense microcolonies (Fig. 3e) of *Nitrosomonas* sp. were found. Outside the biofilm only a few cells were exclusively stained with AmoB antibodies and could be identified as cells of *Nitrosospira* sp. and *Nitrosococcus* sp.. The IF labeling results were confirmed by FISH. Almost all DAPI-stained cells could be detected by probe EUB338. Outside the biofilm about 90% of the cells and about 40% of the cells inside the biofilm were stained with the *Nitrosomonas* sp. specific oligonucleotide probe Nso 190 and NEU. Only a few cells could be labeled by the *Nitrosopira* sp., *Nitrosolobus* sp., and *Nitrosovibrio* sp. specific probe Nsv 443.

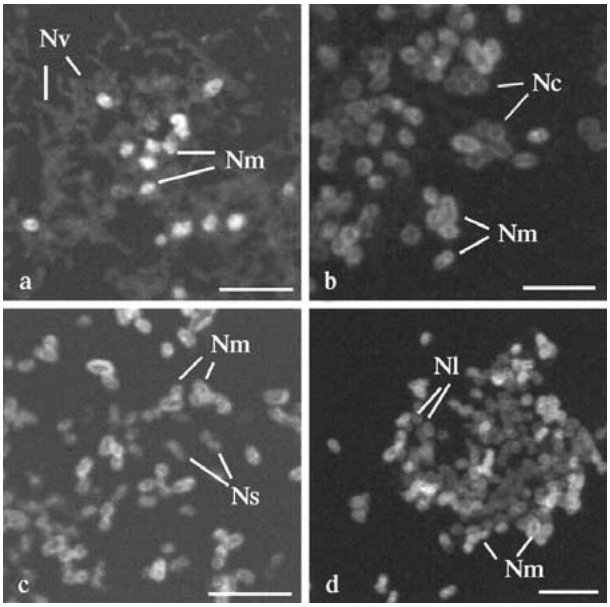

Figure 2. IF labeling with AmoA and AmoB antibodies of defined mixed cultures of *Nitrosomonas eutropha* N904 and (a) *Nitrosovibrio* sp. Nv K7.1 (zoom step 5.0), (b) *Nitrosococcus mobilis* Nc 2 (zoom step 6.2), (c) *Nitrosospira* sp. Nsp G1.6 (zoom step 5.4), (d) *Nitrosolobus multiformis* Nl 13 (zoom step 3.8). Bars = 5 μm.The objective used was a Neoflutar objective (100×/1.4 oil). The images were obtained with a CLSM (model TCS 4D microscope; Leica) by using different zoom steps. Excitation of the green Cy2-labeled anti-rabbit antibodies for the detection of AmoA and red Cy3-labeled anti-chicken antibodies for the detection of AmoB was provided by an argon–krypton laser (488/568 exc./LP 590 em. and BP 520–560 em.). Nc: *Nitrosococcus*; Nl: *Nitrosolobus*; Nm: *Nitrosomonas*; Ns: *Nitrosospira.*

In enrichment cultures prepared from samples of Baumberger sandstone, cells were mostly bound to particles and appeared mainly as single cells. About 68% of the cells were identified by IF labeling with AmoA and AmoB antibodies as cells of *Nitrosomonas.* About 17% of the cells were only stained by AmoB antibodies and had typical *Nitrosospira* sp. and *Nitrosovibrio* sp. morphologies (Fig. 4a). In contrast to the IF labeling, the detection of cells with probes EUB338, Nso 190, NEU, and Nsv 443

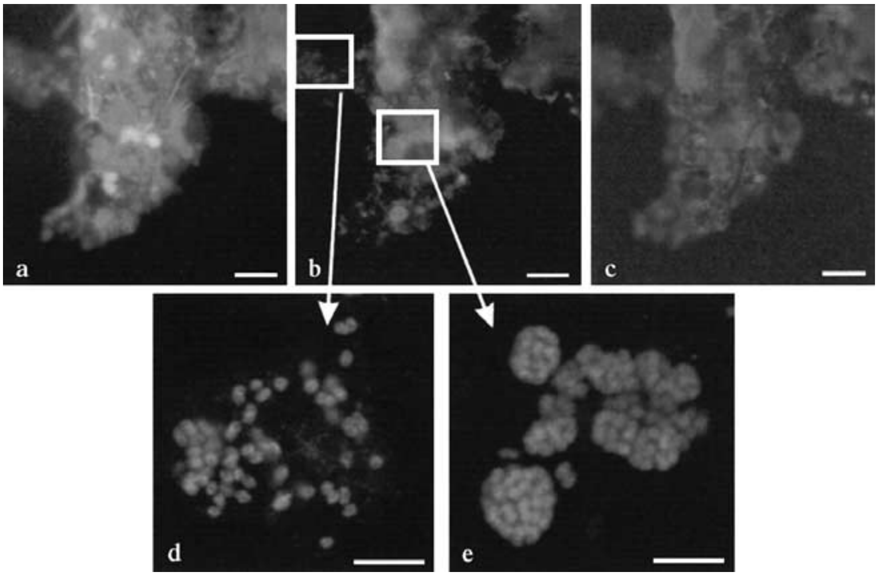

Figure 3. IF labeling with AmoA and AmoB antibodies of an enrichment culture from activated sludge samples from the sewage treatment plant of Dradenau. (a) DAPI staining; (b, d, e) IF labeling with AmoA-specific antibodies; (c) IF labeling with AmoB-specific antibodies. Ammonia oxidizers belonging to the *Nitrosomonas europaea* cluster occurring as (d) single cells (zoom step 4.9) or (e) microcolonies (zoom step 4.7). Bars a–c = 20 µm, d, e = 5 µm. The objective used was a Neoflutar objective (100×/1.4 oil). The images were obtained with a CLSM (model TCS 4D microscope; Leica) by using different zoom steps. DAPI was visualized with Leica filter set A (PB 340–380 exc.; RKP 400; LP 425 em.). IF labeling of the green Cy2-labeled anti-rabbit antibodies for the detection of AmoA and red Cy3-labeled anti-chicken antibodies for the detection of AmoB was visualized by filter set I 3 (BP 450–490 exc.; RKP 510; LP 515 em.) and filter set N 2.1 (BP 515–560 exc.; RKP 580; LP 590 em.), respectively. Details in d, e were provided by an argon–krypton laser (488/568 exc./LP 590 em. and BP 520–560 em.).

failed because of nonspecific reactions of the probe with the stone particles.

In the enrichment cultures of the soil samples, cells were freely suspended or particle bound and were generally not aggregated. About 85% of the cells could be labeled with the AmoB antibodies, and only a few were additionally stained with the AmoA antibodies. Because of the specific IF labeling and the morphology the ammonia oxidizers, cells could be identified as members of the genera *Nitrosospira* and *Nitrosolobus.* Coccoid ammonia oxidizers were also detected that might be small cells of members of the genus *Nitrosolobus* or belong to *Nitrosococcus* sp. (β-Proteobacteria) (Fig. 4b). The dominance of *Nitrosolobus* sp. was confirmed by electron microscopic analyses (Schnier, personal communication). FISH with the probes EUB338, Nso 190, NEU, and Nsv 443 did not succeed in the soil sample because probes bound nonspecifically to soil particles so that ammonia oxidizers could not be detected.

Discussion

Except for the two marine strains of *Nitrosococcus* sp., ammonia oxidizers form a monophyletic assemblage within the β-subclass of Proteobacteria [47]. Therefore, it is assumed that their key enzyme, the ammonia monooxygenase (AMO), is highly conserved. However, little is known about the structure and function of AMO since the enzyme has not been purified [14, 44, 45]. Inactivation experiments suggested that the proteins AmoA and AmoB were possible subunits of AMO [33]. Previously AmoB antibodies had been developed using the whole purified 41-kDa AmoB protein of *Nitrosomonas eutropha* N904, which detected all ammonia oxidizers of the β-subclass of Proteobacteria that were tested [38]. In this study, polyclonal antibodies were produced against the AmoA using a small 15-residue peptide corresponding to the AmoA N-terminal peptide sequence of *Nitrosomonas eutropha* Nm 57. Consequently, the antibodies detect only a small hydrophilic part of the 27-kDa AmoA [33]. The antibodies that recognized the AmoA of *Nitrosomonas eutropha* also detected the AmoA of other *Nitrosomonas* species. Using immunoblot analysis, only members of the *Nitrosomonas europaea* cluster *(N. europaea*, *N. eutropha*, *N. halophila)* [39] reacted with AmoA antibodies, but when IF labeling was used, the AmoA antibodies also detected members of the remaining species of *Nitrosomonas.* However, the species that were not detected in the immunoblot showed only weak fluorescence. The IF method was obviously more sensitive than the Western blot technique, analogous to the findings of Ward and co-workers [53], who used polyclonal antibodies targeting the nitrite reductase. The different IF labeling intensities of *Nitrosomonas* species

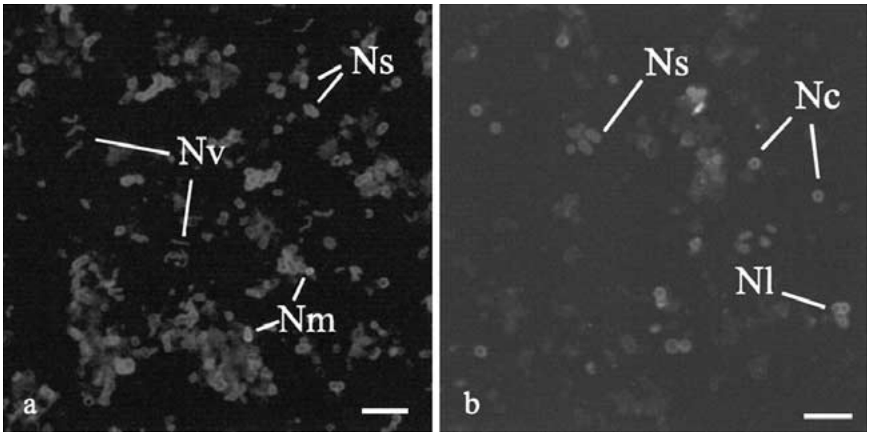

Figure 4. IF labeling with AmoA and AmoB antibodies of enrichment cultures from (a) Baumberger sandstone (zoom step 1.9), (b) garden soil samples (zoom step 2.3). Bars = 5 μm. The objective used was a Neoflutar objective (100×/1.4 oil). The images were obtained with a CLSM (model TCS 4D microscope; Leica) by using different zoom steps. Excitation of the green Cy2-labeled anti-rabbit antibodies for the detection of AmoA and red Cy3-labeled anti-chicken antibodies for the detection of AmoB was provided by an argon–krypton laser (488/568 exc./LP 590 em. and BP 520–560 em.). Nc: *Nitrosococcus*; Nl: *Nitrosolobus*; Nm: *Nitrosomonas*; Ns: *Nitrosospira*; Nv: *Nitrosolobus*.

might result from the differences between the N-terminal AmoA sequences, which could have caused the different binding efficiency of the antibodies. All *Nitrosomonas* strains showing high AmoA-sequence similarity to *N. eutropha*, such as *N. europaea* and *N. halophila*, were intensively stained, whereas strains with low AmoA-sequence similarity such as *N. cryotolerans* and *N. marina* were weakly stained [37, 40]. Only *Nitrosococcus mobilis*, close relative of *Nitrosomonas eutropha* [39, 40], could not be detected by AmoA antibodies. The N-terminal AmoA sequence of *N. eutropha* is identical with the sequence of *N. europaea* [37]. Aside from the two *Nitrosomonas* species, only the sequence of the N terminus of AmoA of one strain of *N. cryotolerans* is known [37], whereas the N-terminal AmoA sequences of the other species of *Nitrosomonas* are known only partially *(Nitrosococcus mobilis,* SwissProt accession number AF037108) or not at all. The N-terminal AmoA sequence of *N. cryotolerans* differs from the sequence of *N. eutropha* [37], which might be the reason for the weak IF labeling using AmoA-antibodies. Recently, Norton and co-workers [37] described the AmoA-sequence of two new isolated strains of *Nitrosomonas* with N-terminal AmoA sequences, which further deviate from the sequence of *N. eutropha* and therefore might not be detected by AmoA antibodies. Although the N-terminal AmoA sequences of strains of the genera *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* correspond with the described sequence of *N. cryotolerans* [37], they did not react with the AmoA antibodies. The third and fourth amino acids of their AmoA are deleted and the glutamic acid in position 7 is replaced by aspartic acid [37]. The reason for this disagreement might be that little strain-specific deviations between the N-terminal AmoA-sequence exist, as described for *Nitrosolobus multiformis* [37], and the *N. cryotolerans* strain we used might correspond more with *N. eutropha* than the used strains of *Nitrosospira* sp., *Nitrosovibrio* sp., and *Nitrosolobus* sp. The different specificity of AmoA antibodies will only be completely explained when the N terminus of the AmoA of the ammonia oxidizer strains that were used in this study has been sequenced. If instead of the N-terminal sequence a more conserved internal or C-terminal region of AmoA had been chosen, these antibodies might be reacted with all ammonia oxidizers of the β-subclass of Proteobacteria. In *Nitrosococcus oceani*, a member of the γ-subclass of Proteobacteria, only three amino acids of the 15 N-terminal AmoA sequence correspond with the AmoA of *N. eutropha* [2, 37], and therefore it did not react with the antibodies. Further, no reaction of AmoA antibodies was found with the closely related particulate methane monooxygenase (pMMO) of methane oxidizing bacteria, because the N-terminal pMMO sequence of *Methylococcus capsulatus* is almost identical to the AmoA of *Nitrosococcus oceani* [2] and therefore clearly differs from the AmoA sequence of *N. eutropha.* The fluorescent signals of all cells correlated with the locations of the AMO in the cytoplasmic and intracytoplasmic membranes [23].

Using both antisera simultaneously, the different IF labeling specificity of AmoA and AmoB antibodies together with the characteristic morphology of the cells enabled us to distinguish between the members of different genera of ammonia oxidizers of β-Proteobacteria in pure cultures as well as in mixed cultures. They could be applied for classification of new isolates of ammonia oxidizers. By IF labeling the isolates could assigned to the genus *Nitrosomonas* or *Nitrosospira.* This clear classification was not possible by using the 16S rRNA probes

NEU, Nso 190, and Nsv 443. This might be due to the choice of the probes. Although probe Nso 190 detected many ammonia oxidizers of the β-subclass of Proteobacteria [35], some *Nitrosomonas* strains were not included [40], and probe NEU is only specific for the detection of a few members of the genus *Nitrosomonas* [40, 50]. Therefore it is not remarkable that strain R1.24, which was classified by IF labeling to genus *Nitrosomonas,* could not be detected by probes Nso 190 and NEU. For a clear classification of the *Nitrosomonas* isolate the probe Nm0 should be used, which cover all strains of *Nitrosomonas* but might not detect the other four genera of ammonia oxidizers [39, 40]. In contrast, no 16S rRNA probe is thus far known that exclusively detects the genus *Nitrosospira* [40]. Therefore no probe exists that enables the clear classification of the isolates G1.6, M1.3, and R6.2, which were assigned by IF labeling to genus *Nitrosospira.* Although probe Nsv 443 has a broader specificity, as it is designed for the detection of *Nitrosolobus* sp., *Nitrosospira* sp., and *Nitrosovibrio* sp. [35, 40], it could not detect the isolates. In contrast to the antibodies, thus far no set of 16S rRNA probes is available for the classification of ammonia oxidizers to the five genera [40].

In addition, the specific immunoreactions were suitable for the identification of ammonia oxidizers enriched from different natural samples. In enrichment cultures of activated sludge from the sewage treatment plant in Dradenau, cells of *Nitrosomonas* sp. were mainly detected, which could be confirmed using FISH analysis with the *Nitrosomonas*- specific oligonucleotide probes NEU and Nsv 443. This finding is in agreement with previous *in situ* IF and FISH analysis, and *Nitrosomonas* sp. was mostly isolated from activated sludge samples [30, 35, 48]. In enrichment cultures derived from sandstone, members of the genus *Nitrosomonas* were also mainly found. However, additional cells were exclusively detected by the AmoB antibodies, and combined with the characteristic morphology the cells could be identified as members of the genera *Nitrosovibrio* and *Nitrosospira.* In previous studies *Nitrosovibrio* sp. and *Nitrosospira* sp. were predominantly isolated from sandstone samples [34, 43]. In enrichment cultures from neutral garden soil (pH 7.6) from the new botanical garden in Hamburg, members of the genera *Nitrosospira* and *Nitrosolobus* were the most abundant ammonia oxidizers. In these cultures, coccoid cells were also detected by AmoB antibodies that might have been small cells of *Nitrosolobus* sp. or might have belonged to members of the genus *Nitrosococcus* (β-Proteobacteria). Electron microscopic investigations of the same cultures revealed characteristic cells with a morphology and ultrastructure of small *Nitrosolobus* sp. cells (Schnier, personal communication), which is in agreement with recent studies in which *Nitrosolobus* [32, 49] and *Nitrosospira* [6, 19, 21, 25] were the most abundant ammonia oxidizers in loamy and arable soils with neutral pH.

In contrast to the AmoA antibodies and the oligonucleotide probes, the AmoB antibodies could not be applied to detect ammonia oxidizers within the biofilm of the activated sludge sample because of nonspecific reactions. However, only slight nonspecific reaction of the antibodies appeared in the presence of stone and soil particles, and the IF cell signals could be easily distinguished from the background, whereas with probes EUB338, Nso 190, NEU, and Nsv 443 the detection of ammonia oxidizers failed in these samples.

The antibodies against the AmoA and AmoB subunits of the AMO detected all ammonia oxidizers of the β-Proteobacteria that were tested and therefore have an advantage over previous antibody detection methods, which were limited in the application to specific serological groups [6, 52] as they recognize specific epitopes of the cell wall [41, 42, 48]. Presumably, the antibodies against the key enzyme, especially the AmoB antibodies, might be used for the detection of all ammonia oxidizers of the β-subclass of Proteobacteria even those that have not been described. Beside the enrichment cultures, the antibodies may be further used for the direct *in situ* detection of ammonia oxidizers in natural samples with a sufficient number of cells. The AmoB antibodies might provide the only tool for detecting all species of ammonia oxidizers of the β-subclass of Proteobacteria simultaneously and exclusively [40].

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